ANALGESIC, ANTI-INFLAMMATORY AND OTHER PHARMACOLOGICAL ACTIVITIES OF METHANOL LEAF EXTRACT OF *OLAX SUBSCORPIOIDEA* OLIV (OLACACEAE) IN LABORATORY ANIMALS

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DECLARATION

I declare that the work in the thesis entitled "Analgesic, Anti-Inflammatory and Other Pharmacological Activities of Methanol Leaf Extract of *Olax subscorpioidea* Oliv (Olacaceae)in laboratory animals" has been carried out by me in the Department of Pharmacology and Therapeutics. The information derived in the literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree at this or any other university.

Saidi ODOMA _____ _____

Name of Student Signature Date

CERTIFICATION

This thesis entitled "ANALGESIC, ANTI-INFLAMMATORY AND OTHER PHARMACOLOGICAL ACTIVITIES OF METHANOL LEAF EXTRACT OF *OLAX SUBSCORPIOIDEA* OLIV (OLACACEAE) IN LABORATORY ANIMALS" by Saidi ODOMA meets the regulations governing the award of the degree of Doctor of Philosophy in Pharmacology of the Ahmadu Bello University and is approved for its contribution to knowledge and literary presentation.

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ABSTRACT

Synthetic analgesic and anti-inflammatory drugs have major side effects such as constipation, nausea and vomiting, sedation and mental clouding, etc.,which have significantly limited their use.There is therefore, an intensification of search for newer analgesic and anti-inflammatory agents from the huge array of medicinal plant resources with better efficacy and fewer side effect profiles. Medicinal plants such as *Olax subscorpioidea* have been used traditionally for the management of pains, inflammatory diseases, yellow fever, cancer and rheumatism. The study aims at establishing the analgesic and anti-inflammatory potentials of methanol leaf extract of *Olax subscorpioidea*; and elucidating its possible mechanism of actions. The methanol extract and its fractions were subjected to phytochemical screening; oral and intraperitoneal median lethal dose (LD_{50}) determination; evaluation of analgesic activities using acetic acid-induced writhing, formalin induced pain and hot plate tests in mice; and evaluation of anti-inflammatory activity using carrageenan-induced hind paw oedema model in rats. The doses (oral) used for these studies were 250, 500 and 1,000 mg/kg for the methanol extract, residual aqueous and butanol fractions; while doses of 150, 300 and 600 mg/kg were used for the hexane fraction. The residual aqueous and butanol fractions (1,000 mg/kg, orally) were subjected to sub-acute inflammation studies using cotton-pellet induced granuloma in rats; also the concentrations of inflammatory cytokines in the tissue exudates of rats following carrageenan induced paw oedema was investigated. The roles of opioidergic, (α_1, α_2 and β)-adrenergic, serotonergic, ATP-sensitive potassium channels and nitric oxide-l-arginine pathways in the analgesic activities of the butanol fraction (1,000 mg/kg, oral) were further investigated. Results of the preliminary phytochemical screening of the methanol extract and the fractions indicated the presence of various phytochemicals such as

carbohydrates, cardiac glycosides, tannins, flavonoids, alkaloids, saponins, steroid and triterpenes. The oral LD_{50} of the methanol extract, residual aqueous and butanol fractions was estimated to be greater than 5,000 mg/kg in both rats and mice; that of hexane fraction was estimated to be 2,200 and 3,800 mg/kg in mice and rats respectively. The intraperitoneal LD_{50} in mice was estimated to be 3,800 mg/kg for the methanol extract; 2,200 mg/kg for the residual aqueous fraction and 1,300 mg/kg for butanol and hexane fractions; it was estimated in rats to be 3,800 mg/kg for the methanol extract, residual aqueous and butanol fractions; and 2,200 mg/kg in the hexane fraction. The acetic acid induced writhes and the formalin induced pain licking effect were significantly ($p<0.05$, $p<0.01$ and $p<0.001$) reduced by the methanol extract and the fractions in a dose-dependent manner. The thermal pain latency was also significantly ($p<0.05$, $p<0.01$ and $p<0.001$) increased by the methanol extract and its fractions (except hexane fraction). The paw oedema was also significantly ($p<0.05$, $p<0.01$ and $p<0.001$) reduced by the methanol extract and the fractions across the time. The residual aqueous and butanol fractions (1,000 mg/kg) significantly ($p<0.01$ and $p<0.001$) reduced granuloma formation in the cotton pellet-induced granuloma studies in rats. The residual aqueous and butanol fractions significantly ($p<0.05$ and $p<0.01$) decreased the concentrations of vascular endothelial growth factor (VEGF); the butanol fraction significantly decreased (($p<0.05$) concentrations of epidermal growth factor (EGF) and interleukin-1α (Il-1α). The residual aqueous and butanol fractions also significantly ($p<0.05$ and $p<0.01$) increased the concentration of Il-1β, IL-5 and interferon-γ (IFN-γ) while the residual aqueous fraction significantly($p<0.05$) increased the concentration of IL-6 in the rats' paw tissue exudates. The pretreatment of mice with l-arginine and metergoline significantly ($p<0.01$ and $p<0.05$, respectively) decreased the analgesic effect of the butanol fraction; while pretreatment with

naloxone, prazosin, yohimbine, propranolol and glibenclamide,each, had no significant effect on its analgesic activity. The results of the studies revealed that *Olax subscorpioidea* possesses marked analgesic and anti-inflammatory activities; the anti-inflammatory activity is mediated via the inhibition of pro-inflammatory cytokines such as IL-1α, IL-1β, VEGF and EGF and/or via the stimulation of the synthesis of anti-inflammatory cytokines such as IL-5, IL-6 and IFN-γ. These results also suggest the possible involvement of serotonergic and nitric oxide pathways in the analgesic effect of *Olax subscorpioidea.*

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List of Abbreviations

°C	Degree Centigrade
μ	Mu
δ	Delta
κ	Kappa
AA	Arachidonic acid
AC	Adenylate cyclase
ANOVA	Analysis of variance
ASA	Acetylsalicylic acid
ATP	Adenosine triphosphate
BPS	British Pain Society
cAMP	Cyclic adenosine monophosphate
CNS	Central Nervous System
COX	Cyclooxygenase
CPN	Current Protocols in Neuroscience
DRG	Dorsal root ganglion
DW	Distilled water
EGF	Epidermal Growth Factor
g	Gram
GIT	Gastrointestinal tract
GLI	Glibenclamide
Hr	Hour(s)
IASP	International Association for the Study of Pain

IL	Interleukin
IL-1ra	Il-1 receptor antagonist
INF	Interferon
iNOS	Inducible nitric oxide synthase
kg	kilogram
L-ARG	L-arginine
LD_{50}	Median Lethal dose
L-NAME	N^G-Nitro-L-Arginine methyl ester
L-NNA	Nomega-nitro-l-arginine
MEOS	Methanol leaf extract of *Olax subscorpioidea.*
MET	Metergoline
mg	Milligram
ml	Millilitre
MOR	Morphine
MPE	Maximum possible effect
n	Number of animals (per group)
NF-κB	Nuclear factor-kappa B
NO	Nitric oxide
NSAIDs	Non-steroidal anti-inflammatory drugs
PAG	Periaqueductal gray matter
PAMPs	Pathogen-associated molecular patterns
PAN	Primary afferent nociceptor
PBS	Phosphate buffered saline

PG	Prostaglandin
PGA	Periaqueductal grey area
Sec	Second(s)
SEM	Standard error of mean
Sub-P	Substance-P
TCAs	Tricyclic antidepressants
TENS	Transcutaneous electrical nerve stimulation
TLC	Thin layer chromatography
TNF	Tumour necrosis factor
T_X	Thromboxane
$^{V}/_{V}$	Volume/volume
VEGF	Vascular Endothelial Growth Factor
WHO	World Health Organization

CHAPTER ONE

1.0 INTRODUCTION

Pain is defined by the International Association for the Study of Pain (IASP) as an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage (Loeser and Treede, 2008). The British Pain Society (BPS) has defined pain as an emotion experienced in the brain; it is not like touch, taste, sight, smell or hearing. It can be perceived as a warning of potential damage, but can also be present when no actual harm is being done to the body (Moffat and Rae, 2010). It is the most common symptom of injuries and diseases (Haddad, 2007). It is a multidimensional sensory experience that is intrinsically unpleasant and associated with hurting and soreness. It may vary in intensity (mild, moderate or severe), quality (sharp, burning or dull), duration (transient, intermittent or persistent) and referral (superficial or deep, localized or diffuse). Although it is essentially a sensation, it has strong cognitive and emotional components; it is linked to or described in terms of suffering. It is also associated with avoidance motor reflexes and alterations in autonomic output. All of these traits are inextricably linked in the experience of pain (Woolf, 2004). Pain triggers various responses in the spinal cord and the brain, including reflexes, conscious perception, cognitive learning and memory processes, emotional reactions such as depression, and drug addiction (Gu *et al.*, 2005).

Inflammation is considered as the primary physiologic defense mechanism that helps the body to protect itself against infection, burns, toxic chemicals, allergens or other noxious stimuli. An uncontrolled and persistent inflammation may act as an etiologic factor for

many chronic illnesses (Kumar *et al.,* 2004). Inflammation is the immune system's response to infection and injury and has been implicated in almost all chronic diseases, such as cancer, cardiovascular diseases, arthritis, inflammatory bowel syndrome, atherosclerosis and autoimmune diseases (Luo *et al*., 2010; Ricciotti and FitzGerald 2011; Viljoen *et al*., 2012). It is an intrinsically beneficial event that leads to removal of offending factors and restoration of tissue structure and physiological function (Ricciotti and FitzGerald, 2011). A disturbance that is successfully cleared results in a return to basal homeostatic set points. When conditions that induce inflammation are persistent or resolution mechanisms fail, a state of chronic inflammation ensues that can lead to loss of normal physiological functions (Hotamisligil and Erbay, 2008). The inflammatory response is characterized by redness, heat, swelling, loss of function and pain (Gautam and Jachak, 2009). Redness and heat result from an increase in blood flow, swelling is associated with increased vascular permeability and pain is the consequence of activation and sensitization of primary afferent nerve fibres (Calixto *et al.,* 2003).

1.1 Statement of Research Problem

Pain is the most common symptom of injuries and diseases (Haddad, 2007). Virtually all known disease conditions are accompanied by pain (Donkor *et al.,* 2013). Pain imposes significant financial burden due to its long-term treatment (Bhangoo and Swanson, 2012). It is one of the most common conditions limiting efficiency and diminishing quality of life (Caraceni *et al.,* 2002; Mert *et al.,* 2013). It is the main reason for visiting the emergency department of a hospital in more than 50% of cases (Cordell *et al.,* 2002) and it is present in 30% of family practice visits (Hasselström *et al.,* 2002).

Several epidemiological studies from different countries have reported widely varying prevalence rates for chronic pain, ranging from 12-80% of the population (Abu-Saad, 2010). It becomes more common as people approach death. Pain affects more people than diabetes, heart disease and cancer combined (Institute of Medicine Report, 2011). It is a leading cause of disability and it is a major contributor to health care costs (National Center for Health Statistics, 2006). It is estimated that 20% of adults suffer from pain and another 10% are diagnosed with chronic pain every year. Pain affects all populations, regardless of age, sex, income, race/ethnicity or geography; it is not distributed equally across the globe. Pain can lead to depression, inability to work, disrupted social relationships and suicidal thoughts (Goldberg and McGee, 2011).

Unresolved inflammatory processes may be involved in the pathogenesis and progression of many inflammatory diseases, including asthma, atherosclerosis, cancer, rheumatoid arthritis, multiple sclerosis, heart disease, gouty arthritis, rhinitis and ischemia–reperfusion injury (Iwalewa *et al*., 2007; Medzhitov, 2008; Medzhitov, 2010; Alessandri*et al*., 2013). The costs of unrelieved pain and inflammatory diseases can result in longer hospital stays, increased rates of re-hospitalization, increased outpatient visits, and decreased ability to function fully leading to lost income. As such, patient's unrelieved chronic pain and inflammatory problems often result in an inability to work and maintain sound health (Strigo *et al.,* 2000).

Some of the analgesic and anti-inflammatory drugs available are often expensive, inaccessible and cause undesired and serious adverse effects (Babu *et al.,* 2009; Donkor *et al.*, 2013). For example, most of the opiate analgesics used clinically activate μ opioid receptors and the various central nervous system (CNS) side effects resulting from the use of opioids have been attributed to the μ opioid receptors. This development has directed research in favour of the more selective, safe and efficacious δ opioid receptors (Amrani, 2011). NSAIDs exert their analgesic effect by inhibiting the enzyme cyclooxygenase (COX), which catalyzes the conversion of arachidonic acid to leukotrienes and prostaglandins (PG), which are known to sensitize nociceptors near the location of the pain. Under physiological conditions, PG mediate many biological functions, such as regulation of immune responses, blood pressure, gastrointestinal integrity and fertility. Dysregulated PG synthesis or degradation has been associated with a wide range of pathological conditions (Ricciotti and FitzGerald, 2011). Therefore, adverse effects of NSAIDs mediated through the gastrointestinal and renal systems are as a result of the inhibition of COX.

1.2 Justification of the Study

Pain, when untreated can negatively affect all aspects of daily life, including physical activities, school attendance, sleep patterns, family interactions and social relationships and can lead to distress, anxiety, depression, insomnia, fatigue or mood changes, such as irritability and negative coping behaviour (WHO, 2012). The problems associated with drugs used in pain management are of serious concern, which necessitate the need for

development of new drugs and variety of treatment option from bioactive constituents obtained from plants used in traditional medicine (Stark *et al*, 2013).

Plants represent still a large untapped source of structurally novel compounds that might serve as lead for the development of novel drugs (Ahmad *et al.,* 1992). Despite the immense technological advancement in modern medicine, many people in developing countries still rely on traditional medicine for their daily health care needs (Louw*et al.,* 2007; Premanathan *et al.,* 2000).

Herbal medicine is thus gaining popularity, but lack of knowledge of the mechanism action and side effects of these preparations may undermine their utilization (Boullata and Nace, 2000). Understanding the cellular and molecular mechanisms of analgesic and anti-inflammatory actions of herbs will permit the discovery of promising targets for the development of new drugs to treat chronic pain and inflammatory diseases (Calixto *et al.,* 2003).

A good number of plant products with anti-inflammatory and analgesic activity have been documented, but very few of these compounds have reached clinical use due to scant scientific evidence that could explain their mode of action (Bellik *et al.,* 2013). The anti-inflammatory and analgesic activities of *O. subscorpioidea* have not been established scientifically. Similarly, the mechanism of analgesic and anti-inflammatory actions of *O. subscorpioidea*has not been studied before; thus, there is need for the studies.

1.3 Aim and Objectives

1.3.1 Aim

The aim of the study is to establish the analgesic and anti-inflammatory potentials of methanol leaf extract of *Olax subscorpioidea* and elucidate its possible mechanisms of analgesic and anti-inflammatory actions.

1.3.2 Specific objectives

1. To identify the class of phytoconstituents present in the methanol leaf extract and fractions of *O. subscorpioidea*.
2. To determine the acute toxicity of the methanol leaf extract and fractions of *O. subscorpioidea*.
3. To assess the analgesic effect of the methanol leaf extract and fractions of *O. subscorpioidea*.
4. To assess the anti-inflammatory effect of the methanol leaf extract and fractions of *O. subscorpioidea*.
5. To determine the possible mechanisms of analgesic and anti-inflammatory activitiesof *O. subscorpioidea*.

1.4 Research Hypothesis

The methanol leaf extract of *Olax subscorpioidea*possess analgesic activity which is mediated through the involvement of nitric oxide-l-arginine, serotonergic, adrenergic and opioidergic pathways; and possess anti-inflammatory activity mediated through the inhibition of pro-inflammatory cytokines and/or stimulation of anti-inflammatory cytokines.

CHAPTER TWO

2.0LITERATURE REVIEW

2.1 Pain

Pain, according to Moffat and Rae (2010), is a complex experience, initiated by sensory information conveyed from an unpleasant stimulus, greatly modified by affective (i.e. emotional), cultural and cognitive perspectives. While the physical processes that relay a stimulus to become the 'feeling of pain' can be described, the nature of pain as a sensation and its overall significance to the individual is unique.

The World Health Organization (WHO, 2012) has described pain as a multidimensional phenomenon with sensory, physiological, cognitive, affective, behavioural and spiritual components. Emotions (affective component), behavioural responses to pain (behavioural component), beliefs, attitudes, spiritual and cultural attitudes about pain and pain control (cognitive component) all alter the way that pain is experienced (sensory component) by modifying the transmission of noxious (unpleasant) stimuli to the brain (physiological component).

Pain is associated with negative emotions and is always highly salient, enabling the organism to either escape or protect the injured body part and thus enhance survival (Baliki *et al*., 2006). But, the truth about pain is that; it is a normal, protective, physiologic response. Without pain, one may be subjected to repeated injury allowing injured areas to heal poorly or not at all; as in the case of diabetics, who gradually lose sensation in their feet as the diabetic neuropathy progresses, often develop skin ulceration. These areas heal poorly without the normal protective reminder that pain provides, often leading to amputation. Also, poorly healing decubitus ulcers that occur in patients who have sustained spinal cord injury are another example of the importance of normal sensation and pain in reminding us to protect injured areas so that healing can proceed without recurrent injury (Rathmell, 2012). However, when pain becomes chronic that is when subjects live with unrelenting pain over many years, it becomes maladaptive and modifies one's outlook to everyday experience and to future expectations, by changing physiological and psychological processes underlying pain perception and pain-related behavior (Baliki *et al*., 2006).

2.1.1 Classification of pain

There are several variables for the classification of pain but no international classification system has been unanimously adopted. The most commonly used system for pain classification is four and they are 1) the pathophysiological mechanism of pain, 2) the duration of pain, 3) the etiology of pain and 4) the anatomic location of pain (Vadivelu *et al.*, 2009; WHO, 2012).

2.1.1.1. Pathophysiological classification of pain

Pathophysiological classification of pain have divided pain into two major types; nociceptive and neuropathic (and uncommonly; physiologic pain). Clinical distinction between these pains is useful because the treatment approaches are different (WHO, 2012).

Nociceptive pain: Nociceptivepain is defined as noxious perception resulting from cellular damage following surgical, traumatic or disease-related injuries. It arises when tissue injury activates specific pain receptors called nociceptors, which are sensitive to noxious stimuli. Nociceptors can respond to heat, cold, vibration, stretch stimuli and chemical substances released from tissues in response to oxygen deprivation, tissue disruption or inflammation (Fein, 2012).

Nociceptive pain has also been termed *inflammatory pain* because peripheral inflammation and inflammatory mediators play major roles in its initiation and development. In general, the intensity of nociceptive pain is proportional to the magnitude of tissue damage and release of inflammatory mediators (Vadivelu *et al*., 2009).

Scientists distinguish between pain and nociception; where nociception refers to signals arriving in the CNS resulting from activation of specialized sensory receptors called nociceptors that provide information about tissue damage. Pain then is the unpleasant emotional experience that usually accompanies nociception (Fein, 2012). Nociceptive pain

can be subdivided into *somatic* and *visceral* pain depending on the location of activated nociceptors.

Somatic painis caused by the activation of nociceptors in surface tissues (skin, mucosa of mouth, nose, urethra, anus, etc) or deep tissues such as bone, joint, muscle or connective tissue. It is well localized and generally follows a dermatomal pattern. It is usually described as sharp, crushing, or tearing in character. For example, cuts and sprains causing tissue disruption produce surface somatic pain while muscle cramps due to poor oxygen supply produce deep somatic pain (Vadivelu *et al*., 2009; WHO, 2012).

Visceral painis caused by the activation of nociceptors located in the viscera (the internal organs of the body that are enclosed within a cavity, such as thoracic and abdominal organs). It can occur due to infection, distension from fluid or gas, stretching or compression, usually from solid tumours. It is generally poorly localized and non-dermatoma and is described as cramping or colicky. Moderate to severe visceral pain is observed in patients presenting with bowel or ureteral obstructions, as well as peritonitis and appendicitis (WHO, 2012).

Visceral pain radiating in a somatic dermatomal pattern is described as referred pain. Referred pain maybe explained by convergence of noxious input from visceral afferents activating second-order cells that are normally responsive to somatic sensation. Because of convergence, pain emanating from deep visceral structures may be perceived as well-

delineated somatic discomfort at sites either adjacent to or distant from internal sites of irritation or injury (Vadivelu *et al*., 2009).

Neuropathic pain: Neuropathicpain is defined as "pain initiated or caused by a pathologic lesion or dysfunction" in peripheral nerves and CNS. It is caused by structural damage and nerve cell dysfunction (WHO, 2012). It is often intense and unrelenting and resistant to relief by available therapies (Fein, 2012). It is usually constant and described as burning, electrical, lancinating, and shooting. Disease states associated with classic neuropathic sysmptoms include infection (e.g. herpes zoster), metabolic derangements (e.g. diabetic neuropathy), toxicity (e.g. chemotherapy), and Wallerian degeneration secondary to trauma or nerve compression (Fein, 2012).

Neuropathic pain can be either *peripheral* (arising as a direct consequence of a lesion or disease affecting the peripheral nerve, the dorsal root ganglion or dorsal root) or *central* (arising as a direct consequence of a lesion or disease affecting the CNS). However, a clear distinction is not always possible (WHO, 2012).

Physiologic pain: physiologic pain defines rapidly perceived non-traumatic discomfort of very short duration. It alerts the individual to the presence of a potentially injurious environmental stimulus, such as a hot object, and initiates withdrawal reflexes that prevent or minimize tissue injury (Vadivelu *et al*., 2009).

Mixed pain: mixed pain is the type of pain in which the neuropathic and nociceptive pain may coexist. In some disease conditions, patients may have mixed pain consisting of somatic, visceral and neuropathic pain all at the same time or each separately at different times. The different pathophysiological mechanisms described above can operate together to produce mixed pain. Examples include trauma that damages tissue and nerves, burns (that affect skin as well as nerve endings), and cancer that causes external nerve compression as well as damaging nerves by infiltration (WHO, 2012).

Clinical distinction between nociceptive and neuropathic pain is based on the anatomic origin of the stimulus, whether it is well-localized or diffuse, and the character of the pain (e.g. sharp, dull or burning). In some types of painful conditions, the pathophysiological mechanisms of pain are not well understood and/or cannot be demonstrated (WHO, 2012).

2.1.1.2 Pain classification based on duration

Pain classification based on duration can be classified into two major types; acute and chronic pain. The acute pain according to IASP 'is pain of recent onset and probable limited duration; it usually has an identifiable temporal and causal relationship to injury or disease'. It is of sudden onset, is felt immediately following injury, is severe in intensity, but is usually short-lasting (less than 30 days). It arises as a result of tissue injury stimulating nociceptors and generally disappears when the injury heals (WHO, 2012). Although acute pain and associated responses can be unpleasant and often debilitating, they serve important adaptive purposes. They identify and localize noxious stimuli, initiate withdrawal responses that limit tissue injury, inhibit mobility thereby enhancing wound

healing, and initiate motivational and affective responses that modify future behavior (Vadivelu *et al*., 2009). Acute pain generally comprises two phases; the first phase (lasting seconds) alerts the individual potentially dangerous stimuli and the second phase (subchronic phase, lasting hours to days) may be regarded as a protective mechanism characterized by guarding of the injured tissue as a means of promoting healing and recovery (Smith and South, 2008).

The chronic pain is defined as pain lasting for long period of time (Smith and South, 2008), it persist 3 to 6 months (or more) beyond the expected period of healing (Vadivelu *et al*., 2009; WHO, 2012). It may begin as acute pain and persist for long periods or may recur due to persistence of noxious stimuli or repeated exacerbation of an injury. It may also arise and persist in the absence of identifiable pathophysiology or medical illness (Smith and South, 2008; WHO, 2012). Chronic pain is often regarded as a maladaptive response that confers no physiological advantage, such that the pain state itself has become the "disease", requiring treatment (Smith and South, 2008). It can negatively affect all aspects of daily life, including physical activities, school attendance, sleep patterns, family interactions and social relationships and can lead to distress, anxiety, depression, insomnia, fatigue or mood changes, such as irritability and negative coping behaviour (WHO, 2012).

2.1.1.3 Etiological classification of pain

Etiological classification of pain is commonly based on the underlying disease being *malignant* or *non-malignant*. It has little relevance to the mechanism and treatment of pain (WHO, 2012).

2.1.1.4 Anatomical classification of pain

Anatomical classification of pain is often classified by body location (e.g. head, back or neck) or the anatomic function of the affected tissue (e.g. myofascial, rheumatic, skeletal, neurological and vascular). However, location and function solely address the physical dimension and do not include the underlying mechanism. As such, although anatomical classifications can be useful for differential diagnoses, these classifications do not offer a framework for clinical management of pain (WHO, 2012).

2.1.2 Pain pathway (mechanism)

There are multiple mechanisms of pain, which include; nociception, peripheral sensitization, phenotypic switches, central sensitization, ectopic excitability, structural reorganization, and decreased inhibition. Nociception is the sole mechanism that causes nociceptive pain and comprises some processes (Woolf, 2004). The pain processes comprise of transduction, transmission, modulation, and perception (Pujol *et al.,* 2007). These processes have been named differently by different researchers. E.g. Woolf (2004) named it transduction, conduction, transmission and perception; Vadivelu *et al.,* (2009) named it transduction, conduction, transmission and modulation and Moffat and Rae (2010) named it transduction, transmission and perception.

2.1.2.1 Transduction

Transduction is the conversion of a noxious stimulus into electrical activity in the peripheral terminals of nociceptor sensory fibers. This process is mediated by specific receptor ion channels expressed only by nociceptors. It defines responses of peripheral nociceptors to traumatic or potentially damaging chemical, thermal, or mechanical stimulation. Noxious stimuli are converted into a calcium ion– (Ca^{2+}) mediated electrical depolarization within the distal fingerlike nociceptor endings. Peripheral noxious mediators are either released from cells damaged during injury or as a result of humoral and neural responses to the injury. Cellular damage in skin, fascia, muscle, bone, and ligaments is associated with the release of intracellular hydrogen (H^+) and potassium (K^+) ions, as well as arachidonic acid (AA) from lysed cell membranes. Accumulations of AA stimulate and upregulate the COX-2 enzyme isoform that converts AA into biologically active metabolites, including prostaglandin E_2 (PGE_2), prostaglandin G_2 (PGG_2) and later, prostaglandin H_2 (PGH_2). Prostaglandins and intracellular H^+ and K^+ ions play key roles as primary activators of peripheral nociceptors. They also initiate inflammatory responses and peripheral sensitization that increase tissue swelling and pain at the site of injury (Woolf, 2004; Vadivelu *et al.,* 2009).

2.1.2.2 Transmission

Transmission is the process by which impulses are sent to the dorsal horn of the spinal cord, and then along the sensory tracts to the brain. The primary afferent neurons are active senders and receivers of chemical and electrical signals. Their axons terminate in the dorsal horn of the spinal cord, where they have connections with many spinal neurons (Pujol *et al*., 2007).

The noxious stimuli activate primary afferent neurons with free nerve endings (Woolf, 2004). The primary afferent nerve fibres can be classified into three types on the basis of their diameter, structure (myelinated or unmyelinated) and conduction velocity. The largest-diameter sensory fibers, termed Aβ fibers. Aβ-fibers have myelinated axons, with a diameter ranging >10 μm, conducting nerve impulses at a rate of 30 to 1000 m/sec. The Aβ-fibers are generally non-noxious special sensory axons that innervate somatic structures of the skin and joints and mediate the fast, pricking quality of pain. Two classes of nociceptive fibers include the thin myelinated Aδ and unmyelinated C-fibers that innervate skin and a wide variety of other tissues. The Aδ fibers has a diameter ranging from 2-6 μm, transmit the "first pain," a rapid-onset (<1 s) well-localized, sharp or stinging sensation of short duration. This perception of "first pain" alerts the person to actual or potential injury, localizes the site of injury, and initiates reflex withdrawal responses. The unmyelinated C fibers, also termed *high threshold polymodal nociceptive fibers*, with a diameter ranging from 0.4-1.2 μm, conduct action potentials slowly at a rate of 0.5 to 2.0 m/sec and respond to mechanical, chemical, and thermal injuries. They are responsible for the perception of "second pain," which has a delayed latency and is described as a diffuse burning, stabbing sensation that is often prolonged and may become progressively more uncomfortable. C-fibers comprise approximately 70% of all nociceptors. A variety of neuropeptides, including substance-P (Sub-P) and calcitonin gene-related peptide (CGRP) have been identified in C-fibers (Ahmed, 2010).

2.1.2.3 Modulation

Modulation is the process of dampening or amplifying these pain related neural signals. Modulation takes place primarily in the dorsal horn of the spinal cord, but also elsewhere, with input from ascending and descending pathways. Rich arrays of opioid receptors (mu, kappa and delta) are present in the dorsal horn. In addition to an ascending tract, the nociceptive system contains descending pathways that send neurons from the frontal cortex and hypothalamus to the midbrain and medulla. These neurons inhibit nociceptive neurons and interneurons in the ascending pathway (Pujol *et al*., 2007).

The gate control theory: The gate control theory is a popular model of pain modulation proposed by Melzack and Wall in 1965 and later revised by Melzack and Casey in 1968. These investigators proposed the existence of an endogenous ability to reduce or increase the degree of perceived pain through modulation of incoming impulses at a gate located in the dorsal horn of the spinal cord. The gate acts on signals from the ascending and descending systems and weighs all of the inputs. The integration of these inputs from sensory neurons, the segmental spinal cord level, and the brain determines whether the gate will be opened or closed, either increasing or decreasing the intensity of the ascending pain signal. The importance of psychological variables in the perception of pain, including motivation to escape pain, and the role of thoughts, emotions, and stress reactions in increasing or decreasing painful sensations is evident in the gate control theory (Fields and Basbaum, 2000).

2.1.2.4 Perception

Perception refers to the subjective experience of pain that result from the interaction of transduction, transmission, modulation and the psychological aspects of the individual (Pujol *et al*., 2007).

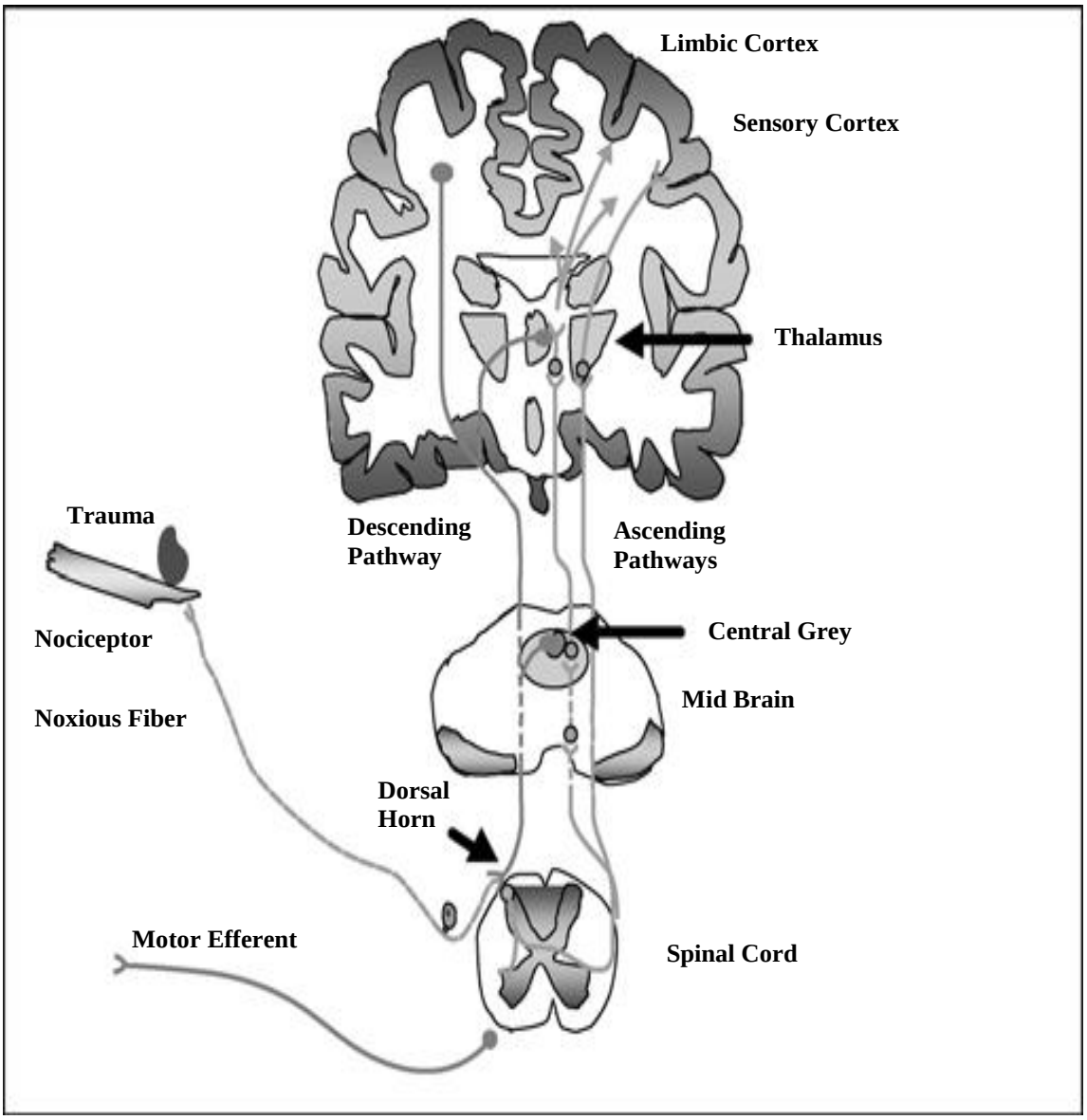


Figure 2.1: Anatomical Overview of Pain Pathways(Adapted from Vadivelu *et al.*, 2009)

Noxious information is conveyed from peripheral nociceptors to the dorsal horn via unmeylinated and myelinated noxious fibers. Second-order spinal neurons send impulses rostrally via two distinct pathways, the neospinothalamic and paleospinothalamic tracts. These cells also activate motor and sympathetic efferents within the spinal cord. Ascending tracts make contacts in the brainstem and midbrain, central gray and thalamus. Projections

are then made with the frontal and limbic cortex. Descending fibers emanating from cortex, hypothalamus and brainstem project to the spinal cord to modulate pain transmission.

2.1.3 Analgesics

Analgesics are medications that relieve or reduce pain without causing loss of consciousness. They are sometimes referred to as painkiller (Lilley *et al.,* 2011; WHO, 2012). There are three classes of analgesics; they are: opioids, non-opioids and adjuvant analgesics.

2.1.3.1 Opioid analgesics

The term opioid refers to any chemical, natural or synthetic, that binds to the opioid receptor and can be antagonized by naloxone; whereas opiates are drugs, i.e. naturally occurring alkaloids, derived from *Opium poppy* (Rosenblum *et al*., 2008; Vallejo *et al*., 2011). Another commonly used nonscientific term for opioids are "narcotics" (Vallejo *et al*., 2011). The term narcotic is a legal designation and should not be used in the clinical setting (Rosenblum *et al*., 2008). It describes opioid-like or, more specifically, morphine-like drugs and other drugs with potential for abuse (Vallejo *et al*., 2011).

Opioids are the most widely used and effective analgesics for the treatment of pain and related disorders. Opiates have been used for thousands of years for the treatment of pain and they are frequently used in the treatment of numerous other disorders including diarrhea, cough, post-operative pain and cancer (Al-Hasani and Bruchas, 2011). Opioid

analgesics are considered to be a mainstay in the treatment of moderate to severe pain that does not respond to non-opioids alone; because they are effective, fairly easy to titrate and have a favorable risk to benefit ratio (Pujol *et al*., 2007).

Opioid receptors: Opioids produce effects on neurons by acting on receptors located on neuronal cell membranes (Chahl, 1996). Several types of opioid receptors exist but three have been characterized. The characterized opioid receptors are: mu (μ), delta (δ) and kappa (κ), (Rosenblum *et al*., 2008; Amrani, 2011). These receptors belong to the large family of receptors which possess 7 transmembrane-spanning domains of amino acids. Each of the receptors displays distinctive distribution patterns and pharmacological profiles (Amrani, 2011). Studies have shown that the naturally occurring opioid peptide, endorphins, interacts preferentially with μ receptors, the enkephalins with δ receptors and dynorphin with κ receptors. Morphine has considerably higher affinity for μ receptors than for other opioid receptors. The opioid antagonist, naloxone, inhibits all opioid receptors, but has highest affinity for μ receptors. All 3 receptors produce analgesia when an opioid binds to them. However, activation of κ receptors does not produce as much physical dependence as activation of μ receptors. The opioid receptors are coupled to guanine nucleotide binding proteins known as G-proteins. G-proteins consist of 3 subunits (α, β and γ). When the receptor is occupied, the α subunit is uncoupled and forms a complex which interacts with cellular systems to produce an effect (Figure 2.2). Several types of G-proteins have been found. The types to which the opioid receptors are coupled produce inhibitory effects in neurons (Chahl, 1996; Amrani, 2011).

*Sites of action of opioids on neurons:*Opioids have actions at two sites, the presynaptic nerve terminal and the postsynaptic neuron. The postsynaptic actions of opioids are usually inhibitory. The presynaptic action of opioids is to inhibit neurotransmitter release, and this is considered to be their major effect in the nervous system. However, the final effect of an opioid in the brain is the result, not only of its action at multiple presynaptic sites on both inhibitory and excitatory neurons, but also of its postsynaptic effects(Chahl, 1996).

The nervous system comprises neurons of many different types which differ in size, shape, function and the chemical nature of the neurotransmitters released from their terminals to carry information to other neurons. Morphine, by an action on μ receptors, inhibits release of several different neurotransmitters including noradrenaline, acetylcholine and the neuropeptide, sub-P (Pujol *et al*., 2007).

Opioids and pain pathways: Pain is normally associated with increased activity in primary sensory neurons induced by strong mechanical or thermal stimuli, or by chemicals released by tissue damage or inflammation. Primary sensory neurons involved in pain sensation release predominantly sub-P and glutamate in the dorsal horn of the spinal cord. Nociceptive information is transmitted to the brain via the spinothalamic tracts. This ascending information can activate descending pathways, from the midbrain periaqueductal grey area (PGA), which exert an inhibitory control over the dorsal horn (Chahl, 1996).

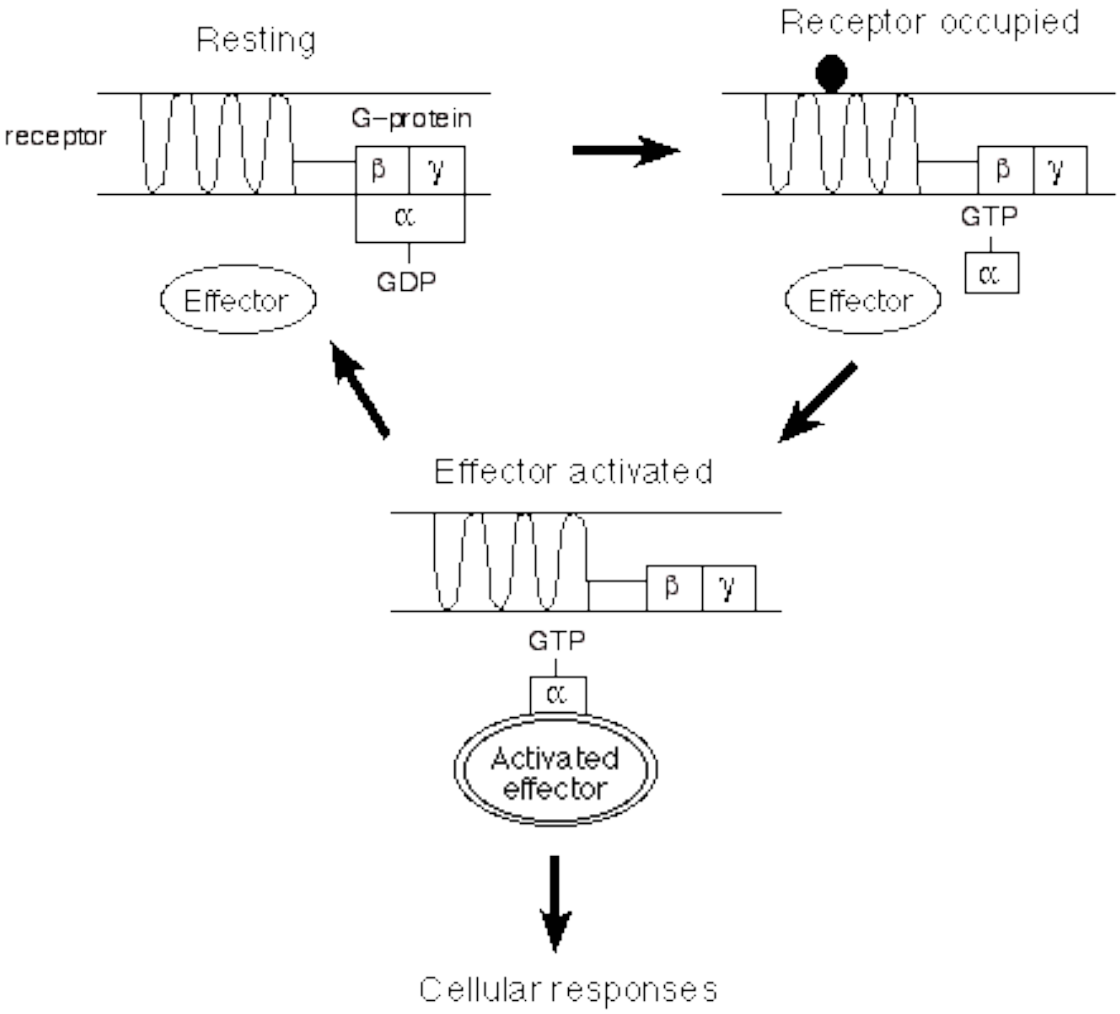


Figure 2.2: The Function of G-proteins (adapted from Chahl, 1996)

Under resting conditions, guanosine diphosphate (GDP) is associated with the α subunit. When the opioid binds to the receptor, GDP dissociates from the α subunit and guanosine triphosphate (GTP) takes its place. This produces a conformational change that causes the opioid to dissociate from the receptor. The α subunit bound to GTP also dissociates from the β and γ subunits and interacts with the system within the cell that produces the effect (the effector). The intrinsic enzymatic activity of the α subunit causes GTP to be converted back to GDP and the a subunit now reassociates with the b and g subunits to return the complex to its normal state.

Opioid receptors are present in many regions of the nervous system that are involved in pain transmission and control, including primary afferent neurons, spinal cord, midbrain and thalamus. The physiological role of naturally occurring opioid peptides in regulating pain transmission is not clear. However, under pathological conditions, the endogenous opioid system is activated. The opioid drugs produce analgesia by actions at several levels of the nervous system, in particular, inhibition of neurotransmitter release from the primary afferent terminals in the spinal cord and activation of descending inhibitory controls in the midbrain (Chahl,1996).

Mechanism of action of Opioid analgesics: Opioids exert their analgesic effect by binding to specific opioid receptors. The opioid receptors involved in pain modulation are situated in both the central nervous system and the peripheral nervous system. These receptors also bind to endogenous opioid peptides, which are involved in pain modulation and numerous other functions in the body (Rosenblum *et al*., 2008).Opioids exhibit their analgesic effects by:

1) inhibition of calcium influx in the presynaptic membrane by closing of voltage sensitive calcium channels; thus, inhibiting neurotransmitter release; such as sub-P,

2) hyperpolarization of presynaptic cells by stimulation of potassium efflux leading to increased potassium outflow preventing nociceptive afferent information from spreading to adjacent neurons and

3) central modulation of nociceptive information at the limbic system by reducing cyclic adenosine monophosphate (cAMP) production via inhibition of adenylyl cyclase.

Overall, this results in reduced neuronal cell excitability leading to a reduction in transmission of nerve impulses along with inhibition of neurotransmitter release (McDonald and Lambert, 2005; Vallejo *et al*., 2011).

Opioid inhibition of neurotransmitter releases: Neurotransmitter release from neurons is normally preceded by depolarization of the nerve terminal and Ca^{2+} entry through voltage-sensitive Ca^{2+} channels. Drugs may inhibit neurotransmitter release by a direct effect on Ca^{2+} channels to reduce Ca^{2+} entry, or indirectly by increasing the outward K^{+} current, thus shortening repolarisation time and the duration of the action potential. Opioids produce both of these effects because opioid receptors are apparently coupled via G-proteins directly to K^{+} channels and voltage-sensitive Ca^{2+} channels. Opioids also interact with other intracellular effector mechanisms, the most important being the adenylate cyclase system (Chahl, 1996).

Decreased Ca^{2+} entry: Voltage-sensitive channels are activated only when there is depolarisation of the neuron. Three types of voltage-sensitive Ca^{2+} channels are known, the L-type (large conductance) sensitive to calcium channel blockers, the T-type (small conductance) and the N-type (intermediate conductance). Opioids inhibit N-type Ca^{2+} channels and thus inhibit neurotransmitter release. This effect alone does not account fully for the effect of opioids on neurotransmitter release (Akil and Simon,1993).

Increased outward movement of K^{+}: Many types of K^{+} channels are now known, some of which are voltage-sensitive and others which are sensitive to intracellular substances.

Opioids open voltage-sensitive K^+ channels and thus increase outward movement of K^+ from neurons. This effect occurs in several brain regions as well as in the spinal cord and myenteric plexus. Increased outward movement of K^+ is the most likely mechanism for the postsynaptic hyperpolarisation and inhibition of neurons induced by opioids throughout the nervous system. However, it remains to be definitively established that this mechanism is also involved in the presynaptic action of opioids to inhibit neurotransmitters release (Reisine and Bell, 1993).

Inhibition of adenylate cyclase: Adenylate cyclase is an enzyme that breaks down adenosine triphosphate (ATP) to form cyclic adenosine monophosphate (cAMP). All 3 types of opioid receptors couple to adenylate cyclase. Inhibition of adenylate cyclase may result in inhibition of neurotransmitter release (Figure 2.3)(Chahl, 1996).

Adverse effect of opioid analgesics: Adverse effect of opioidsinclude constipation, nausea and vomiting, sedation and mental clouding, respiratory depression, dry mouth, urinary retention, pruritus, myoclonus, dysphoria, euphoria, sleep disturbances, sexual dysfunction, and inappropriate secretion of antidiuretic hormone etc (Rosenblum *et al*., 2008).

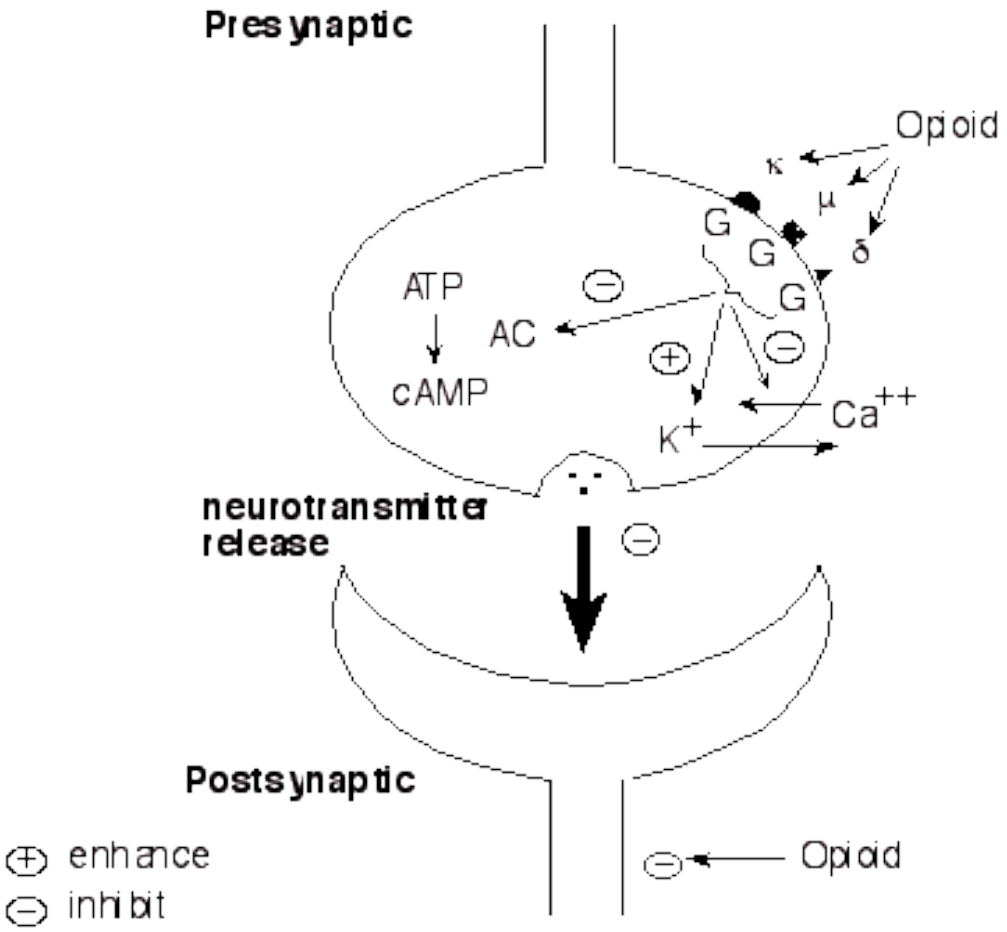


Figure 2.3: Mechanism of Analgesic Action of Opioids(adapted from Chahl, 1996)

Opioids have been proposed to inhibit neurotransmitter release by inhibiting calcium entry, by enhancing outward movement of potassium ions, or by inhibiting adenylate cyclase (AC), the enzyme which converts adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP).

2.1.3.2 Non-opioid analgesics

The non-opioids analgesics include drugs such as acetaminophen and non-steroidal anti-inflammatory drugs (NSAIDs).

NSAIDs: NSAIDs are large chemically diverse group of drugs that are analgesics and also possess anti-inflammatory and antipyretic activity but are not steroids (Lilley*et al*., 2011). The analgesic effect of NSAIDs is not necessarily secondary to an anti-inflammatory response because they have clear analgesic effects in the absence of inflammation (Calixto *et al*., 2000). NSAIDs are used primarily for treatment of mild to moderate pain and provide additive analgesia when combined with opioids prescribed for more severe pain conditions or inflammatory pain conditions. NSAIDs are also used for the management of oedema and tissue damage resulting from inflammatory joint disease (arthritis). A number of these drugs possess antipyretic activity in addition to having analgesic and anti-inflammatory actions, and thus have utility in the treatment of fever. In contrast to opioids, NSAIDs have a distinct ceiling effect for analgesia, that is, increasing the dose beyond a certain threshold does not increase analgesia (but can increase toxicity). NSAIDs do not produce physical or psychological dependence (Pujol *et al*., 2007).

Mechanism of NSAIDs: The major mechanism by which the NSAIDs elicit their therapeutic effects (analgesic, anti-inflammatory and antipyretic activities) is inhibition of prostaglandin (PG) synthesis. Specifically NSAIDs competitively (for the most part) inhibit COX, the enzymes that catalyze the synthesis of cyclic endoperoxides from arachidonic acid (AA) to form prostaglandins (Figure 2.4).

Two COX isoenzymes have been identified: COX-1 and COX-2. COX-1, expressed constitutively, is synthesized continuously and is present in all tissues and cell types, most notably in platelets, endothelial cells, the gastrointestinal (GI) tract, renal microvasculature, glomerulus, and collecting ducts. Thus COX-1 is important for the production of prostaglandins of homeostatic maintenance, such as platelet aggregation, the regulation of blood flow in the kidney and stomach and the regulation of gastric acid secretion. Inhibition of COX-1 activity is considered a major contributor to NSAIDs' GI toxicity. COX-2 is considered an inducible isoenzyme, although there is some constitutive expression in the kidney, brain, bone, female reproductive system, neoplasias, and GI tract (Pujol *et al*., 2007).

Generally, the NSAIDs inhibit both COX-1 and COX-2. Most NSAIDs are mainly COX-1 selective (e.g., aspirin, ketoprofen, indomethacin, piroxicam, sulindac). Others are considered slightly selective for COX-1 (e.g. ibuprofen, naproxen, diclofenac) and others may be considered slightly selective for COX-2 (e.g. etodolac, nabumetone, and meloxicam). The mechanism of action of celecoxib and rofecoxib is primarily selective inhibition of COX-2; at therapeutic concentrations, the COX-1 isoenzyme is not inhibited thus GI toxicity may be decreased(Smith, 2009).

Adverse effects of NSAIDs: Adverse effects of NSAIDs include; renal impairment, bleeding, gastric ulceration, and hepatic dysfunction. Some less common side effects include confusion, precipitation of cardiac failure, pedal oedema, and exacerbation of hypertension (Katz*et al*., 2002).

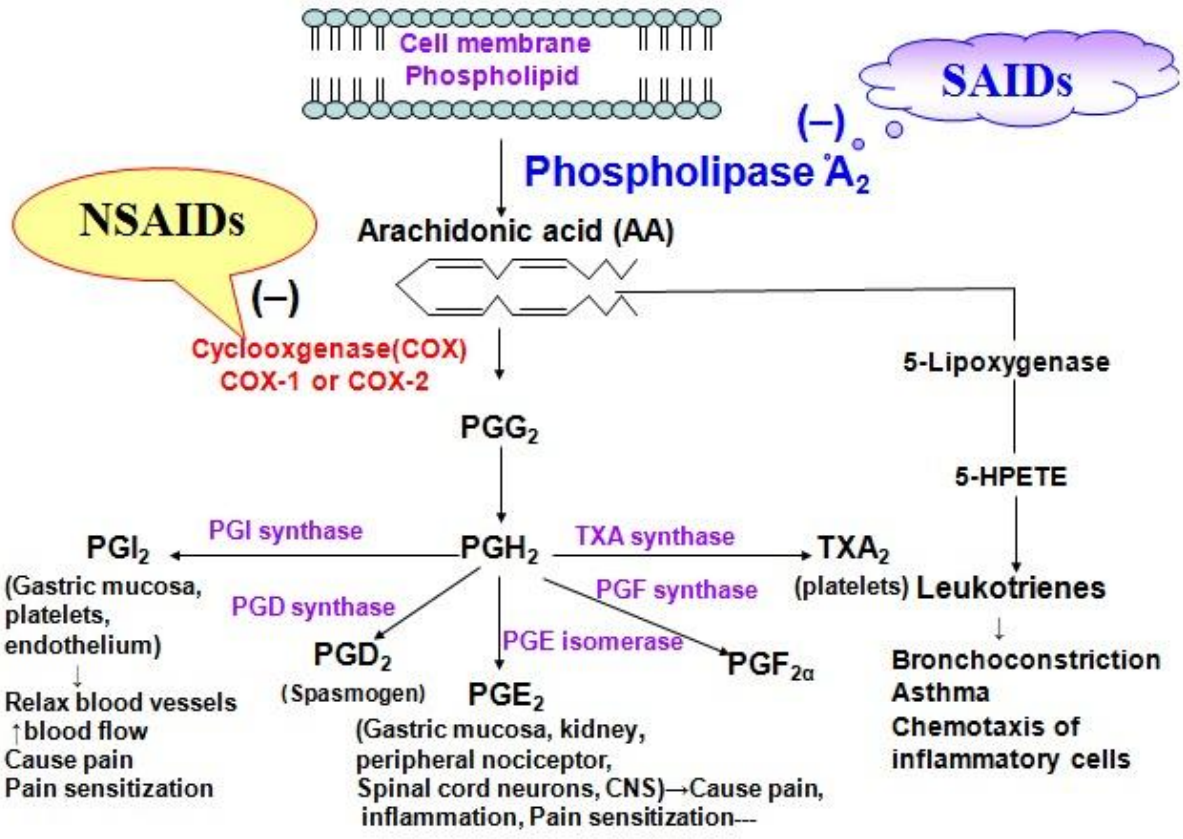


Figure 2.4: Enzymatic Pathway of Prostaglandin (PG) Formation from Arachidonic Acid and its Inhibition by the NSAIDs

Cyclooxygenase (COX), which is present in different isozymes COX-1 and 2, oxygenates arachidonic acid to form PGG_2 which is further reduced to PGH_2. PGH_2 is a highly unstable endoperoxide that is rapidly converted by specific synthases to PGs of the E_2, F_2 and D_2 series and also to PGI_2 (prostacyclin) and thromboxane (T_X) A_2. Both PGI_2 and T_XA_2 have a very short half-life (30 seconds and 3 minutes, respectively) and are rapidly hydrolyzed to the inactive compounds 6-keto-$PGF_{1\alpha}$ and T_XB_2, respectively. NSAIDs=non-steroidal anti-inflammatory drugs, SAIDs=steroidal anti-inflammatory drugs, HPETE=hydroperoxyeicosatetraenoic acid.

Acetylsalicylic Acid: Acetylsalicylic acid, or aspirin, is one of the oldest non-opioid analgesics and considered to be a member of the class of NSAIDs medications. Aspirin is commonly used to treat minor to moderate types of pain, including arthritic conditions. It can sometimes be as effective as other non-opioid analgesics in relieving pain. Gastrointestinal disturbances (usually upper gastrointestinal) and bleeding due to platelet aggregation inhibition are the most common adverse effects seen with aspirin therapy (Stolfi*et al.*, 2013).

Acetaminophen: Acetaminophen has analgesic and antipyretic properties similar to that of aspirin, without the anti-inflammatory effect. Acetaminophen is used in the treatment of mild to moderate pain and is often the recommended first-line analgesic therapy for the treatment of osteoarthritic pain. Its mechanism of action is not well-defined, although it is thought to be associated with eicosanoid systems and/or nitric oxidecontaining pathways (Smith, 2009).

2.1.3.3. Adjuvant analgesics

Adjuvant analgesics are a miscellaneous group of pain-relieving drugs whose primary indication traditionally is not for the treatment of pain. They are used to provide treatment for specific types of pain, and at times, to augment the analgesic effect of opioids and/or reduce the side effects of analgesics. Some of the most commonly used adjuvant drugs include corticosteroids, anticonvulsants, antidepressants, local anesthetics, neuroleptic agents, and hydroxyzine (Pujol *et al*., 2007).

Corticosteroids: Steroids inhibit the arachidonic acid cascade and thereby inhibit synthesis of prostaglandins and leukotrienes. The steroids block the chemicals that sensitize the primary afferent nociceptor (PAN) and thereby raise the threshold at which the PAN is transduced. Corticosteroids are often used in palliative care, where they have a number of beneficial effects, including pain reduction, improved appetite, weight gain, antiemetic action and mood elevation. Steroids are used in the treatment of neuropathic pain due to cord compression, brachial or lumbosacral plexus invasion or peripheral nerve infiltration. Additionally, they are helpful in treating headache due to increased intracranial pressure, some types of arthritic pain, and pain after visceral obstruction. Chronic use of corticosteroids produces weight gain, Cushing's syndrome, proximal myopathy, hyperglycemia, insulin resistance diabetes mellitus, osteoporosis, anxiety effects, mental changes, and increased risk of gastrointestinal bleeding (Donihi *et al*., 2006). Dexamethasone is an example of corticosteroids.

Anticonvulsants: Anticonvulsants are used for some neuropathic pain conditions to relieve lancinating or stabbing pain. Anticonvulsants inactivate the sodium channels in afferent fibers and thereby inhibit the action potential. They have the ability to suppress discharge in pathologically altered neurons, thus inhibiting neural hyper-excitability, which may be responsible for their usefulness in treatment of neuropathic pain conditions. Anticonvulsant drugs (e.g. phenytoin, carbamazepine, sodium valproate clonazepam, and baclofen) prevent transmission via this type of mechanism. Anticonvulsants such as gabapentin is indicated for treatment of post-herpetic neuralgia but also has been widely studied in treatment of other types of neuropathic pain. Gabapentin's mechanism of action is more

complex than other anticonvulsants. Gabapentin is associated with the typical side effects of all central nervous system–acting drugs, including sedation, dizziness and confusion (Butler *et al.*, 2004).

Antidepressants: Antidepressants enhance the descending-pain inhibitory system by preventing cellular re-uptake of serotonin norepinephrine and dopamine at synapses, (which may increase their analgesic effects, as well as improve mood favorably). These transmitters typically are released from the descending-fiber cells and rapidly transported back into the cell. Rapid re-uptake limits the time that both serotonin and norepinephrine are available for receptor binding and thereby to inhibit transmission of nociceptive signals. Tricyclic antidepressants (TCAs), such as amitriptyline, imipramine, nortriptyline, and desipramine, which have moderate serotonin effects and weak to potent norepinephrine effects are useful agents for neuropathic pain, cancer pain, and non-neuropathic pain. Common side effects of TCAs include dry mouth, insomnia, sedation, urinary retention, depression, constipation, and orthostasis. They may also be associated with cardiovascular side effects, such as increased blood pressure, and conduction blockade. They may also lower seizure threshold (Katz, 2002).

Topical Analgesics: Topical analgesics are targeted toward a specific area of pain. Typically, topical analgesics (e.g. lidocaine patch, capsaicin) are applied directly onto the painful area. Topical analgesics are used for both neuropathic and musculoskeletal pain. Capsaicin, derived from the active ingredient in chili peppers, is available over the counter

and is widely used. Capsaicin depletes sub-P from nerve terminals and is thought through this mechanism to decrease peripheral pain transmission (Pujol *et al.,* 2007).

Neuroleptic Agents: Neuroleptic agents have been used as adjuvant analgesics for many decades; however, their role in the treatment of chronic pain is limited at present. Methotrimeprazine is the only neuroleptic with definite analgesic properties and is occasionally used for patients with opioid tolerance or side effects. Common side effects of neuroleptics include sedation and hypotension. Prolonged use of phenothiazines is associated with tardive dyskinesia. Furthermore, extrapyramidal symptoms can occur, usually in younger patients (Katz, 2002).

Antihistamines: Hydroxyzine is an antihistamine with anticholinergic (drying) and sedative properties that is used to treat allergic reactions. In addition to its antihistamine effects, hydroxyzine has mild analgesic, antiemetic, anxiolytic, and sedative effects(Butler *et al.,* 2004).

Adjuvants for Bone Pain: Strontium (a radioisotope) and bisphosphonates are analgesic adjuvants used for metastatic bone pain. Radioisotopes work by delivering radiation to the bone. Although, they are effective for the pain of widespread bony metastases, they are complicated by bone marrow suppression (Pujol *et al.,* 2007).

2.1.4 Non-pharmacological management of pain

The armamentarium of pain treatment tools is vast and varied. The most appropriate interventions will depend on a number of variables, including the location and nature of the

patient's pain and its psychosocial context, the availability of specific interventions, the patient's preferences, the treatment provider's clinical orientation, and the relative risks and benefits of particular interventions (Savage *et al*., 2008).

The non-pharmacological therapy is considered to help the standard pharmacological treatment in pain management. While medical drugs are being used for treating the somatic (physiological and emotional) dimension of the pain non-pharmacological therapies aim to treat the affective, cognitive, behavioral and socio-cultural dimensions of the pain. The methods used are divided into three groups, they are; peripheral therapies (physical agents/skin stimulation methods), cognitive-behavioral therapies and other therapies. Some of these methods require special training (Demir, 2012).

2.1.4.1 Peripheral therapies (physical agents/skin stimulation)

Skin stimulation that provides analgesia is defined as stimulating the patient's skin in a harmless manner to treat the pain. Skin stimulation attempts (physical therapies) can be classified as hot-cold treatments, exercise, positioning, movement restriction-resting, acupuncture, hydrotherapy, Transcutaneous Electrical Nerve Stimulation (TENS), massage and therapeutic touch. If used in an appropriate manner these methods are effective on secondary pathologies such as inflammation, oedema, progressive tissue damage, muscle spasm and function loss which takes part in acute pain. (Demir, 2012).

Transcutaneous Electrical Nerve Stimulation (TENS): The American Physical Therapy Association has defined TENS as applying electrical stimulation to the skin to manage the

pain (Sluka and Walsh, 2003). Usually, it may be used in addition or instead of pharmacological agents to manage acute, chronic and post-operative pain. It is an electro-analgesia method. TENS, which functions in that way, has an effect to reduce the narcotic drugs usage and pain level (Demir, 2012).

Hot-cold treatment: Hot treatment moves the reflex arcs that inhibit the pain by means of heat receptors and reduces pain by vasodilatation effect. It is cheap and easy to use and it has a minimum amount of side effects when used regularly. It can be applied deeply or on surfaces. Application to the surface includes hot compresses, warm baths and paraphine usage (Demir, 2012).

Acupuncture: Acupuncture is accepted as a scientific treatment method that provides the body to restore its balance by means of stimulating some special points on the body with needles (Demir, 2012).

Exercise: Exercise includes active-passive movements, bed movements and ambulation. Exercise increases the movement and provides continuity thus increasing the blood flow, preventing spasm and contractures of the muscles and relieving the pain (Demir, 2012).

Cognitive-behavioral therapies: Cognitive-behavioral therapies are a part of multimodal approach in pain management. These attempts affect not only the pain level but also helps the patients to establish a management feeling of their selves while dealing with pain and develop management behaviors and improved self-esteem. The classes of treatments under

cognitive-behavioral therapies include; relaxation (respiration techniques and dreaming), distraction, praying, meditation, yoga, hypnosis, etc. (Delaune and Ladner, 2002).

2.2 Inflammation

Inflammation is a salutary host response against invading pathogens or following sterile tissue injury. It is a biological reaction to a disrupted tissue homeostasis. Basically, it is a tissue-destroying process that involves the recruitment of blood-derived products, such as plasma proteins, fluid and leukocytes, into perturbed tissue. This migration is facilitated by alterations in the local vasculature that lead to vasodilation, increased vascular permeability, and increased blood flow (Medzhitov, 2008; Ashley *et al*., 2012).

The inflammatory response is a spatially and temporally orchestrated event in which cells and mediators collaborate to neutralize and eliminate the damaging stimuli to allow maintenance of homeostasis. Although the primary functions of inflammation are to rapidly destroy or isolate the underlying source of the disturbance, remove damaged tissue, and then restore tissue homeostasis which is a physiological and beneficial process. But, non-resolving inflammatory processes may be involved in the pathogenesis and progression of many inflammatory diseases, including asthma, atherosclerosis, rheumatoid arthritis, multiple sclerosis, rhinitis and ischemia–reperfusion injury (Medzhitov, 2008; Medzhitov, 2010; Alessandri *et al*., 2013).

2.2.1 The inflammatory pathway

The inflammatory pathway (or mechanism of inflammation) consists of a tightly regulated cascade of immunological, physiological and behavioral processes that are orchestrated by soluble immune signaling molecules called cytokines (Ashley *et al*., 2012). The inflammatory pathway is classified into five, they are; inducers, sensors, mediators, effectors and resolution of inflammation (Medzhitov, 2008;Medzhitov, 2010; Ashley *et al*., 2012).

2.2.1.1 Inducers of inflammation

Inducers of inflammation are defined as the signals that initiate the inflammatory response. They activate specialized sensors, which then elicit the production of specific sets of mediators. These inflammatory inducers may either be exogenous or endogenous (Medzhitov, 2008).

Exogenous inducers of inflammation: The inducer of inflammation is classified exogenous if the signals that initiate the inflammatory response originate from outside the organism or system. Exogenous inducers are further classified into two groups: *microbial* and *non-microbial* (e.g. allergens, irritants and toxic compounds). The microbial inducers are further classified into two: pathogen-associated molecular patterns (PAMPs) and virulence factors. The first class of microbial inducer, PAMPs, is a limited and defined set of conserved molecular patterns that is carried by all microorganisms of a given class (whether pathogenic or commensal). PAMPs are defined in the sense that the host has evolved a corresponding set of receptors (known as pattern-recognition receptors) that

detect their presence. The second class of microbial inducer comprises a variety of virulence factors and is therefore restricted to pathogens. In contrast to PAMPs, they are not sensed directly by dedicated receptors. Instead, the effects of their activity, particularly their adverse effects on host tissues, are responsible for triggering the inflammatory response (Medzhitov, 2008).

Endogenous inducers of inflammation: The inducer of inflammation is classified endogenous if the signals that initiate the inflammatory response originate from within the organism or system (example include stressed or malfunction tissues).

2.2.1.2 Sensors of inflammation

The inflammatory inducers activate specialized sensors which detects and recognize invading pathogens or tissues damage. There is no clear-cut definition for inflammatory sensors. The sensors may sometimes be receptors or messengers. The inducers in most cases determine what sensor to respond. For example, the pore-forming exotoxins produced by Gram positive bacteria are detected by the NALP3 (NACHT-, leucine-richrepeat- and pyrin-domain-containing protein) inflammasome, which is sensitive to the efflux of K^+ ions that results from pore formation. Similarly, the proteolytic activity of proteases produced by helminthes is sensed by basophils by an unknown sensor. Many damage signals are recognized by germ-line encoded receptors, such as transmembrane Toll-like receptors and intracellular nucleotide binding domain and leucine-rich-repeat-containing receptors (NOD-like receptors) (Medzhitov, 2008; Proell *et al*., 2008; Ashley *et al*., 2012).

2.2.1.3 Mediators of inflammation

Inducers of inflammation trigger the production of numerous inflammatory mediators, which in turn alter the functionality of many tissues and organs. Many of these inflammatory mediators have effects in common on the vasculature and on the recruitment of leukocytes. Inflammatory mediators can be classified into seven groups according to their biochemical properties; they are: vasoactive amines (e.g. histamine and serotonin), vasoactive peptides (e.g. sub-P), fragments of complement components (anaphylatoxins), lipid mediators (e.g. eicosanoids and platelet-activating factors), cytokines (e.g. tumour-necrosis factor-α (TNF-α), interleukin (IL)-1 and IL-6), chemokines and proteolytic enzymes (e.g. elastin, cathepsins and matrix metalloproteinases) (Medzhitov, 2008).

2.2.1.4 Effectors of inflammation

The effectors of an inflammatory response are the tissues and cells, the functional states of which are specifically affected by the inflammatory mediators. The inflammatory mediators facilitate the recruitment of effector cells, such as monocytes and neutrophils, to the site of disturbance. The net effect of these interactions culminates in the stereotypical cardinal signs of local inflammation: heat, swelling, redness, pain, and loss of function (Medzhitov 2008; Medzhitov 2010; Ashley *et al*., 2012).

2.2.1.5 Resolution of inflammation

Resolution is the last phase of inflammation, it is critical for limiting collateral damage to the host. After the first few hours of inflammation, a coordinated program of resolution is set into motion by tissue-resident and recruited macrophages. During acute inflammation, these cells produce pro-inflammatory prostaglandins and leukotrienes, but rapidly switch to lipoxins, which block further neutrophil recruitment and instead favor enhanced infiltration of monocytes important for wound healing. The resolution leads to the followings: 1) termination of the inflammatory response (mainly by diminishing granulocyte recruitment and reversing vasodilatation and vascular permeability); 2) switching from pro-inflammatory mediator generation to production of pro-resolution mediators; 3) turning off signaling pathways associated with cytokine production and leukocyte survival; 4) apoptosis of recruited inflammatory cells; 5) phagocyte clearance of apoptotic cells (especially by macrophages in a non-phlogistic process) and; 6) switching from pro-inflammatory cell phenotypes to pro-resolution phenotypes (especially relevant to macrophages) (Serhan and Savill 2005; Medzhitov 2008; Medzhitov, 2010; Ashley *et al*., 2012; Alessandri *et al*., 2013).

Summarily, Inducers are the signals that initiate the inflammatory response. They activate specialized sensors, which then elicit the production of specific sets of mediators. The mediators, in turn, alter the functional states of tissues and organs (which are the effectors of inflammation) in a way that allows them to adapt to the conditions indicated by the particular inducer of inflammation. After few hours of inflammation, macrophages will set in a coordinated program of resolution, in which there will be transformation from pro-inflammatory to anti-inflammatory mediators, from prostaglandins to lipoxins and finally from inflammation to resolution.

2.2.2 Anti-inflammatory drugs

Drugs used for the management of inflammation include the NSAIDs and the steroids. Many steroids, specifically glucocorticoids and mineralocorticoids reduce inflammation or swelling by binding to corticoid receptors. These drugs are often referred to as corticosteroids. Long-term corticosteroids use has several severe side effects e.g. hyperglycemia, insulin resistance, diabetes mellitus, osteoporosis, anxiety effects etc. (Donihi *et al.,* 2006).

Mode of anti-inflammatory activity of NSAIDs has earlier being explained. Other mechanisms that may contribute to NSAIDs anti-inflammatory activity include the reduction of superoxide radicals, induction of apoptosis, inhibition of adhesion molecule expression, decrease of nitric oxide synthase, decrease of pro-inflammatory cytokine levels (e.g. tumor necrosis factor-α, interleukin-1), modification of lymphocyte activity and alteration of cellular membrane functions (Modi *et al.,* 2012).

2.3 Cytokines

Cytokines are a family of glycosylated or non- glycosylated polypeptides and proteins. They are soluble hormone-like proteins that allow for communication between cells and the external environment (Tayal and Kalra, 2008). They are secreted by cells in response to a stimulus which modulates the behavior of target cells (Dixon and Philips; 1993).Their secretion is typically transient (Joseph *et al.,* 2002). The cytokine network is a complex and dynamic system, involved in numerous biological responses in the human body

(Joseph *et al.*, 2002). They function as intercellular chemical messengers. They are crucial to innate and adaptive inflammatory responses, cell growth and differentiation, cell death, angiogenesis and developmental as well as repair processes (Oppenheim, 2001). Cytokines include lymphokine (cytokines made by lymphocytes), monokine (cytokines made by monocytes), chemokine (cytokines with chemotactic activities) and interleukin (cytokines made by one leukocyte and acting on other leukocytes). Their actions may include acting on the cells that secrete them (autocrine action), on nearby cells (paracrine action) or in some instances on distant cells (endocrine action)(Zhang and An, 2007).

2.3.1 Pathophysiological significance of cytokines

Cytokine levels differ dramatically from baseline in acute and chronic pathological conditions. In many disease states, marked local inflammatory responses cause cytokines to spill into general circulation, resulting in detectable levels in biological fluids, such as serum and plasma. Changes in the circulating levels of these proteins have been linked to many disease states, making them valuable functional biomarkers. Excessive or diminished cytokine levels are associated with many clinical conditions and diseases. Such conditions include; CNS disorders, autoimmunity, cardiac diseases, fibromyalgia, toxicity, diabetes, bacterial infections, viral infections, tumours, allergies and asthma (Khare and Khare, 2014).

2.3.2 Cytokines, pain and inflammation

Pro-inflammatory cytokines are produced predominantly by activated macrophages and are involved in the up-regulation of inflammatory reactions. Various evidences have shown

that certain pro-inflammatory cytokines (e.g. IL-1β and TNF-α) are involved in the process of pathological pain (Zhang and An, 2007).

IL-1β is released primarily by monocytes and macrophages as well as by non-immune cells, such as fibroblasts and endothelial cells, during cell injury, infection, invasion and inflammation. It has also been found that IL-1β is expressed in nociceptive dorsal root ganglion (DRG) neurons (Copray *et al.,* 2001). IL-1β has been found to increase the production of sub-P and prostaglandin E_2 (PGE_2) in a number of neuronal and glial cells (Jeanjean *et al.,* 1995; Schweizer *et al.,* 1988). IL-1 receptor antagonist (IL-1ra), a specific IL-1ra, competitively binds to the same receptor as IL-1β but does not transduce a cellular signal, thereby blocking IL-1β-mediated cellular changes. Administrations of IL-1ra and other anti-inflammatory cytokines have been demonstrated to prevent or attenuate cytokine mediated inflammatory hyperalgesia (Maier *et al.,* 1993) and nerve-injury induced mechanical allodynia (Sweitzer *et al.,* 2001). Increased IL-1β production is associated with sepsis, type-2 diabetes, leukemia, atherosclerosis, schizophrenia, depression, sleep disorders, colitis, periodontitis, rheumatoid arthritis, myasthenia gravis and other inflammatory disease (Khare and Khare, 2014).

IL-6 has both pro and anti-inflammatory properties (Matthews *et al*., 2010; Shaikh, 2011; Scheller *et al*., 2011). Its pro-inflammatory activities include contributing to the development of neuropathic pain behavior (Ramer *et al.,* 1998). In addition, intrathecal infusion of IL-6 induces tactile allodynia and thermal hyperalgesia in intact and nerve-

injured rats, respectively (Zhang and An 2007). But it is said to possess more of anti-inflammatory properties (Matthews *et al*., 2010; Shaikh, 2011).

TNF-α, plays a well-established key role in some pain models; TNF acts on several different signaling pathways through two cell surface receptors, TNFR1 and TNFR2 to regulate apoptotic pathways, nuclear factor-κB (NF-κB) activation of inflammation, and activate stress-activated protein kinases. Intraplantar injection of TNF-α also produces mechanical (Cunha *et al.*, 1992) and thermal hyperalgesia (Perkins and Kelly 1994). TNF-α plays a pivotal role in the pathogenesis of various diseases which include bacterial infection, viral replication, septic shock, rheumatoid arthritis, multiple sclerosis, celiac disease, type 1 and type 2 diabetes, crohn's disease, systemic lupus erythematosus, depression - schizophrenia - other inflammatory autoimmune and diseases (Khare and Khare, 2014).

2.4 Traditional Medicine

Traditional medicine as defined by the World Health Organization (WHO) is the sum total of all the knowledge, beliefs and practices that are used in diagnosis, prevention and elimination of physical, mental or social imbalance and rely exclusively on practical experiences and observation handed down from generation to generation (WHO, 2002). The elements of traditional medicines include therapies such as herbal medicine, massage, homeopathy, mud bath, music therapy, wax bath, reflexology, dance therapy, hydrotherapy, mind and spirit therapies, self-exercise therapies radiation and vibration,

osteopathy, chiropractic, aromatherapy, preventive medicine, radiant heat therapy, therapeutic fasting and dieting spinal manipulation, psychotherapy, etc (Adeshina, 2008).

The use of medicinal plants in curing diseases is as old as man. Since the beginning of human civilization, medicinal plants have been used by mankind for its therapeutic value (Aibinu *et al*., 2007). The medicinal plants which are found in our environment enjoy wide acceptability by the population and serve as cheaper alternatives to orthodox medicine. Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from natural sources. Many of these isolations were based on the uses of the agents in traditional medicine. Plant derived natural products such as flavonoids, terpenes and alkaloids have received considerable attention due to their diverse pharmacological properties including anti-inflammatory, antipyretic and analgesic activities (Shukla *et al*., 2010). Consumption of natural products reduces the risk of developing pathological conditions, including cancer, nervous system disorders, cardiovascular, genetic and inflammatory diseases. Plants contain numerous bioactive molecules that can improve the body's resistance to cellular stress and prevent the cytotoxicity of various agents (Iwalewa *et al*., 2007; Newman and Cragg, 2007; Jurenka, 2009).

Natural products and their derivatives have traditionally been the most common sources of drugs and still represent a fairly large percentage of the pharmaceutical market. It has long been recognized that natural product structures have the characteristics of high chemical diversity, biochemical specificity and other molecular properties that make them

favourable as lead structures for drug discovery (Okoye and Osadebe, 2010). It is believed to be the most abundant, affordable, reliable, trusted and well understood form of health care in virtually all African villages (Abalaka *et al*., 2009) and 80% of African populations use some form of traditional herbal medicine (WHO, 2002; Willcox and Bodeker, 2004; Owolabi *et al*., 2007).

Before the advent of orthodox medicine, African people relied on herbs growing in and around them to take care of their health problems and in some cases, as a simultaneous source of food (Abalaka *et al*., 2009). Orthodox medicine somewhat minimized the herbal health care system but the development of resistance against orthodox medicine by pathogens, high costs as well as the lack of availability of some of these drugs has, in recent times, begun to reverse this trend (Awodele *et al.,* 2012). This development is fortified by the notion that all herbal products are safe, effective and have minimal or no side effects (Adam *et al.,* 2011).

2.4.1 Medicinal plants reported to have analgesic and anti-inflammatory activities

Many plants have been scientifically evaluated to possess analgesic and anti-inflammatory properties. These plants include; *Newbouldia laevis* (Usman *et al.,* 2008), *Tacazzea apiculata* (Ahmed, *et al*., 2007), *Ganoderma applanatum* (Ede *et al*., 2012), *Tamarindus indica* (Ukwuani and Hassan, 2014), *Vitis vinifera* (Singh *et al*., 2009, *Kalanchoe pinnata* (Matthew *et al*., 2013), *Carissa edulis* (Hassan *et al*., 2010), *Cassia occidentalis* (Vijayabhaskar *et al*., 2013), *Securinega virosa* (Yerima *et al.,* 2009), *Ficus ingens* (Aiyelero *et al.,* 2009), *Argyreia argentea* (Rahman *et al.,* 2010), *Zingiber officinale*,

Ananas comosus, *Calotropis procera*, *Mangifera indica* and *Sida cordifolia* (Anilkumar, 2010), *Dalbergia saxatilis* (Ismail *et al.,* 2015) etc.

2.4.2 Plants that have contributed to the development of modern analgesic and anti-inflammatory drugs

Over the years, natural products have contributed enormously to the discovery of drugs for use in modern medicine. It is estimated the about 40% of all medicines in the market today have been derived from natural sources, 25% being from plants, 13% from microorganisms and 3% from the animals.From the principal plants that have contributed to the development of modern analgesic and anti-inflammatory drugs are; *Papaver seminiferum, salix specie* (with over 350 species among which is *Salix alba*), *cannabis sativa* (with over 60 cannabinoids; some of which are analgesic and anti-inflammatory agents), *Capsicum specie* (e.g *C. plaster*, *C. annuum*),*Panax ginseng, tanacetum parthenium, Acotinum specie, Siphocampylus verticillatus, Drymis winteri, Hedyosmum brasiliense, Phyllanthus species, Protium species* etc (Calixto *et al.,* 2009).

There are over 120 distinct chemical substances derived from plants that are considered important drugs currently in use in one or more countries in the world. Among these drugs are potent analgesic and anti-inflammatory agents; they include aesin (derived from *Aesculus hippocastanum*), borneol (from several *Artemisia* species), bromelain (from *Ananas comosus*), codeine and morphine (both are from *Papaver somniferum*), rotundine (from *Stephania sinica*) and tetrahydropalmatine (from *Corydalis ambigua*) (Calixto *et al.,* 2009).

2.4.3 *Olax subscorpioidea* Oliv

Olax subscorpioidea Oliv belongs to the family Olacaceae. Olacaceae is a family of 25 genera and over 250 species (Hua-xing and Gilbert, 2003). *O. subscorpioidea* grows freely and its wildlings usually preserved (Kayode *et al.,* 2008). It grows up to 10 m high and bole to 60 cm girth with long thin, often drooping branches (Burkill, 1997; Ayandele and Adebiyi, 2007). It is a shrubby plant commonly found in tropics, especially Africa. It is widely distributed in Nigeria, Democratic Republic of Congo (previously known as Zaire) and Senegal (Ayandele and Adebiyi, 2007).

2.4.3.1 Botanical classification

- Kingdom: Plantae
- Subkingdom: Tracheobionta
- Superdivision: Spermatophyta
- Division: Magnoliophyta
- Class: Maagnoliopsida
- Subclass: Rosidae
- Order: Santalales
- Family: Olacaceae
- Genus: *Olax*
- Specie: *subscorpioidea*

It is known with various local names. Such names include *Ifon, Ufon* (Yoruba), *Gwàànón kùrmìì, Gwàànón rààfìì*(Hausa), *Igbulu, Atu,-ogili, Osaja* (Igbo) *Ukpakon* (Edo) (Okoli *et*

al., 2007), *Mtungapwezi* (Swahili), *Aziza* (Nsukka) (Ukwe *et al.*, 2010) and *Ocheja* (Igala).Traditionally, it is widely employed in the treatment of various ailments (Burkill, 1997). The roots has been used to reduce pregnancy associated fat, constipation (Okoli *et al*., 2007), management of cancer (Soladoye *et al*., 2010) and rheumatism (Ogunmefun and Gbile 2012). The leaf has been used for yellow fever, jaundice, venereal diseases and guinea worm infestation (Okoli *et al*., 2007). The leaf decoction is used by the Igala people of Kogi State, North Central Nigeria in the management of swelling and pains (Bamidele Dada, Herbalist; personal communication, 2012). Scientifically, the root of *O. subscorpioidea* has been reported to possess anti-ulcer properties (Ukwe *et al*., 2010), anthelmintic (Koné *et al*., 2012) and the stem possess antimicrobial activities (Ayandele and Adebiyi, 2007). The leaf has been reported to possess antimalaria activities (Kipre *et al*., 2009; Kipre *et al*., 2015), hepatoprotective and antioxidant (Konan *et al.*, 2013; Konan *et al.*, 2015) properties. Preliminary phytochemical studies show that the stem of *O. subscorpioidea* contains alkaloids, flavonoids and steroids (Ayandele and Adebiyi, 2007) and the root contains glycosides, alkaloids, steroids and terpenoids (Ukwe *et al*., 2010).



Figure 2.5: *Olax subscorpioidea* in its Natural Habitat

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Animals

Adult Wistar rats (120-170g) and Swiss Albino mice (20-30g) of both sexes were obtained from the Animal House Facility of the Department of Pharmacology and Therapeutics, Ahmadu Bello University, Zaria, Nigeria. The animals were maintained under standard environmental conditions (light/dark cycle) and fed with standard rodent pellet diet and water *ad libitum.* All the experiments (with the exception of the determination of inflammatory cytokines) were carried out in the Main Laboratory of the Department of Pharmacology and Therapeutics, Ahmadu Bello University, Zaria; the determination of inflammatory cytokines in the carrageenan induced paw oedema was carried out in the Research Laboratory of the Department of Pharmacology, Therapeutics and Toxicology, University of Lagos, Idi-Araba Lagos, Nigeria. The experiments were carried out in accordance with the criteria outlined in the Guide *for the Care and Use of Laboratory Animals* by the National Institute of Health (Publication No. 80-23, revised 1996) and were approved by the Departmental Animal Ethic Committee of the Department of Pharmacology and Therapeutics, Ahmadu Bello University, Zaria, Nigeria (DAC/IW-OT/137/14).

3.2 Materials and Equipment

The materials and equipments used for the studies include digital weighing balance (AE240 dual range, Mettler instrument corporation, USA), digital vernier calliper

(Battenfeld Technologies Inc. USA), electric hot plate (DB-1A, Life Assistance Scientific, U.K), animal cages, dissecting kits, stop watch, observation chamber, funnel, cotton wool, desiccators, water bath, mortar and pestle, scissors, syringes (1ml, 2ml and 5ml), Petri dish, cannula, hand gloves, eppendorf tubes, sample bottles and laboratory record book.

3.3 Chemicals/Reagents

Drugs and chemicals used for the studies include glacial acetic acid (May and Baker limited, Dagenham, England), methanol, hexane, butanol, ethyl-acetate, carrageenan, tween 80, formalin solution,chloroform (Sigma Aldrich, St. Louis Mo, USA), l-arginine, naloxone hydrochloride, metergoline, prazosin, glibenclamide, yohimbine hydrochloride, nomega-nitro-l-arginine (L-NNA), propranolol (Abcam Plc, Cambridge, UK), morphine sulphate (Martindale Pharmaceuticals, U.K), ketamine hydrochloride (RotexMedica, Germany), acetylsalicylic acid (Bayer, Leverkusen, Germany), hydrochloric acid, sulphuric acid (May and Baker, UK), ferric chloride anhydrous (Avishkar, India), ammonia (Lobachemie, India), normal saline and distilled water were used in the study, all the reagents were of analytical standard grade.

3.4 Collection and Identification of Plant Material

The plant samples were collected from a farm in the premises of College of Health Sciences, Kogi State University, Anyigba, Kogi State, North Central Nigeria, in March 2013. The sample was identified by a taxonomist, Dr Emmanuel I. Aigbokhan of the Department of Biological Sciences, Faculty of Natural Sciences, Kogi State University,

Anyigba, Kogi State, Nigeria, where a voucher specimen number (KSUH-277-2013-01) was deposited for future references.

3.5 Extraction and Fractionationof Plant Material

The extraction and fractionation was carried out according to the method described by Kupchan*et al*. (1973). The leaves of *O. subscorpioidea* were shade dried until constant weight was obtained and then reduced into fine powder with the aid of a mortar and pestle. One kilo gram (1kg) of the powdered leaf material was extracted exhaustively with aqueous-methanol (80% methanol in water) using continuous soxhlet apparatus for 48 hr. The solvent was removed by placing the extract on water bath set at 50°C, then the extract was placed in a bottle container and stored in a desiccator prior to use; it is henceforth referred to as methanol leaf extract of *Olax subscorpioidea* (MEOS).Solutions of the extract were prepared freshly with distilled water for each study. The percentage yield of the extract was calculated using the formula:

$$\text{Percentage yield} = \frac{\text{weight of extract}}{\text{weight of powdered material}} \times 100$$

One hundred grams (100 g) of MEOS was suspended in water (500 ml) and successively partitioned with hexane (5 × 500 ml), ethyl-acetate (5 × 500 ml) and butanol (5 × 500 ml) to afford the corresponding fractions.The hexane and ethyl-acetate were evaporated to dryness while the butanol and residual aqueous were removed by placing the fractions on water bath set at 50°C. Each of the fractions was placed in a container and stored in a desiccator prior to use. Solutions of the fractions were prepared freshly with distilled water for each study. The percentage yield for each of the fractions was calculated using the above formula.

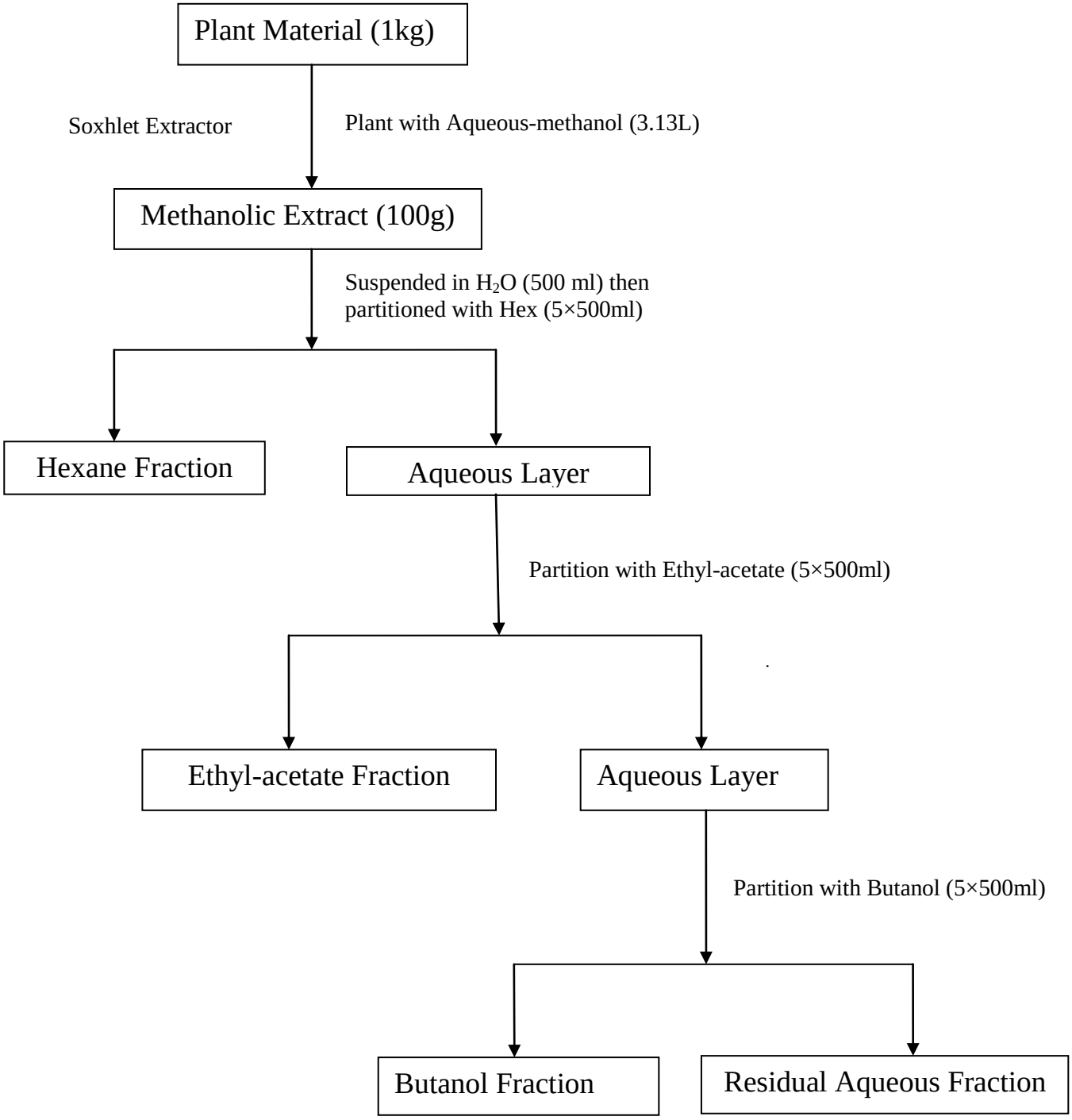

Plant Material (1kg)
Soxhlet Extractor
Plant with Aqueous-methanol (3.13L)
Methanolic Extract (100g)
Suspended in H_2O (500 ml) then partitioned with Hex (5×500ml)
Hexane Fraction
Aqueous Layer
Partition with Ethyl-acetate (5×500ml)
Ethyl-acetate Fraction
Aqueous Layer
Partition with Butanol (5×500ml)
Butanol Fraction
Residual Aqueous Fraction

Figure 3.1: Extraction and Fractionation Chart

3.6 Qualitative Phytochemical Analysis

The MEOS and fractions were subjected to phytochemical screening in accordance with standard protocol as described by Sofowora (2008) and Evans (2009).They were screened for the presence of alkaloids, flavonoids, saponins, cardiac glycosides, tannins, anthraquinones, carbohydrates, steroids and triterpenes.

3.6.1 Test for alkaloids

The MEOS and each of its fractions were individually dissolved in 2 N HCl. Each of the mixtures was filtered and the filtrate was divided into 3 equal portions. Each of the portions was further treated with different reagents.Few drops of Dragendorff's reagent were added to the first portion; an orange precipitate indicates the presence of alkaloid.Another portion of the filtrate was treated with equal amount ofWagner's reagent; a reddish brown precipitate solution indicates the presence of alkaloid.The last portion was treated with equal amount ofMayer's reagent; a white or cream precipitate indicates the presence of alkaloids.

3.6.2 Test for flavonoids(Shinoda and sodium hydroxide tests)

About 0.5 g of the MEOS and each of its fractions was dissolved in 2 ml of 50% methanol. Few magnesium chips and 3 drops of hydrochloric acid were added and the pink or tomato red colour within few minutes indicates the presence of flavonoids. Also, few drops of aqueous sodium hydroxide were added to about 5 ml solution of MEOS or each of its fractions; a yellow colouration indicates the presence of flavonoids.

3.6.3 Test for saponins(frothing test)

Small quantity of MEOS and each of its fractions was individually dissolved in 5 ml of distilled water and shaken vigorously in a test tube for 30 seconds and allowed to stand for 30 minutes. The occurrence of frothing column or honey comb-like for of at least 1 cm in height and persisting for at least 15 min indicates the presence of saponins.

3.6.4 Test for cardiac glycosides(Kella-killiani test)

Two milliliters (2 ml) of MEOS or each of the fractions was dissolved in glacial acetic acid containing 5% ferric chloride ($FeCl_3$) and 1 ml of sulphuric acid (H_2SO_4) was added to the solution; the appearance of reddish-brown, greenish blue color within few minutes or formation of pure ring colour indicates the presence of cardiac glycoside.

3.6.5 Test for tannins(ferric chloride test)

Ten milliliters (10 ml) of distilled water was added to 0.5 ml of MEOS or each of the fractions' solution and then filtered; few drops of $FeCl_3$ solution were added to the filtrates; a blue or green precipitate indicates the presence of tannins.

3.6.6 Test for triterpenes (Lieberman-Burchard test)

One milliliter (1 ml) of acetic anhydride was added to 1 ml of MEOS or each of the fractions' solution. Few drops of sulphuric acid were then added to the solution. A blue green ring indicated the presence of triterpenes.

3.6.7 Test for steroids(Salkowski test)

Two milliliters (2 ml) of chloroform and few drops of sulphuric acid were carefully added to about 2 ml of MEOS or each of the fractions' solutions from the side of the test tube to form a lower layer. Appearance of reddish brown ring indicated the presence of steroids.

3.6.8 Test for anthraquinones(Bontrager test)

Five milliliters (5 ml) of chloroform was added to MEOS or each of the fractions and was shaken for at least 5 min. This was filtered and the filtrate shaken with equal volume of 10% ammonia solution. A bright pink colour in the aqueous (upper) layer indicates the presence of anthraquinones.

3.6.9 Test for carbohydrates(Molish test)

Few drops of Molish reagent were added to MEOS or each of the fractions, and then concentrated acid was added down the side of the test tube to form a lower layer. A reddish coloured ring at the interphase signifies the presence of carbohydrates.

3.7 Acute Toxicity Studies

The oral and intraperitoneal median lethal doses (LD_{50}) of MEOS and its fractions in rats and mice were estimated according to the method of Lorke (1983). Briefly, the method is divided into two phases, in the first phase, 3 groups of 3 animals each (mice/rats) were administered the MEOS or each of the fractions 10, 100 and 1,000 mg/kg. The animals

were observed for signs of toxicity and death for the first 4 hours and intermittently for 24 hours. In the second phase, 3 animals were each administered graded doses of MEOS or (each of the) fractions based on the result from the first phase and were also observed for signs of toxicity and death for the first 4 hours and intermittently for 24 hours. The LD_{50} value was estimated by calculating the geometric mean of the lowest dose that caused death and the highest dose for which the animals survived.

$$LD_{50} = \sqrt{(\text{Highest non lethal dose}) \, X \, (\text{Lowestlethal dose})}$$

3.8 Evaluation of Analgesic Activities in Mice

3.8.1 Acetic acid induced writhing in mice

The method previously described by Koster *et al.* (1959) was adopted for this study. Randomly selected groups of mice (n=6) were orally administered with distilled water (10 ml/kg), MEOS, residual aqueous or butanol fraction(250, 500 and 1,000 mg/kg), hexane fraction (150, 300 and 600 mg/kg) and acetylsalicylic acid (ASA, 300 mg/kg). Sixty minutes after oral administration, acetic acid 0.6% $^{v}/_{v}$ (10 ml/kg) was administered to each mouse and was placed in observation cage. Five minutes after acetic acid injection the number of writhes was counted for each mouse for a period of 10 minutes. A reduction in the number of writhes as compared to the vehicle treated animals was considered as evidence for the presence of analgesia and expressed as percent inhibition of writhes.

Percentage Inhibition (%) =

$$\frac{\text{Mean No. of writhes (control)} - \text{Mean No. of writhes (Test)}}{\text{Mean No. of writhes (control)}} \times 100$$

3.8.2 Formalin-induced pain test in mice

The method of Dubuisson and Dennis (1977) and modified by Tjølsen *et al*. (1992) was adopted in this study. Randomly selected groups of mice (n=5) were orally administered distilled water (10 ml/kg), MEOS (250, 500 and 1,000 mg/kg)and morphine (10 mg/kg). Sixty minutes post treatment, 20 µl of freshly prepared 2.5% formalin in saline was injected subcutaneously into the right hind paw of each mouse. The mice were placed individually in an observation chamber and monitored. The severity of pain response was recorded based on the following scale:

0= the mouse walked or stood firmly on the injected paw,

1= the injected paw was favoured or partially elevated,

2= the injected paw was clearly lifted off the floor and

3= the mouse licked, chewed or shook the injected paw.

Anti-nociceptive effect was determined in two phases,the early phase (0-5 min) and the late phase (15-60 minutes) with a 10 minutes lag period in-between both phases.

In a separate experiment, randomly selected groups of mice (n=6) were orally administered distilled water (10 ml/kg), residual aqueous or butanol fraction(250, 500 and 1,000 mg/kg), hexane fraction (150, 300 and 600 mg/kg) and morphine (10 mg/kg). Sixty minutes post treatment, 20 µl of freshly prepared 2.5% formalin in saline was injected subcutaneously into the right hind paw of each mouse. The mice were placed individually in an observation chamber and monitored and the time (sec) spent in licking or biting the injected paw, indicative of pain, was recorded. The responses of the mice were observed for the first 5 min (early phase) and 15–40 minutes (late phase) post formalin injection (Khanavi *et al*., 2012).

3.8.3 Hot plate test in mice

The method of Eddy and Leimbach (1953) was adopted. Randomly selected groups of mice (n=6) were orally administered distilled water (10 ml/kg), MEOS, residual aqueous or butanol fraction (250, 500 and 1,000 mg/kg), hexane fraction (150, 300 and 600 mg/kg) and morphine(10 mg/kg).Mice were individually placed on a hot plate (45 ± 1°C), before drug treatment so that each animal serves as its own control. The time until the animal either licked the paw, fluttered any of the paws or jumped was taken as reaction time and recorded. A cut-off time of 20 sec was used to avoid paw tissue damage. The latency was observed and recorded after 60, 90, 120 and 150 minutes. The prolongation of the latency times was taken as an analgesic response (per cent maximum possible effect {%MPE}).

$$\%MPE = \frac{Test - Baseline}{Cutoff - Baseline} X\ 100$$ Where;

Test = latency to respond after treatment

Baseline = latency to respond prior to treatment and

Cut-off (20 sec) = preset time at which the test was ended in the absence of a response.

3.9Evaluation of Anti-inflammatory Activities

3.9.1 Acute anti-inflammatory study in rats

The acute anti-inflammatory study wascarried out using the carrageenan induced paw oedema in rats method as previously described by Winter *et al*. (1962). Randomly selected groups of rats (n=6) were orally administered distilled water (1 ml/kg), MEOS, residual

aqueous or butanol fraction (250, 500 and 1,000 mg/kg), hexane fraction (150, 300 and 600 mg/kg) and ASA (300 mg/kg). Sixty minutes post treatment, each rat was injected with 0.1 ml of 1% carrageenan into plantar surface of rat right hind paw. The hind paw oedema was measured and recorded at times 0, 1, 2, 3, 4 and 5 hours using vernier caliper to determine the diameter of the oedema. The increase in paw diameter (oedema index) for each rat was calculated as the difference in paw diameter before carrageenan injection and after carrageenan injection at each time interval, while the percent inhibition of oedema was calculated for each group with respect to its vehicle-treated control group using the following relationship:

$$\frac{\text{Mean increase in paw volume of control} - \text{Mean increase in paw volume of treated}}{\text{Mean increase in paw volume of control}} \times 100$$

3.9.2 Sub-acute anti-inflammatory study in rats

The sub-acute anti-inflammatory study wascarried out using the cotton pellet induced granuloma formation in rats method as previously described by Winter and Porter (1957).Male rats were randomly divided into four groups (n=5), the groin region of each rat was shaved under aseptic conditions, sterile pre-weighed cotton pellet (50 mg) soaked in 0.2 mL of distilled water containing penicillin (0.1 mg) and streptomycin (0.13 mg) was implanted subcutaneously in the groin under ketamine (15 mg/kg) anesthesia through a single needle incision. After the implantation of the cotton pellets, rats were orally administered distilled water (1 ml/kg), residual aqueous/butanol fraction (1,000 mg/kg) and ASA(300 mg/kg) for 9 consecutive days from the day of cotton pellet implantation. On day 10, the pellets were dissected out, weighed and oven-dried at 60 °C and the dried weights were determined. The weight of the cotton pellet before implantation was

subtracted from the weight of the wet and the weight of the dried granuloma pellets. The increase in the pellet weight was considered as granuloma tissue deposit.

3.9.3 Investigating the involvement of inflammatory cytokines in the anti-inflammatory activities of *Olax subscorpioidea*

The method previously described by Santos *et al*. (1999) was used to investigate the role of inflammatory cytokines. Four randomly-selected groups of rats (n=6) were orally administered distilled water 1ml/kg, residual aqueous/butanol fraction 1,000 mg/kg and ASA 300 mg/kg. Sixty min post treatment, each rat was injected with 0.1 ml of 1% carrageenan into plantar surface of the rat's right hind paw (Winter *et al*., 1962).4 hours after carrageenan injection rats were anaesthetized using chloroform and the hind paws were removed at the level of the calcaneus bone. The paws were homogenized using phosphate buffered saline (PBS) and centrifuged at 10,000 revolutions per minute for 10 minutes at 4°C before collecting the exudates (oedema fluid). The concentrations of inflammatory cytokines (in the exudates) were measured using rat cytokine 27-plex discovery assay by Eve Technologies (Calgary, Alberta, Canada). The cytokines determined includes TNF-α,IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10 and IFN-γ.

3.10 Investigation of Mechanisms of Analgesic Activities

The most active fraction of *Olax subscorpioidea*extract was subjected to mechanistic studies.The participation of various pain pathways in the analgesic activitiesthe butanol fraction (BFOS)was investigated using mouse model of acetic acid induced writhing as

previously described by Rangel *et al*. (2012). The pathways investigated and the reagents used for the studies were as follows:

a) opioidergic, using naloxone (a nonselective opioid receptor antagonist, 2 mg/kg, *i.p*),

b) α_1-adrenergic, using prazosin (an α_1-adrenoceptor antagonist, 1 mg/kg, *i.p*),

c) α_2-adrenergic, using yohimbine (an α_2-adrenoceptor antagonist, 1 mg/kg, *i.p*),

d) β-adrenergic, using propranolol (a non-selective β-adrenergic blocker, 20 mg/kg, *i.p*)

e) serotonergic, using metergoline (a non-selective serotonin receptor antagonist, 2 mg/kg, *i.p*),

f) potassium ATP, using glibenclamide (a K_{ATP} channel blocker, 5 mg/kg, *i.p*), and

g) nitric oxide-l-arginine using l-arginine (a nitric oxide precursor, 50 mg/kg, *i.p*).

For each of the mechanistic study, mice were randomly divided into 6 groups (n=6) for each of the pathways (except nitric oxide-l-arginine pathway which had 8 groups). All the groups were treated as follows:

Group I: distilled water (10 ml/kg, p.o) alone

Group II: BFOS (1,000 mg/kg, p.o) alone

Group III: morphine (10 mg/kg, p.o) alone

Group IV: receptor antagonist/blocker (reagents and doses mentioned above depending on the pathway involved).

Groups V: pretreated with receptor antagonist/blocker 30 minutes before administering BFOS (1,000 mg/kg, oral).

Group VI: pretreated with receptor antagonist/blocker 30 minutes before administering morphine (10 mg/kg, oral).

Group VII (NO-l-arginine pathway): nomega-nitro-l-arginine (L-NNA, an inhibitor of nitric oxide synthesis, 50 mg/kg, i.p.) alone and

Group VIII: pretreated with l-arginine (50 mg/kg, i.p) 30 min before L-NNA (50 mg/kg i.p).

Sixty minutes post treatment (30 minutes for groups IV, VII and VIII), mice were challenged with acetic acid induced writhing test adopting the method previously described by Koster *et al.* (1959).

3.11 Statistical Analysis

Results were expressed as mean ± standard error of mean and presented as graphs and tables. Data were analyzed using either one way or repeated measures analysis of variance (ANOVA) followed by Dunnett or Bonferroni test for multiple comparison. Kruskal-Wallis followed by Mann Whitney testwas used to analyze the formalin-induced pain where scores were ranked. Results were considered significant when $p \leq 0.05$.

CHAPTER FOUR

4.0 RESULTS

4.1 Percentage Yield of the Leaf Extract and Fractions of *Olax subscorpioidea*

One thousand gram(1,000 g) of the powdered leaf material of *Olax subscorpioidea* afforded 314 g methanol extract (MEOS, 31.4%$^{w}/_{w}$). The MEOS further afforded hexane, ethyl-acetate, butanol and residual aqueous fractions of 28, 2, 26 and 22g respectively(Table 4.1).

Table 4.1: Percentage Yield of the Fractionsfrom 100g of Methanol Extract of *Olax subscorpioidea*

Fractions	**Percentage yield (%)$^{w}/_{w}$**
Hexane fraction	28
Ethyl-acetate fraction	2
Butanol fraction	26
Residual aqueous fraction	22

4.2 Phytochemical Constituents

The results of the preliminary phytochemical screening of the MEOS and its fractions indicated the presence of various phytochemicals such as carbohydrates, cardiac glycosides, tannins, flavonoids, alkaloids, saponins, steroid and triterpenes. Flavonoids was present in MEOS and all its fractions while anthraquinones was absent in MEOS and its fractions. The residual aqueous and butanol fractions lacked steroid and triterpenes, while the ethyl acetate and hexane fractions were devoid of alkaloids, carbohydrates, saponins, tannins and carbohydrates (Table 4.2).

Table 4.2: Phytochemical Constituents of Methanol Extract and Fractions of *Olax subscorpioidea*

Chemical constituents	Methanol extract	Hexane fraction	Ethyl acetate fraction	Butanol fraction	Residual aqueous fraction
Alkaloids	+	-	-	+	+
Anthraquinone	-	-	-	-	-
Steroid and triterpenes	+	+	+	-	-
Cardiac glycosides	+	-	-	+	+
Saponins	+	-	-	+	+
Tannins	+	-	-	+	+
Flavonoids	+	+	+	+	+
Carbohydrates	+	-	-	+	+

+= present, -= absent

4.3 Median Lethal Dose

In the acute toxicity test, the oral median lethal doses (LD_{50}) of MEOS, residual aqueous and butanol fractions were estimated to be greater than 5,000 mg/kg in both rats and mice. Those of the hexane fraction were estimated to be 2,200 and 3,800 mg/kg in mice and rats respectively. The intraperitoneal (*i.p*) LD_{50} of MEOS in mice was estimated to be 3,800 mg/kg; that of the residual aqueous fraction were estimated to be 2,200 mg/kg, while that of the butanol and hexane fractions were found to be 1,300 mg/kg. The *i.p* LD_{50} in rats was estimated to be 3,800 mg/kg in MEOS, residual aqueous and butanol fractions while it was estimated to be 2,200 mg/kg in the hexane fraction.

4.4 Analgesic Activities of *Olax subscorpioidea*

4.4.1 Effect of methanol extract and fractions of *Olax subscorpioidea* on acetic acid induced writhing test in mice

The MEOS and its fractionselicited significant ($p<0.05$,$p<0.01$ and $p<0.001$) and dose-dependent analgesic activities compared to the control. MEOS (250, 500 and 1,000 mg/kg) produced a reduction in the number of writhes with percentage inhibition of 29.65, 51.75 and 65.70% respectively (Figure 4.1). The residual aqueous and butanol fractions (1,000 mg/kg) had their maximal analgesic effectsof 69.76 and 66.86% inhibition of writhes respectively. The hexane fraction (600 mg/kg) produced a significant effect of 37.79% inhibition (Figure 4.2). However, ASA produced significant ($p<0.001$) analgesic activity with percent inhibition of 81.97%.

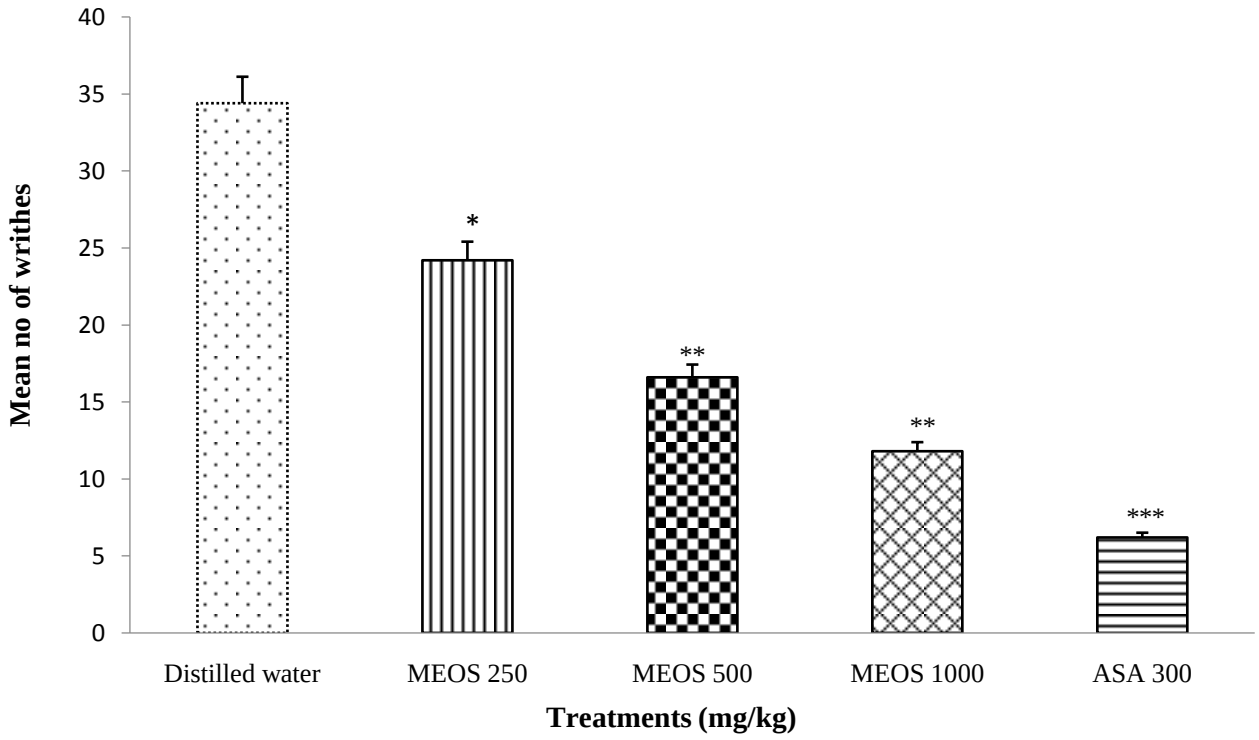


Figure 4.1: Effects of Methanol Extract of *Olax subscorpioidea* on Acetic Acid Induced Writhing Test in Mice

Values presented as Mean ± SEM, * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ versus control (one-way ANOVA followed by Dunnett's post-hoc test), MEOS=Methanol leaf extract of *O. subscorpioidea*,ASA= acetylsalicylic acid, n=6.

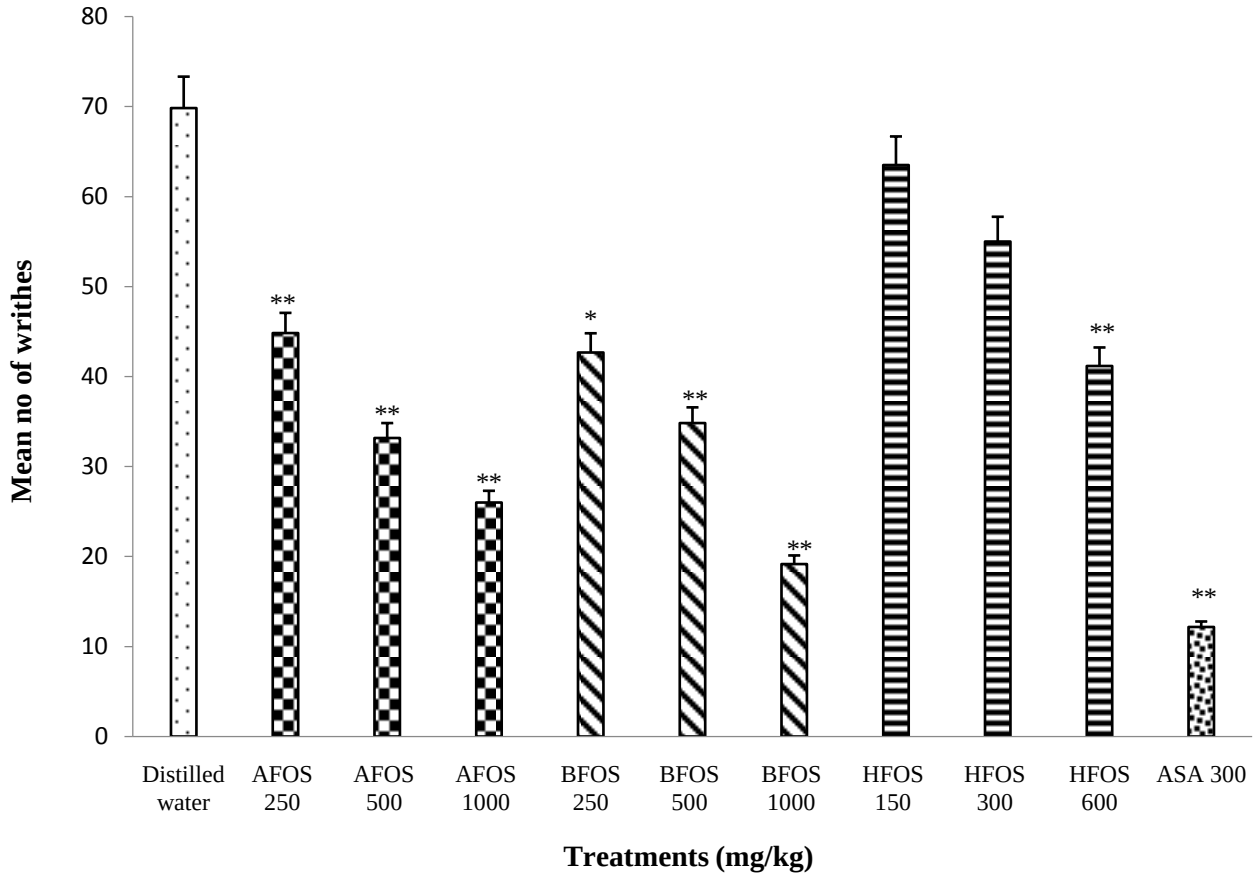


Figure 4.2: Effects of Residual Aqueous, Butanol and Hexane Fractions of *Olax subscorpioidea* on Acetic Acid Induced Writhing Test in Mice

Values presented as Mean ± SEM,* $p<0.01$, ** $p<0.001$ versus control (one-way ANOVA followed by Bonferroni's post-hoc test), AFOS=residual aqueous fraction, BFOS=butanol fraction), HFOS=hexane fraction, ASA=acetylsalicylic acid, n=6.

4.4.2 Effect of methanol extract and fractions of *Olax subscorpioidea* on formalin induced pain test in mice

The MEOS(1,000 mg/kg) significantly ($p<0.05$ and $p<0.01$) inhibited both phases of the formalin induced pain in mice (Table 4.3). The residual aqueous, butanol and hexane fractions also showed significant ($p<0.05$ and $p<0.001$) reduction in the paw licking effect when compared to the control. The residual aqueous and butanol fractions (1,000 mg/kg) exhibited significant reduction in the paw licking effect with percent inhibition of 58.86 and 71.09% in the early phase respectively and 62.7772.56% in the late phase respectively. The hexane fraction (600 mg/kg) produced inhibition of35.42 and 41.04% in the early and late phases respectively. Morphine, produced significant ($p<0.001$) inhibition of both phases (Figure 4.3).

Table 4.3: Effect of Methanol Extract of *Olax subscorpioidea* on Formalin-Induced Pain in Mice

Treatments (mg/kg)	Median Pain Scores	
	Early Phase	**Late Phase**
Distilled water 10 ml/kg	3.0	3.0
MEOS 250	2.0	1.0*
MEOS 500	2.0	1.0*
MEOS 1000	1.0*	1.0**
Morphine 10	1.0**	1.0**

Data presented as Median score, *$p<$ 0.05 **$p<$ 0.01 versus control (Kruskal-Wallis – followed by Mann Whitney test), MEOS= Methanol leaf extract of *O. subscorpioidea*,n=5

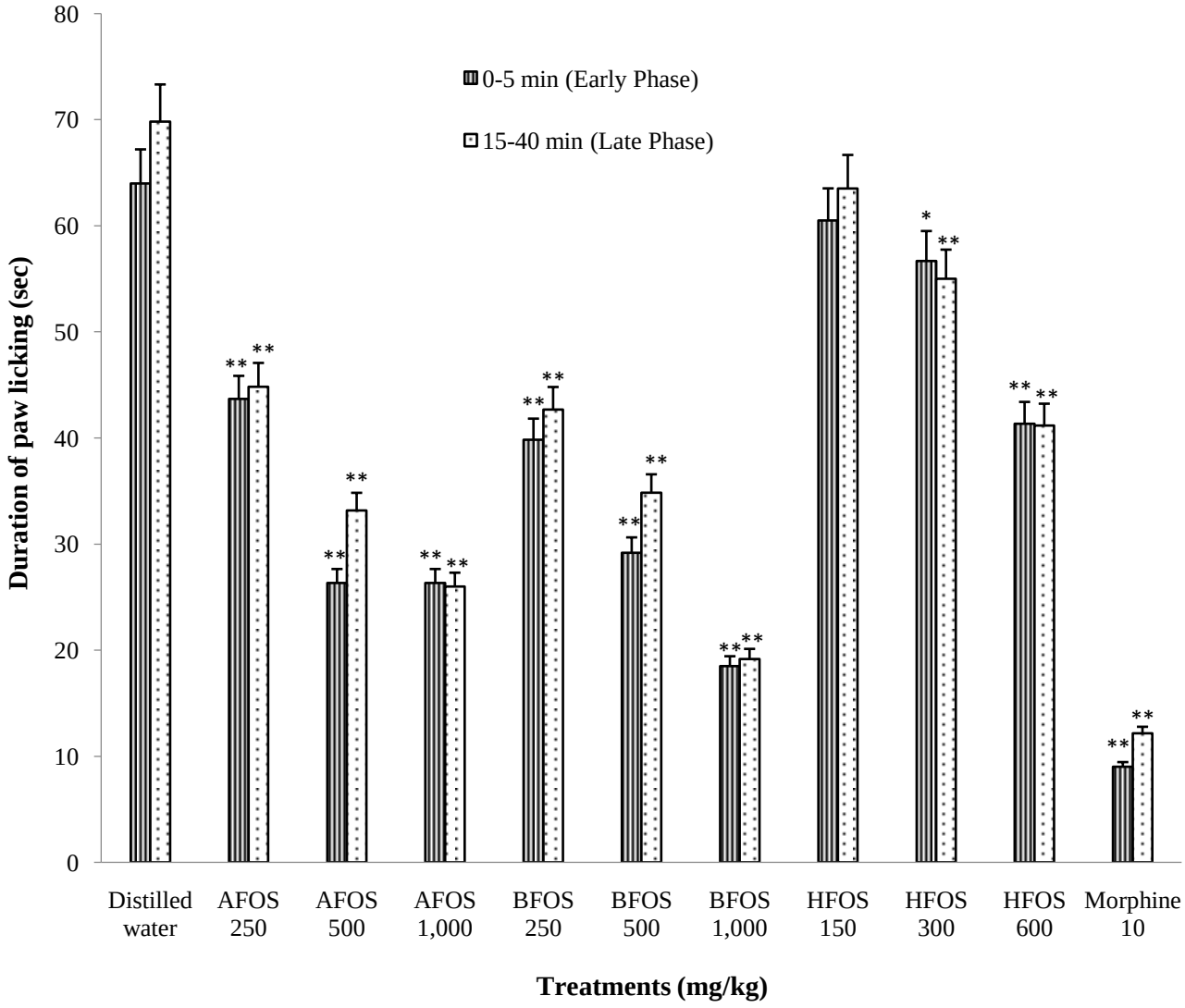


Figure 4.3: Effects of Residual Aqueous, Butanol and Hexane Fractions of *Olax subscorpioidea* on Formalin-Induced Pain in Mice

Values presented as Mean ± SEM,* $p<0.05$, ** $p<0.001$ versus control (one-way ANOVA followed by Bonferroni's post-hoc test), AFOS=residual aqueous fraction, BFOS=butanol fraction, HFOS=hexane fraction, n=6.

4.4.3 Effect of methanol extract and fractions of *Olax subscorpioidea* on hot plate test in mice

The MEOS and its residual aqueous and butanol fractions produced significant ($p<0.05$, $p<0.01$ and $p<0.001$) time and dose-dependent increase in pain latency when compared with the control (Figure 4.4 and Table 4.4). MEOS, residual aqueous and butanol fractions (1,000 mg/kg) produced a peak effect (69.55, 39.98 and 52.99% MPE respectively) at 150 minutes after treatment. However, the hexane fraction did not produce any significant analgesic effect in this model.

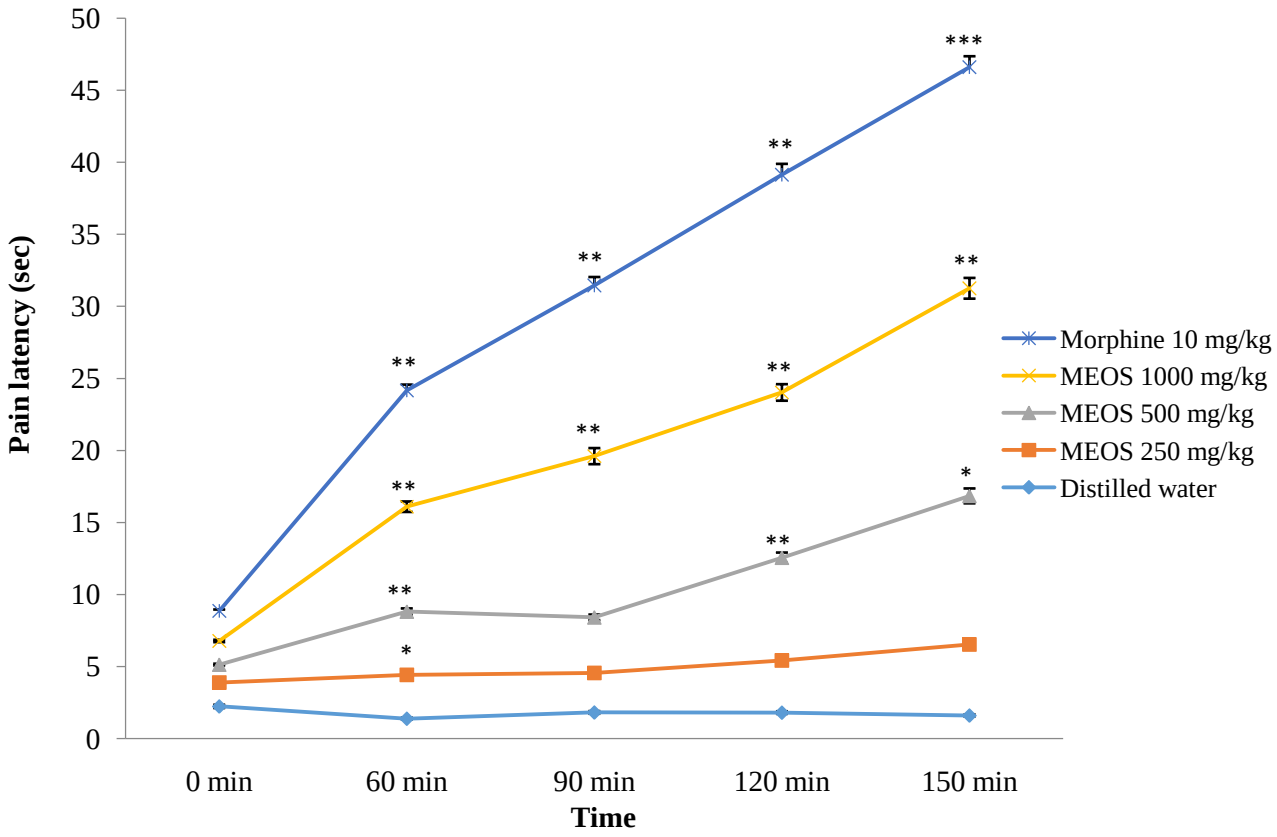


Figure 4.4: Effect of Methanol Extract of *Olax subscorpioidea* on Hot Plate Test in Mice

Values presented as Mean ± SEM, * p<0.05, ** p<0.01, *** p<0.001 versus control (repeated measures ANOVA followed by Bonferroni's post-hoc test), MEOS= Methanol leaf extract of *Olax subscorpioidea*,n=6.

Table 4.4: Effect of *Olax subscorpioidea* Fractions on Hot Plate Test in Mice

Treatments (mg/kg)	Percent Maximum Possible Effect (%MPE)			
	60 min	90 min	120 min	150 min
Distilled water	-	-	-	-
AFOS 250	2.02	8.39	12.86	14.66
AFOS 500	4.46	15.20	28.89	31.31
AFOS 1,000	3.00	12.36	29.08	39.98
BFOS 250	1.96	8.87	9.41	13.93
BFOS 500	6.06	13.25	18.23	27.04
BFOS 1,000	5.70	19.00	27.52	52.99
HFOS 150	4.67	5.32	11.88	10.59
HFOS 300	2.04	4.30	7.01	8.06
HFOS 600	4.41	6.86	8.87	10.88
Morphine 10	33.42	54.46	72.71	74.00

Values presented asPercent Maximum Possible Effect (%MPE),AFOS=residual aqueous fraction, BFOS=butanol fraction, HFOS=hexane fraction, n=6

4.5 Anti-inflammatory Activitiesof *Olax subscorpioidea*

4.5.1 Effect of methanol extract and fractions of *Olax subscorpioidea* oncarrageenan-induced paw oedema in rats

The MEOSand its fractions significantly ($p<0.05$,$p<0.01$and$p<0.001$) decreased paw oedema at all the tested doses with maximum inhibition at the 5th hour. At the 5th hour, the MEOS (250, 500 and 1,000 mg/kg) had 26.24, 41.83 and 47.52% inhibition respectively (Figure 4.5). The residual aqueous (250, 500 and 1,000 mg/kg) fraction evoked 70.54%, 81.86% and 87.87% inhibition respectively.The butanol fraction (250, 500 and 1,000 mg/kg) exhibited 71.04%, 82.12% and 82.43% inhibition respectively. The hexane fraction (150, 300 and 600 mg/kg) produced 61.39%, 64.36% and 67.33% inhibition respectively. The standard drug, ASA (300 mg/kg) had 69.55% inhibition (Table 4.5).

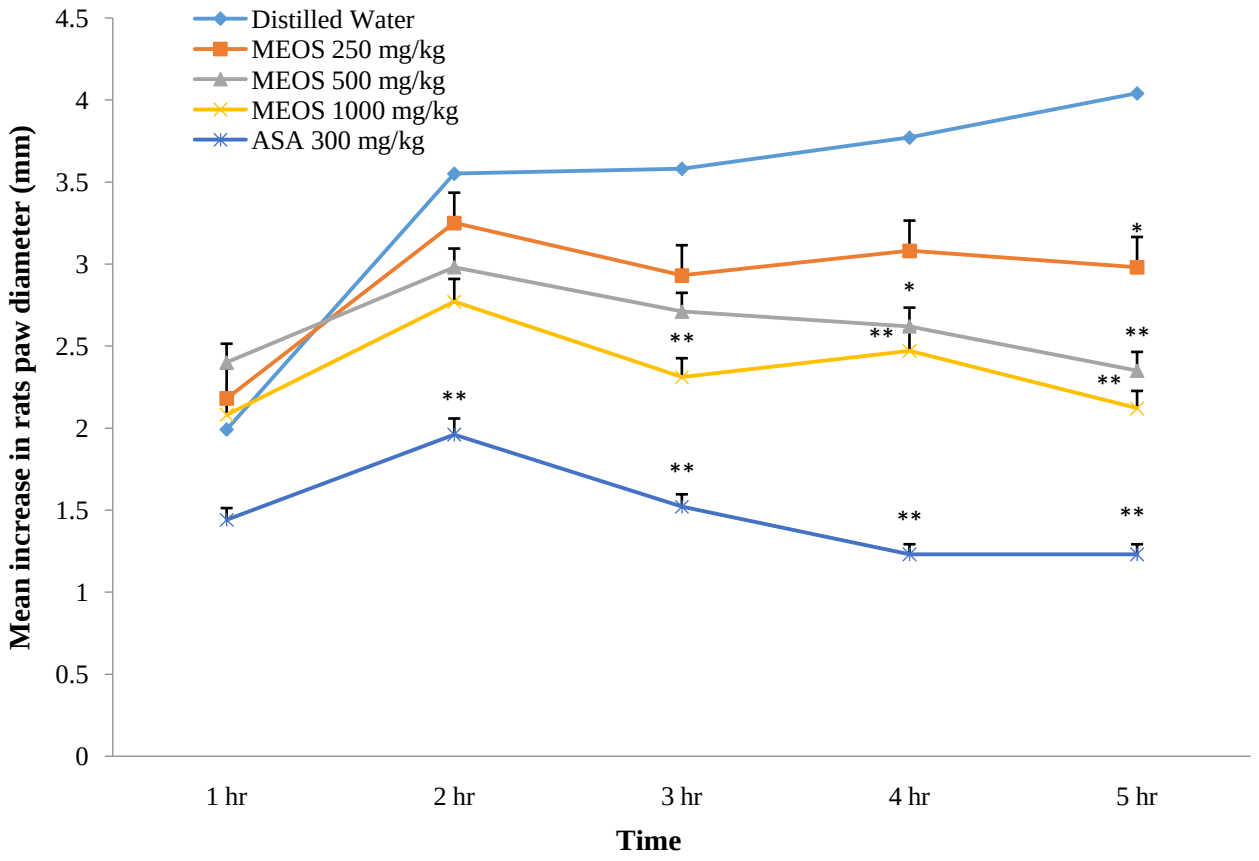


Figure 4.5: Effects of Methanol Extract of *Olax subscorpioidea* on Carrageenan-Induced Rats Paw Oedema

Values presentedas Mean ± SEM, * $p<0.05$, ** $p<0.01$ versus control (repeated measures ANOVA followed by Bonferroni's post-hoc test), ASA= acetylsalicylic acid, n=6

Table 4.5: Effect of *Olax subscorpioidea* Fractions on Carrageenan-Induced Paw Oedema in Rats

Treatments (mg/kg)	Mean Increase in Rats Paw Diameter (mm)				
	1 hr	**2 hr**	**3 hr**	**4 hr**	**5 hr**
Distilled water 1 ml/kg	1.99 ± 0.26	3.55 ± 0.43	3.58 ± 0.29	3.77 ± 0.22	4.04 ± 0.29
AFOS 250	1.64 ± 0.16**(17.58)**	2.48 ± 0.22[a]**(30.14)**	2.12 ± 0.24[c]**(40.78)**	1.43 ± 0.14[c]**(62.07)**	1.19 ± 0.11[c]**(70.54)**
AFOS 500	1.38 ± 0.08**(30.65)**	1.84 ± 0.20[c]**(48.17)**	1.62 ± 0.19[c]**(54.75)**	1.15 ± 0.26[c]**(69.50)**	0.74 ± 0.19[c]**(81.68)**
AFOS 1,000	0.83 ± 0.13[c]**(58.29)**	1.81 ± 0.07[c]**(49.10)**	1.31 ± 0.19[c]**(63.41)**	1.01 ± 0.14[c]**(73.21)**	0.50 ± 0.16[c]**(87.87)**
BFOS 250	1.30 ± 0.12**(34.67)**	1.89 ± 0.25[c]**(46.76)**	1.50 ± 0.18[c]**(58.10)**	1.13 ± 0.04[c]**(70.03)**	1.17 ± 0.15[c]**(71.04)**
BFOS 500	1.09 ± 0.08[b]**(45.23)**	1.44 ± 0.15[c]**(59.44)**	1.51 ± 0.11[c]**(57.82)**	1.11 ± 0.15[c]**(70.56)**	0.72 ± 0.11[c]**(82.18)**
BFOS 1,000	0.88 ± 0.14[c]**(55.78)**	1.16 ± 0.22[c]**(67.32)**	1.25 ± 0.11[c]**(65.08)**	0.75 ± 0.16[c]**(80.11)**	0.71 ± 0.19[c]**(82.43)**
HFOS 150	1.56 ± 0.14**(21.61)**	2.10 ± 0.09[c]**(40.85)**	2.36 ± 0.13[b]**(34.08)**	1.92 ± 0.12[c]**(49.07)**	1.56 ± 0.19[c]**(61.39)**
HFOS 300	1.10 ± 0.21[c]**(44.72)**	1.87 ± 0.19[c]**(47.32)**	2.25 ± 0.19[c]**(37.15)**	1.91 ± 0.17[c]**(49.34)**	1.44 ± 0.17[c]**(64.36)**
HFOS 600	0.88 ± 0.11[c]**(55.78%)**	1.75 ± 0.12[c] **(50.70%)**	2.24 ± 0.19[c] **(37.43%)**	1.78 ± 0.12[c] **(52.79%)**	1.32 ± 0.12[c]**(67.33%)**
ASA 300	1.44 ± 0.21**(27.64%)**	1.96 ± 0.11[c]**(44.79%)**	1.52 ± 0.16[c]**(57.54%)**	1.23 ± 0.16[c] **(67.37%)**	1.23 ± 0.21[c] **(69.55%)**

Values presented as Mean ± SEM, [a]$p<0.05$, [b]$p<0.01$, [c] $p<0.001$ versus control (repeated measures ANOVA followed by Bonferroni's post-hoc test), AFOS= residual aqueous fraction, BFOS= butanol fraction, HFOS= hexane fraction,ASA= acetylsalicylic acid, n=6, figures in parentheses (bold) are percentage inhibition of inflammation.

4.5.2 Effect of residual aqueous and butanol fractions of *Olax subscorpioidea* on cotton pellet induced granuloma formation in rats

The residual aqueous and butanol fractions of *O. subscorpioidea*(1,000 mg/kg) demonstrated significant ($p<0.01$ and $p<0.001$ respectively) inhibitory activity on the granuloma formation whencompared with the control. The butanol fraction evoked percent inhibition of 42.49% and 61.39% on the wet and dried granuloma formation respectively. The residual aqueous fraction produced 23.64% and 35.44% inhibition; while ASA exerted 41.35% and 50.63% inhibition on the wet and dried granuloma formation respectively (Table 4.6).

Table 4.6: Effect of Residual Aqueous and Butanol Fractions of *Olax subscorpioidea* on Cotton Pellet-Induced Granuloma Formation in Rats

Treatments (mg/kg)	Wet granuloma weight (mg)	Percentage inhibition of granuloma (%)	Dry granuloma weight (mg)	Percentage inhibition of granuloma (%)
Distilled water 1ml/kg	438.20 ± 14.87		63.20 ± 2.46	
AFOS 1,000	334.60 ± 18.42*	23.64	40.80 ± 2.18*	35.44
BFOS 1,000	252.00 ± 16.30**	42.49	24.40 ± 4.62**	61.39
ASA 300	257.00 ± 16.9**	41.35	31.20 ± 4.32**	50.63

Values presented as Mean ± SEM, * $p<0.01$, ** $p<0.001$ versus control (one-way ANOVA followed by Dunnett's post-hoc test), AFOS=residualaqueous fraction, BFOS=butanol fraction,ASA=acetylsalicylic acid, n=5.

4.5.3 Effect of residual aqueous and butanol fractions of *Olax subscorpioidea* on pro-inflammatory cytokines

The residual aqueous and butanol fractions of *Olax subscorpioidea*(1,000 mg/kg) significantly ($p<0.05$and$p<0.01$) decreased the concentrations of IL-1α, VEGF and EGF.The concentration of IL-1β was significantly ($p<0.05$) increased by both fractions. The standard drug, ASA, significantly ($p<0.05$) decreased the concentrations of VEGF. No statistical significant difference was observed in the concentrations of IL-2 and TNF-α for both fractions when compared with the control(Figure 4.6).

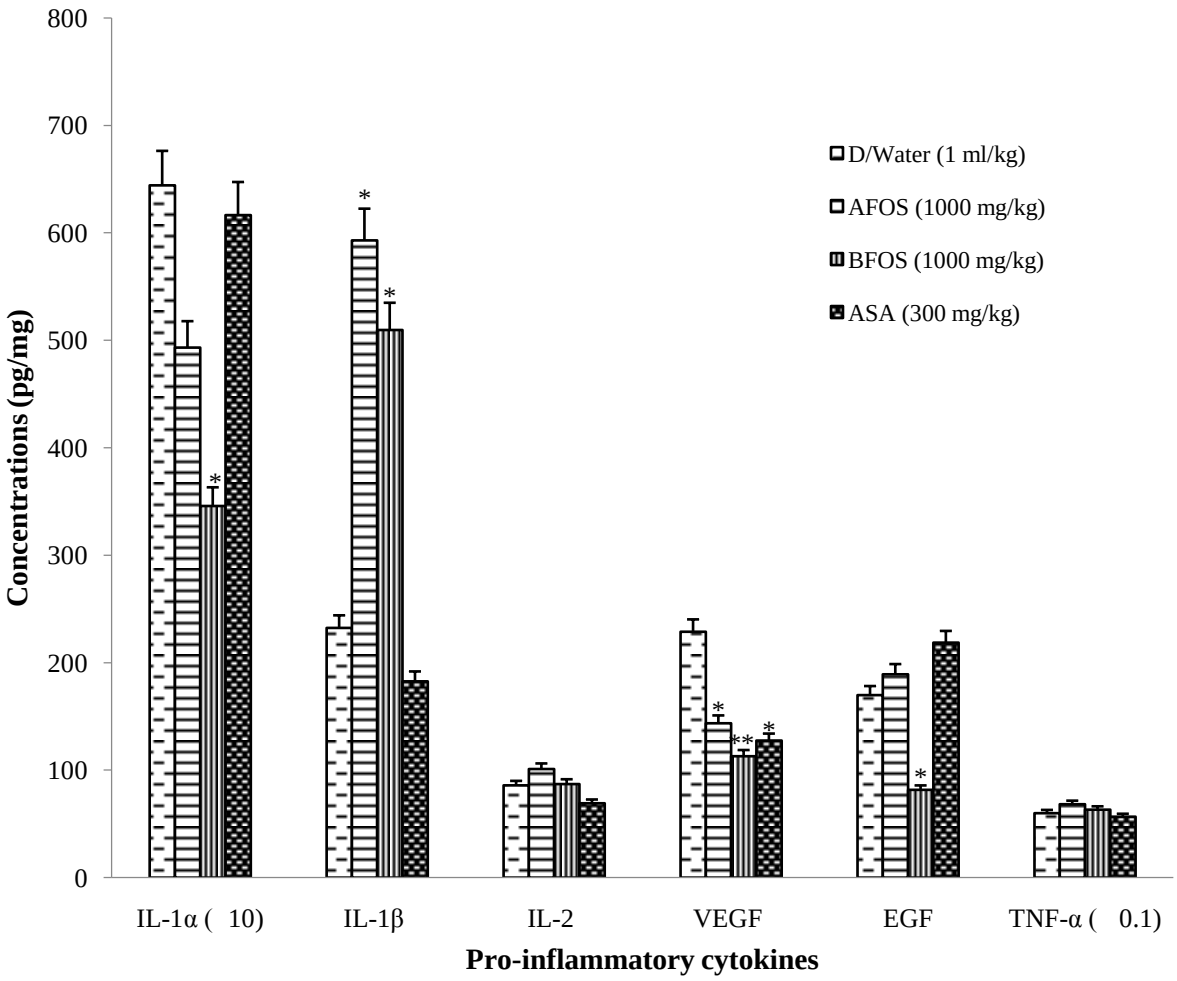


Figure 4.6: Effect of Residual Aqueous and Butanol Fractions of *Olax Subscorpioidea*on Pro-Inflammatory Cytokines

Values presented as Mean ± SEM, * $p<0.05$, ** $p<0.01$ versus control (one-way ANOVA followed by Dunnett's post-hoc test), AFOS =residual aqueous fraction, BFOS=butanol fraction, ASA-acetylsalicylic acid, IL=Interleukin, TNF=tumor necrosis factor, VEGF=Vascular Endothelial Growth Factor, EGF=Epidermal Growth Factor, n=6.

4.5.4 Effect of residual aqueous and butanol fractions of *Olax subscorpioidea* on anti-inflammatory cytokines

The residual aqueous and butanol fractionsof *Olax subscorpioidea* (1,000 mg/kg) significantly ($p<0.05$ and $p<0.01$) increased the concentrations of IL-5, IL-6 and IFN-γ(Figure 4.7). No statistical significant difference was observed in the concentrations of IL-4, IL-10 and IL-13 for both fractions when compared with the control.

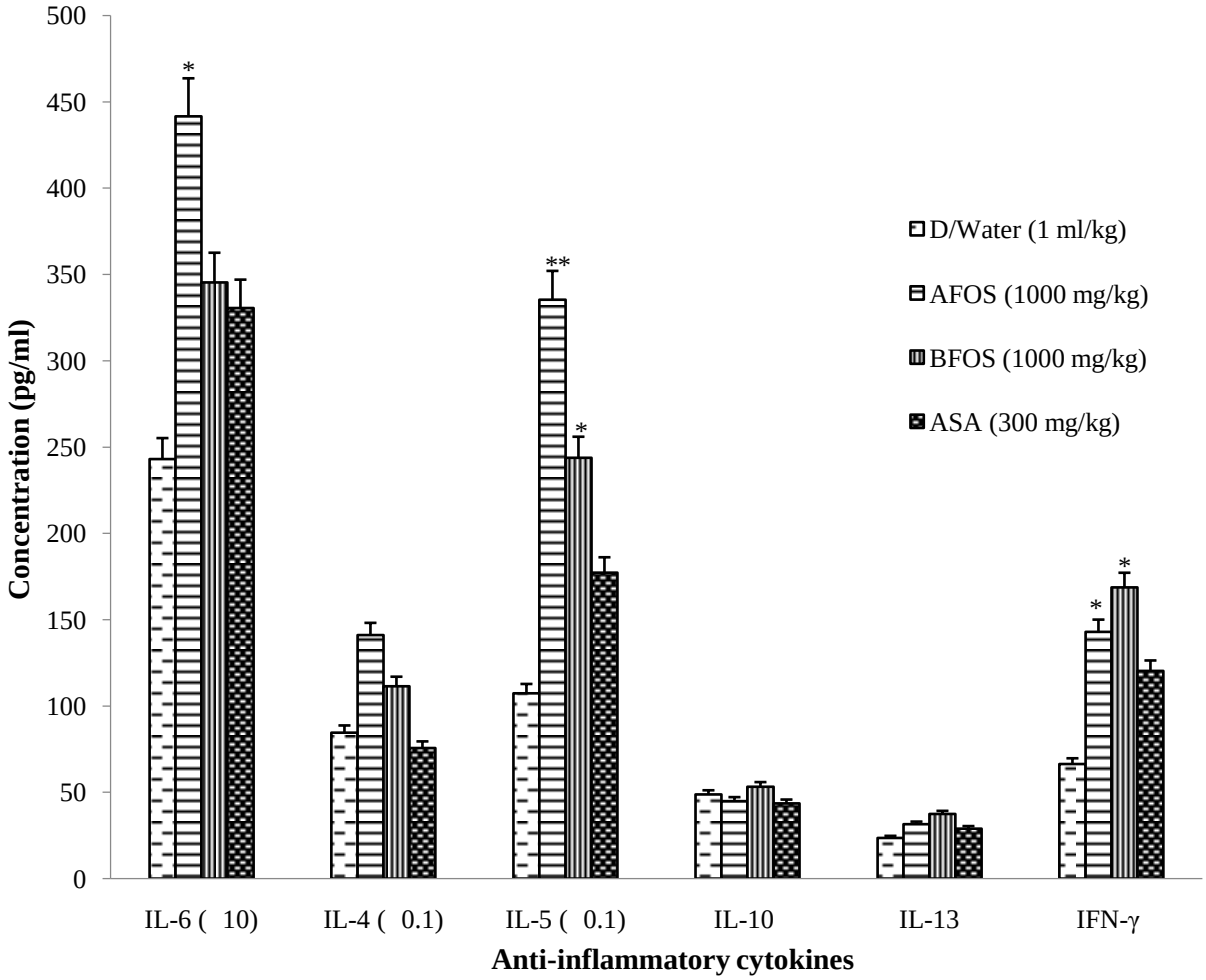


Figure 4.7: Effect of Residual Aqueous and Butanol Fractions of *Olax subscorpioidea* on Anti- Inflammatory Cytokines

Values presented as Mean ± SEM, * $p<0.05$, ** $p<0.01$ versus control (one-way ANOVA followed by Dunnett's post hoc test), AFOS=residual aqueous fraction, BFOS=butanol fraction, ASA=acetylsalicylic acid, IL=Interleukin, IFN=Interferon, n=6.

4.6 Mechanisms of Analgesic Activities of *Olax subscorpioidea*

Treatment of animals with the butanol fraction of *Olax subscorpioidea*(BFOS) and morphine significantly($p<0.001$) decreased the number of writhes induced by acetic acid when compared with the control. The pretreatment of animals with naloxone, prazosin, yohimbine, propranolol or glibenclamide each did not significantly decrease or increase the number of writhes produced by BFOS. However, the pretreatment of animals with either metergoline orl-arginine each significantly($p<0.05$ and $p<0.01$ respectively) increased the number of writhing activity of BFOS.

The pretreatment of animals with naloxone, yohimbine, propranolol,metergoline orl-arginine each significantly ($p<0.05$ and $p<0.01$) increased the number of writhes produced by morphine. Prazosin, propranolol and L-NNA, when administered alone, each significantly ($p<0.001$) decreased the number of acetic acid-induced writhes when compared with the control(Figure 4.8 – Figure 4.14).

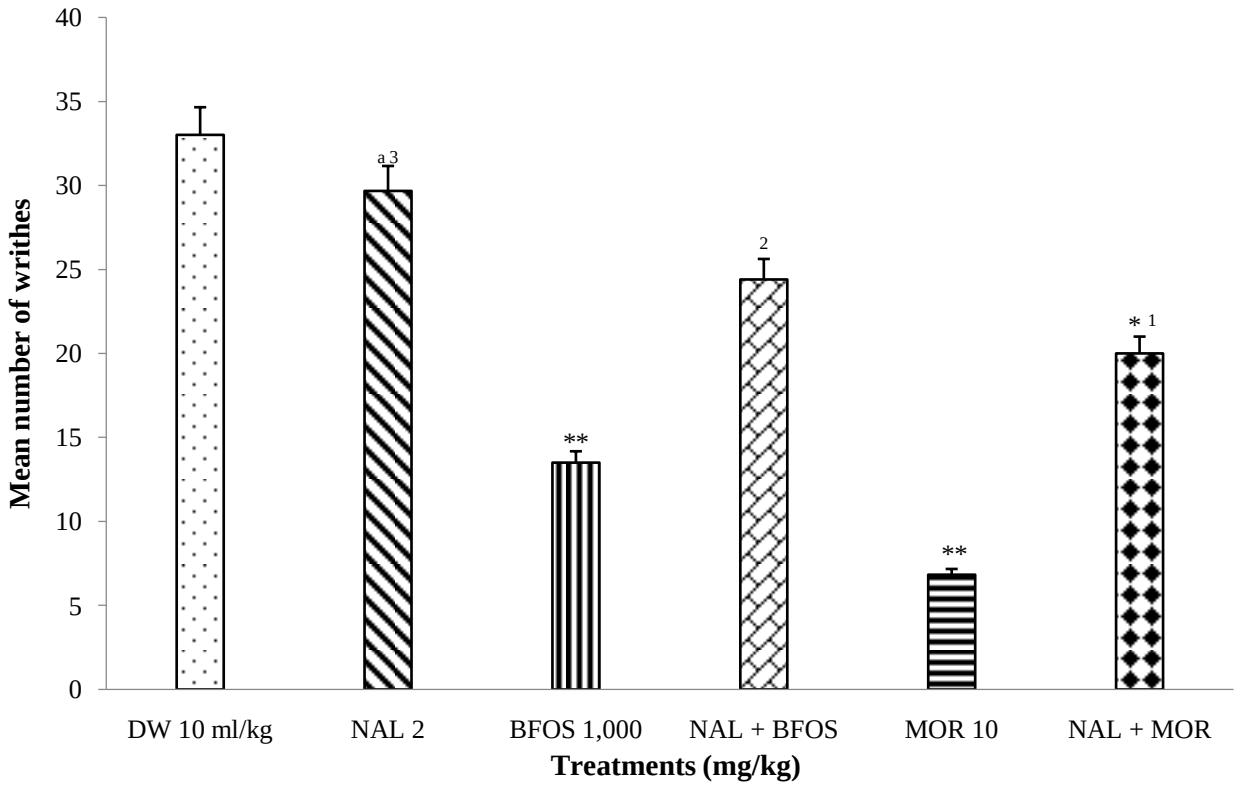


Figure 4.8: Effect of Naloxone on Analgesic Activity of Butanol Fraction of *Olax subscorpioidea* on Acetic Acid-Induced Writhing Testin Mice

Values presented as Mean± SEM; * $p<0.05$, ** $p<0.001$ versus control, $^{a}p<0.01$, versus BFOS and ^{1} <0.05, ^{2} $p<0.01$, ^{3} $p<0.001$ versus MOR (one-way ANOVA followed by Bonferroni's post-hoc test), DW=Distilled water, BFOS=Butanol fraction of *O. subscorpioidea*, NAL=Naloxone, MOR=Morphine, n=6

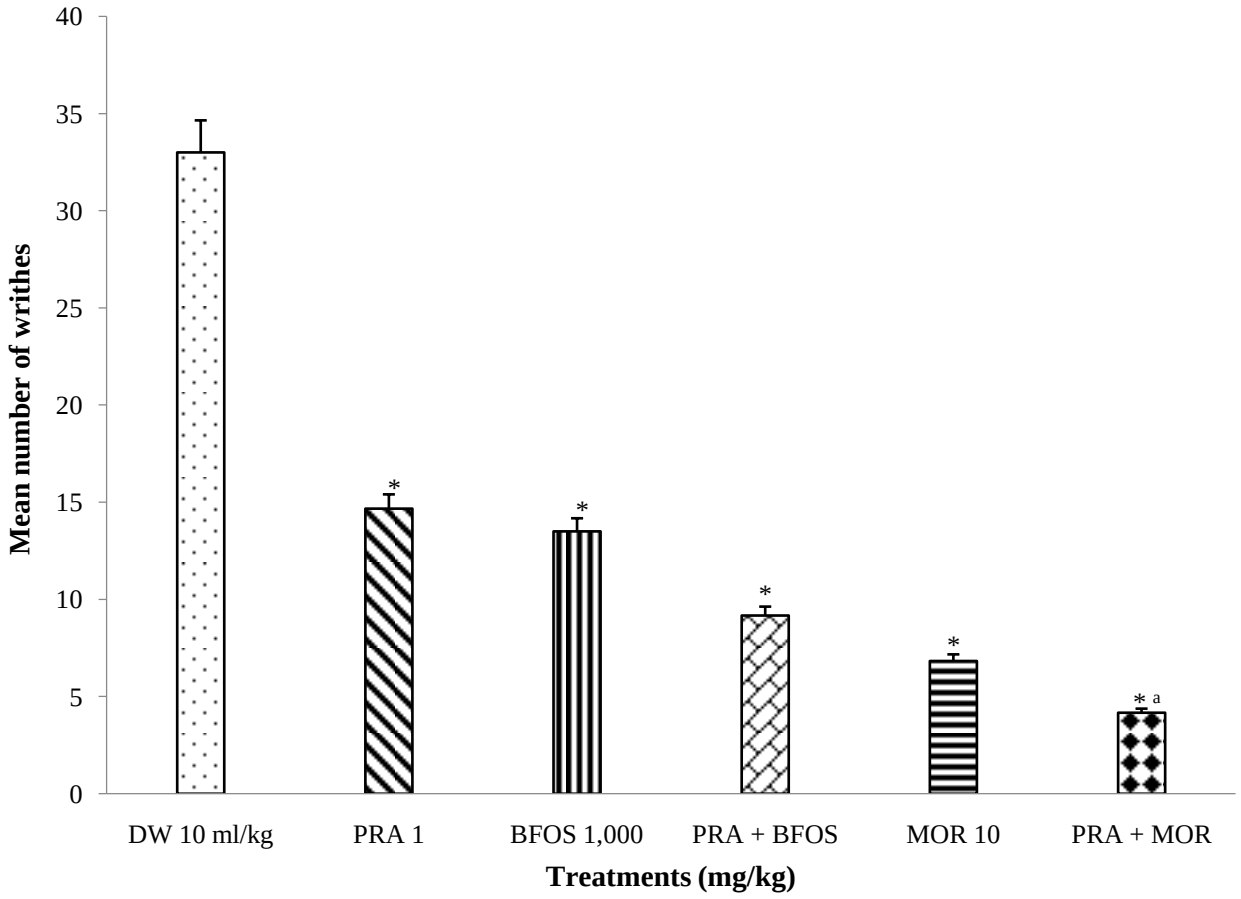


Figure 4.9: Effect of Prazosin on Analgesic Activity of Butanol Fraction of *Olax subscorpioidea* on Acetic Acid-Induced Writhing Testin Mice

Values presented as Mean± SEM; * $p<0.001$ versus control and $^{a}p<0.05$ versus BFOS (one-way ANOVA followed by Bonferroni's post-hoc test), DW=Distilled water, BFOS=Butanol fraction of *O. subscorpioidea*, PRA=Prazosin, MOR=Morphine, n=6

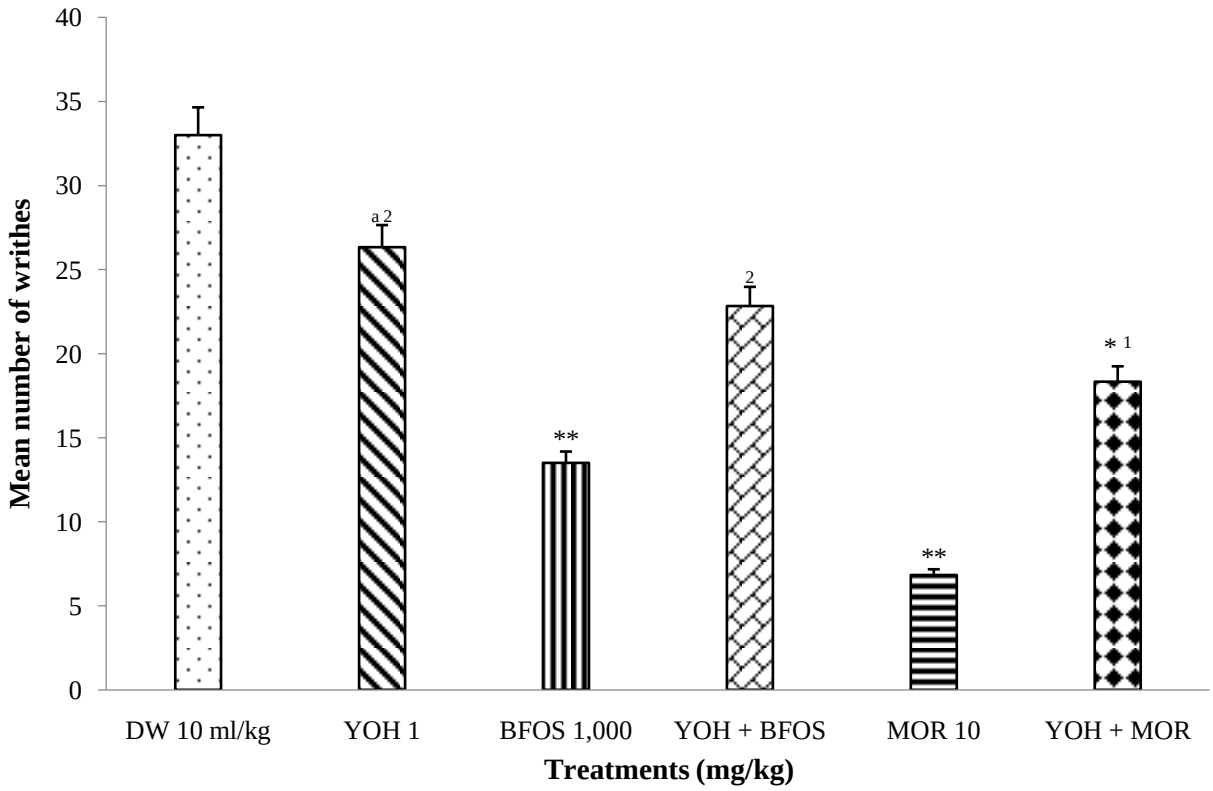


Figure 4.10: Effect of Yohimbine on Analgesic Activity of Butanol Fraction of *Olax subscorpioidea* on Acetic Acid-Induced Writhing testin Mice

Values presented as Mean± SEM; * $p<0.01$, ** $p<0.001$ versus control, [a] $p<0.01$ versus BFOS and [1] $p<0.05$, [2] $p<0.001$ versus MOR (one-way ANOVA followed by Bonferroni's post-hoc test). DW= Distilled water, BFOS= Butanol fraction of *O. subscorpioidea,* YOH= Yohimbine, MOR= Morphine, n=6

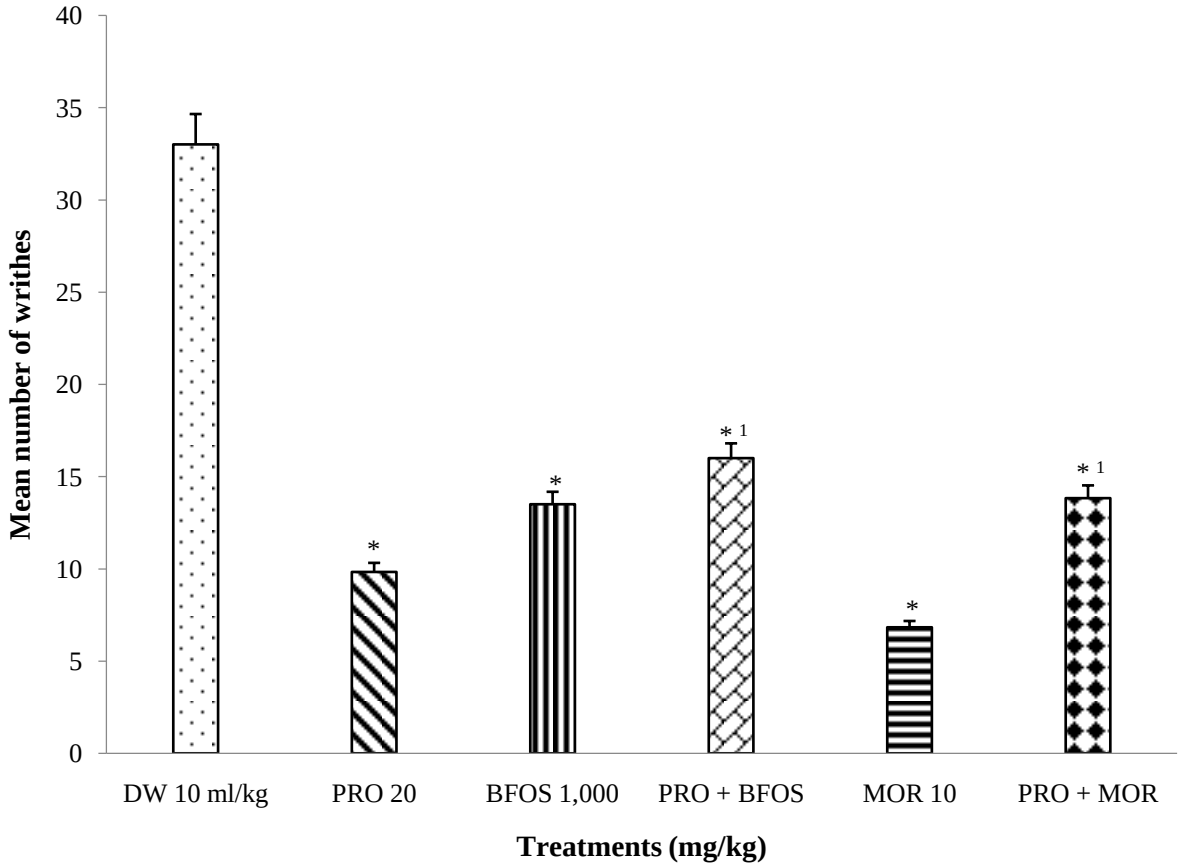


Figure 4.11: Effect of Propranolol on Analgesic Activity of Butanol Fraction of *Olax subscorpioidea* on Acetic Acid-Induced Writhing testin Mice

Values presented as Mean± SEM; * $p<0.001$ versus control, $^{1}p<0.05$ versus MOR (one-way ANOVA followed by Bonferroni's post-hoc test), DW=Distilled water, BFOS=Butanol fraction of *O. subscorpioidea*, PRO=Propranolol and MOR=Morphine, n=6.

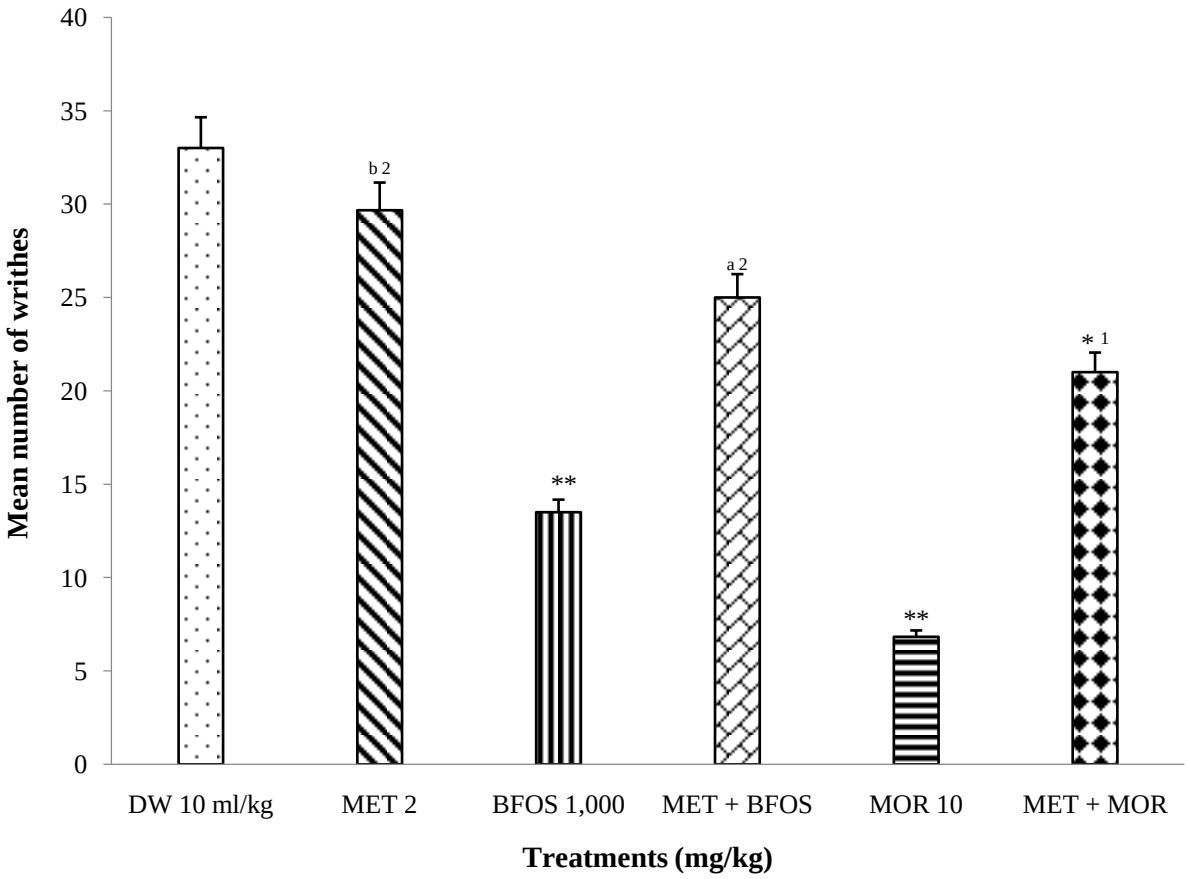


Figure 4.12: Effect of Metergoline on Analgesic Activity of Butanol Fraction of *Olax subscorpioidea* on Acetic Acid-Induced Writhing testin Mice

Values presented as Mean ± SEM, * $p<0.01$, ** $p<0.001$ versus control, [a] $p<0.05$ [b]$p<0.001$ versus BFOS and [1] $p<0.01$, [2] $p<0.001$ versus MOR (one-way ANOVA followed by Bonferroni's post-hoc test), DW=Distilled water, BFOS=Butanol fraction of *O. subscorpioidea*, MET=Metergoline, MOR=Morphine, n=6.

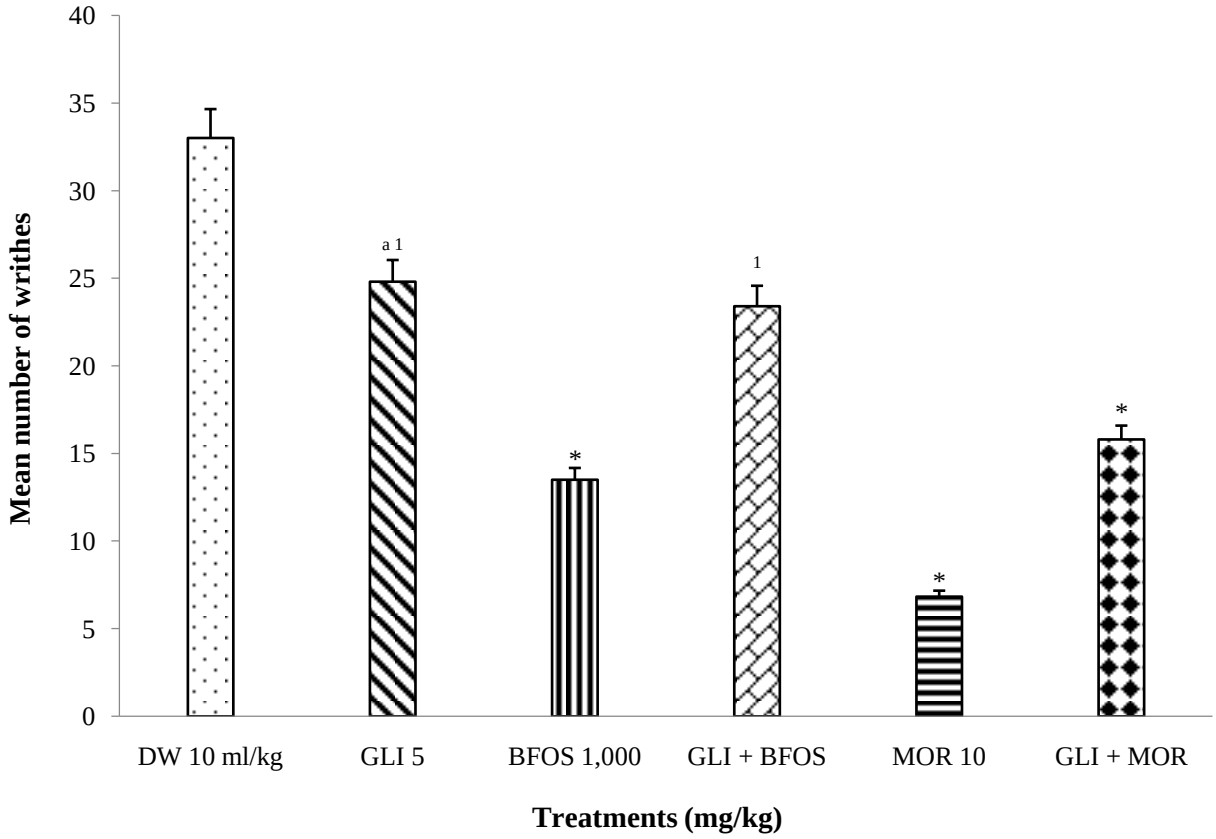


Figure 4.13: Effect of Glibenclamide on Analgesic Activity of Butanol Fraction of *Olax subscorpioidea* on Acetic Acid-Induced Writhing testin Mice

Values presented as Mean ± SEM, * $p<0.001$ versus control, [a]$p<0.05$, versus BFOS, [1] $p<0.001$ versus MOR (one-way ANOVA followed by Bonferroni's post-hoc test), DW=Distilled water, BFOS=Butanol fraction of *O. subscorpioidea,* GLI=Glibenclamide, MOR= Morphine, n=6.

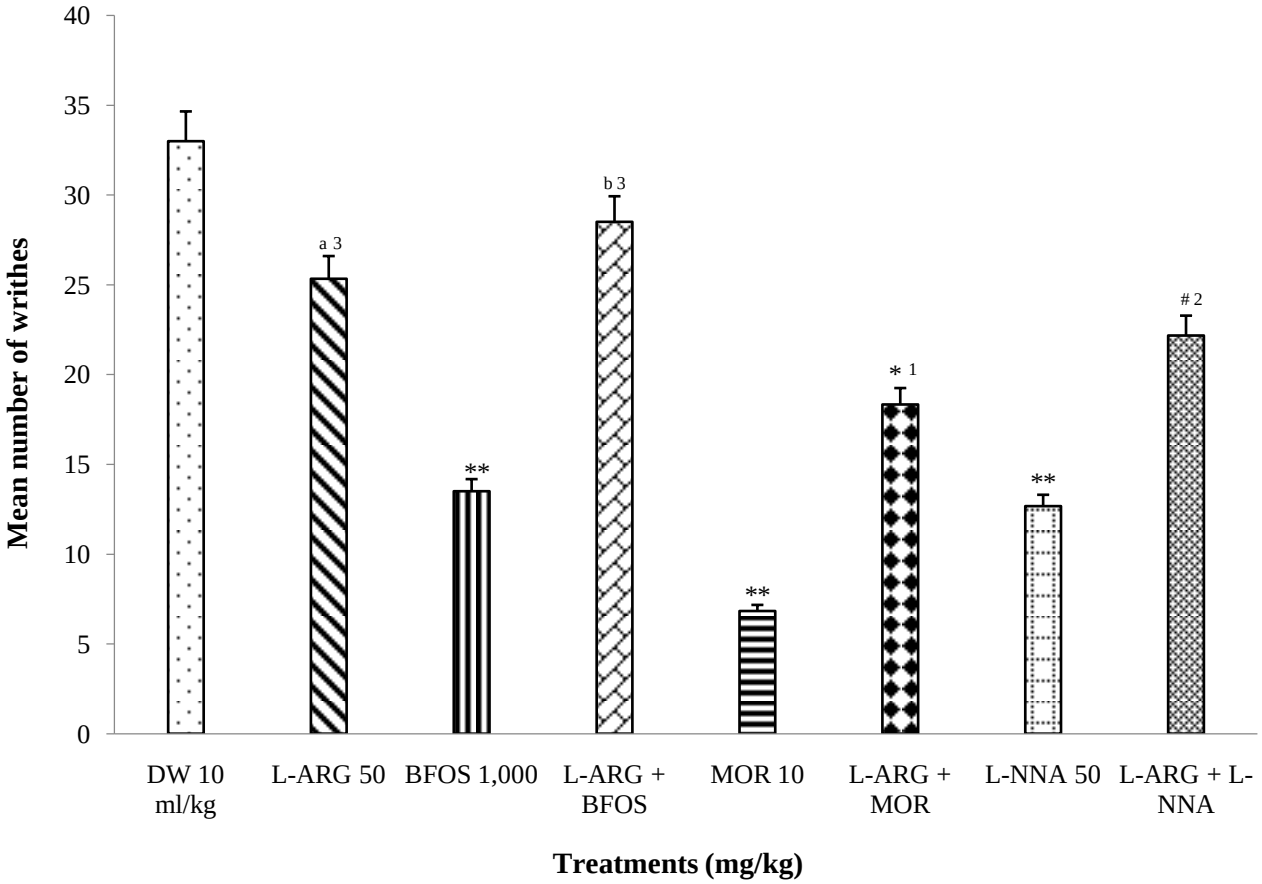


Figure 4.14: Effect of L-arginine on Analgesic Activity of Butanol Fraction of *Olax subscorpioidea* on Acetic Acid-Induced Writhing testin Mice

Values presented as Mean ± SEM, * $p<0.01$, ** $p<0.001$ versus control, [a] $p<0.05$, [b] $p<0.01$ versus BFOS, [1] $p<0.05$, [2] $p<0.01$, [3] $p<0.001$ versus MOR and [#] $p<0.05$ versus L-NNA (one-way ANOVA followed by Bonferroni's post-hoc test), DW=Distilled water, BFOS=Butanol fraction of *O. subscorpioidea*, L-ARG=L-arginine, L-NNA=Nomega-nitro-l-arginine, MOR=Morphine, n=6.

CHAPTER FIVE

5.0 DISCUSSION

Olax subscorpioidea is a naturally growing shrubby plant used in folk medicine for the management of pains, cancer, yellow fever, rheumatism and microbial infections. The present studies investigated the analgesic and anti-inflammatory activity of its methanol leaf extract (MEOS), residual aqueous, butanol and hexane fractions. Also, sub-acute anti-inflammatory activities of the most active fractions of the extract (residual aqueous and butanol fractions) were investigated. Furthermore, the involvements of inflammatory cytokines in the anti-inflammatory activity of both fractions were also examined. Finally, the possible roles of opioidergic, (α_1, α_2 and β)-adrenergic, serotonergic, ATP-sensitive potassium channels and nitric oxide-l-arginine pathways in the analgesia produced by the butanol fraction of *Olax subscorpioidea* were elucidated.

The oral administration of MEOS, residual aqueous and butanol fractions up to 5,000 mg/kg in mice and rats caused no death and also no physical sign of toxicity was observed. These suggest that MEOS as well as the residual aqueous and butanol fractions may be relatively safe (Matsumura, 1975; Lorke, 1983; Loomis and Hayes, 1996) when administered orally. However, the hexane fraction is slightly toxic orally in mice (LD_{50}: 2,200 mg/kg) and rats (LD_{50}: 3,800 mg/kg) according to the classification of toxicity (Matsumura, 1975; Loomis and Hayes, 1996). Acute toxicity studies are usually carried out to determine the range of doses that could be toxic to the animal; it could also be used

to estimate the therapeutic index (LD_{50}/ED_{50}) of drugs and xenobiotics (Maikai *et al*., 2008).

The acetic acid-induced writhing reaction in mice, described as a typical model for inflammatory pain, has long been used as a screening tool for the assessment of analgesic or anti-inflammatory properties of new agents (Meotti *et al*., 2006). It is the most commonly used substances for the stimulation of visceral pain in mice (Ma and Zhang, 2011). This method is very sensitive and able to detect anti-nociceptive effects of compound(s) at dose level that may be inactive in other methods like tail-flick test (Usman *et al*., 2008; Yerima *et al*., 2009). The irritant indirectly triggers the release of nociceptive endogenous mediators (bradykinin, serotonin, and prostaglandin) and pro-inflammatory cytokines (TNF-α and IL-1β) to cause painful sensation (Kakoti *et al*., 2013).

The abdominal constriction response induced by acetic acid is a sensitive procedure to evaluate peripherally acting analgesics like NSAIDs (Aiyelero *et al*., 2009; Mishra *et al*., 2011; Kakoti *et al*., 2013) and centrally acting analgesics like morphine (Donkor *et al*., 2013; Kakoti *et al*., 2013). ASA and other NSAIDs reduce writhes induced by acetic acid by inhibiting COX in peripheral tissues by blocking the release and/or synthesis of inflammatory mediators (Donkor *et al*., 2013). The central acting analgesics such as morphine produce their analgesic effect by reducing the sensory effect of the noxious stimulus (Stevenson *et al.,* 2006). MEOS and its fractions exhibit high level of analgesic activities by effectively inhibiting pain induced by the acetic acid. The ability of MEOS and the fractions to inhibit the acetic acid induced pain suggests that MEOS and its fractions may be eliciting their analgesic effect by inhibiting COX in the peripheral tissues

possibly by blocking the release and/or synthesis of inflammatory mediators; they may also be acting centrally by reducing the sensory effect of acetic acid.

The formalin test is a tonic model of continuous pain resulting from formalin-induced tissue injury (Ellis *et al*., 2008). It is a useful model, particularly for the screening of novel compounds, since it encompasses inflammatory, neurogenic, and central mechanisms of nociception (Tjølsen *et al.,*1992; Ellis *et al*., 2008). It provides a more valid model for clinical pain compared to models such as the hot plate and tail flinch tests (Meunier *et al*., 1998). It differs from most other nociceptive tests in that it is a chemical, little or no restraining of experimental animals is needed during testing and the nociceptive stimulus and response are persistent rather than transient (Current Protocols in Neuroscience, CPN, 2002). Also the formalin test enables evaluation of analgesic activity towards moderate, continuous pain generated by tissueinjury.

The formalin-induced nociceptive behavior is bi-phasic (acute and chronic). The acute phase lasts for about 5 min and is probably due to direct chemical stimulation of nociceptors. The chronic phase lasts about 20 to 40 minutes (starts about 15 to 20 minutes after formalin injection) and it is suggested that peripheral, inflammatory processes are involved (Meunier *et al*., 1998).Centrally acting analgesics (e.g. morphine) inhibit both phases, while peripherally acting analgesics (e.g. ASA) inhibit only the chronic phase (Vogel, 2008). The ability of MEOS and its fractions to inhibit both phases of formalin-induced pain suggests that they may be acting through both peripheral and central mechanisms.

The hot plate thermal model is used to assess central antinociceptive effect of drugs (Bhalke and Pal, 2012). The paws of mice are very sensitive to heat at temperatures which are not damaging to the skin (Vogel, 2008). The responses are jumping, withdrawal of the paws and licking of the paws. The time until these responses occur is prolonged after administration of centrally acting analgesics, whereas peripheral analgesics do not generally affect these responses (Vogel, 2008). The centrally acting agents like morphine activate the release of endogenous peptide by periaqueductal gray matter (PAG), which are carried to the spinal cord to inhibit the pain muscle transmission within the dorsal horn (Katzung, 2005). The ability of MEOS, its residual aqueous and butanol fractions to increase the latency in the hot plate model indicates that MEOS and both fractions may possess central analgesic activity. The inability of the hexane fraction to significantly increase the latency suggests that it may lack or have limited central analgesic activity; and this may not be unrelated with the fact that the hexane fraction was devoid of some phytochemical constituents such as saponins, tannins, and cardiac glycosides which are present in the residualaqueous and butanol fractions and have been reported to possess analgesic activity (Calixto *et al.,* 2000).

Carrageenan is the phlogistic agent of choice for testing anti-inflammatory drugs as it is none antigenic and is devoid of apparent systemic effect (Chakraborty *et al.,* 2006). The oedema induced by carrageenan is simple, rapid and gives reliable result with most of the clinically active anti-inflammatory drugs (Kaneira *et al*., 2007). Carrageenan induced inflammation is most commonly used as an experimental model for evaluating the anti-inflammatory potency of compounds or natural products (Winter *et al*., 1962).After the

carrageenan injection, oedema develops mainly in two phases: the first 30 minutes after the injection, the second beginning at the end of the first hour and lasting until the third hour after injection (Ma and Zhang, 2011). The first phase has been attributed to the release of histamine, serotonin and bradykinin on vascular permeability and the later phase has been due to over production of prostaglandin in tissues (Bhalke and Pal, 2012). The anti-inflammatory activities observed in MEOS and its fractions suggest that the MEOS and the fractions might be producing their anti-inflammatory effect via the inhibition of inflammatory mediators such as histamine, serotonin and prostaglandins.

Cotton pellet granuloma study is a sub-acute inflammation model. In this model, inflammation and granuloma develops during the period of several days (Balasubramanian *et al*., 2005). The model is widely used to assess the transudative and proliferative components of chronic inflammation (Winter and Porter, 1957). The weight of the wet cotton pellets correlates with transude material and the weight of dry pellet correlates with the amount of granulomatous tissue (Verma *et al*., 2010). The inflammation observed in this model is of non-immunological type of inflammation mediated by the activation of the chemical mediators of inflammation, mostly kinins (Sur *et al*., 2003). Kinins cause both vasodilatation and increase vascular permeability in the early stages of inflammation (Elango *et al*., 2012). NSAIDs exert their antiproliferative activity by decreasing granuloma tissue, preventing formation of collagen fibers and suppressing mucopolysaccharides (Verma *et al*., 2010). Thus, the reduction observed in the granuloma formation suggests that the residual aqueous and butanol fractions of *O. subscorpioidea*

might have inhibited the sub-acute inflammation by decreasing granuloma tissue, preventing the formation of collagen fibers and suppressing mucopolysaccharides.

Cytokines are key modulators of inflammation; they participate in acute and chronic inflammation in a complex network of interactions. The functional definition of a pro-inflammatory cytokine is the ability of the cytokine to induce inflammation and that of an anti-inflammatory cytokine is the ability of the cytokine to inhibit the synthesis of major pro-inflammatory cytokines such as IL-1 and TNF (Shaikh, 2011) and/or stimulate the production or synthesis of pro-inflammatory cytokines receptor antagonists (e.g. IL-1ra and TNF-ra).

IL-1 triggers fever by enhancing prostaglandin-E_2 (PG-E_2) synthesis by the vascular endothelium of the hypothalamus and can stimulate T cell proliferation. It also elicits the release of histamine from mast cells at the site of inflammation. Histamine in turn triggers early vasodilation and increase of vascular permeability (Shaikh, 2011). IL-5 is involved in eosinophil differentiation and activation and stimulation of immunoglobulin (Ig) class switching to IgA (Shaikh, 2011, Khare and Khare, 2014**)**. It also increases the activation of B cell proliferation, and enhancement of T cell cytotoxicity. The combined production of IL-4 and IL-5 by CD4+ TH2 cells therefore results in IgE and IgA production and mast cell and eosinophil stimulation (Shaikh, 2011). IL-6 has both pro and anti-inflammatory properties (Matthews *et al*., 2010; Shaikh, 2011; Scheller *et al*., 2011) but possesses more of anti-inflammatory properties (Matthews *et al*., 2010; Shaikh, 2011). It inhibits the production of TNF and IL-1 and other pro-inflammatory cytokines which limits the acute

inflammatory response via negative feedback mechanism (Shaikh, 2011). Interferons (IFN) are induced in response to viral infections. IFN possess anti-viral and anti-proliferative activities. Type I IFN (which includes IFN-α and IFN-β) induce anti-proliferative and anti-viral activity whereas type II IFN (IFN-γ) has weaker anti-viral activity but more potent immunomodulatory properties (Tayal and Kalra, 2008). IFN-γ activates macrophages which in turn kill intracellular pathogens and it also has a unique effect of activating phagocytes (Tayal and Kalra, 2008; Shaikh, 2011). Vascular Endothelial Growth Factor (VEGF) is a sub-family of growth factors. It is an angiogenic cytokine (Ikram *et al*., 2004). It regulates vascular permeability that leads to oedema and swelling of tissues and also stimulates angiogenesis in the adult. Angiogenesis is required for the progression of tumors from a benign to a malignant phenotype and for metastasis. These make VEGF the key factor involved in nearly all human tumors (Masood *et al*., 2001). Epidermal Growth Factor (EGF) mediates the activation of nuclear factor-κB (NF-κB) and inflammation *in vitro* and *in vivo*. The mutations, amplifications or misregulations of EGF receptor (EGFR) have been implicated in about 30% of all epithelial cancers. EGFR have also been reported to play an important role in the pathogenesis of asthma (Xu *et al*., 2011).

The reduction observed in the concentrations of pro-inflammatory cytokines such as IL-1α, VEGF and EGF in the residual aqueous and butanol fractions treated groups may be due to direct inhibition by the fractions or by IL-6 (and/or other anti-inflammatory cytokines). The increase observed in the concentration of the IL-1β (a pro-inflammatory cytokine) in the residual aqueous and butanol fractions treated groups may be due to direct stimulation of IL-1 receptor antagonist (IL-1ra) by the fractions or indirectly via the IL-6 (and/or other

anti-inflammatory cytokines). IL-1ra is a specific IL-1 receptor antagonist; it competitively binds to the same receptor as IL-1β, thereby blocking IL-1β-mediated cellular changes thus increasing the concentration of IL-1β (Zhang and An, 2007; Tayal and Kalra, 2008). IL-4, IL-6, IL-10 and IL-13 have been reported to inhibit the synthesis of IL-1, yet they stimulate the synthesis of IL-1ra (Shaikh, 2011). The residual aqueous and butanol fractions *O. subscorpioidea* were able to stimulate the production of IL-6 which in turn inhibit the production of pro-inflammatory cytokines or stimulate the production of pro-inflammatory cytokines receptor antagonists. The ability of the residual aqueous and butanol fractions to reduce the concentrations of VEGF and EGF may also signify the plants potential as an anti-tumour agent. Oloyede *et al.* (2012) reported the use of the *O. subscorpioidea* leaf as one of the seven medicinal plants combined to form a local decoction (Joloo) used traditionally for the management and treatment of breast tumour in Southwestern Nigeria. This plant may also serve as a prophylactic agent for malignancies in which chronic inflammation, supported by pro-inflammatory cytokines; precede tumour appearance (Abrham *et al*., 2010).

In this study, the concentrations of TNF-α (a potent pro-inflammatory cytokine) and IL-10 (a potent anti-inflammatory cytokine) were not affected. This may be due to the complexity in regulatory networks that govern inflammatory cytokine secretion upon specific stimulation, which might contribute to the different effect of the various cytokines tested (Abrham *et al*., 2010). This also testifies to the fact that measuring (targeting) just one inflammatory cytokine (in studies of this nature) may not give a reliable result.

The results obtained in the present work suggest the involvement of two main systems in the analgesic activity of the butanol leaf fraction of *O. subscorpioidea* in the acetic acid-induced writhing test in mice. The analgesic activity of the extract was found to be related to serotonergic and nitric oxide-l-arginine pathways; and it may partially be related to opioidergic, α_2-adrenergic and ATP-sensitive potassium channels receptors too. However, the analgesic activity of the fraction may not involve α_1 and β-adrenergic receptors.

In this study, pretreatment of mice with prazosin (a selective α_1-adrenoceptor antagonist) and propranolol (a β-adrenoceptor antagonist) each failed to reverse the analgesic activity of the butanol leaf fraction. These suggest the non-involvement of the α_1-adrenoceptor and β-adrenoceptor pathways in the analgesic activity of the fraction. Also, the pretreatment of mice with naloxone (a non-selective opioid receptor antagonist), yohimbine (a α_2-adrenoceptor antagonist) and glibenclamide (a potassium channel blocker) each reversed the analgesic action of the plant fraction but not significantly. These suggest a partial involvement of these pathways in the analgesic activity of the fraction. But pretreatment of animals with l-arginine (a nitric oxide precursor) and metergoline (a serotonin receptor antagonist) significantly reversed the analgesic action of the butanol fraction. These suggest the involvement of the serotonergic and nitric oxide-l-arginine pathways in the analgesic activity of the butanol fraction of *O. subscorpioidea*.

The results of the present study provide evidence supporting the involvement of the serotoninergic system in the antinociceptive effect of *O. subscorpioidea* as revealed by the finding that pretreatment of animals with metergoline significantly reversed its butanol

fraction's analgesic activities. Serotonergic systems comprise one of the major components of descending pain inhibitory pathways (Dogrul and Seyrek, 2006; Fields *et al*. 2006; Yoshimura and Furue, 2006). Serotonin released from platelets is able to activate nociceptors (Lang *et al*. 1990). Studies have suggested pronociceptive effects for serotonin (Zeitz *et al*., 2002; Pickering *et al*. 2003) and that the antinociceptive activities of various analgesics depend on integrity of descending serotonergic system (Millan, 2002; Dogrul and Seyrek, 2006). Metergoline administered alone has no analgesic activity and this is in agreement with previous reports (Dogrul and Seyrek, 2006).

Another interesting finding of the present study was the demonstration that the L-arginine-nitric oxide pathway is likely to be involved in the activity of *O. subscorpioidea.* This conclusion derives from the fact that the pretreatment of mice with the substrate of nitric-oxide synthase, l-arginine, largely reversed the analgesia caused by *O. subscorpioidea* and morphine as well the analgesia caused by nomega-nitro-l-arginine (L-NNA), a nitric oxide inhibitor, when assessed in the acetic acid-induced writhing test in mice. Nitric-oxide (NO) is produced from l-arginine by a chemical reaction catalyzed by the enzyme inducible nitric oxide synthase (iNOS) in living systems. After stimulation with bacterial lipopolysaccharide (LPS), many cells including macrophages express the iNOS which is responsible for the production of large amount of NO (Makchuchit *et al*., 2010). NO-l-arginine pathway has been shown to participate in thermal inflammatory hyperalgesia and the nociceptive transmission of neuropathic pain; it is said to play a critical role in the glutamate and N-methyl-D-aspartate (NMDA) mediated nociceptive response (Gultekin and Ahmedov, 2006). Over production of NOhave also been reported in a number of

clinical disorders including convulsions, pain, schizophrenia and neurodegenerative disorders like Alzheimer's and Parkinson's (Kiran and Srikanth, 2014).The analgesic activity of L-NNA is in agreement with other studies (Duarte and Ferreira, 2000).

However, the analgesic activity elicited by the butanol fraction seems to be independent of interaction with α_1-adrenergic and β-adrenergic receptors. These notions are because prazosin and propranolol (both) failed to reverse the analgesic activity of the butanol fraction when animals were pretreated with either of the antagonists. Though, prazosin potentiated the analgesic effect of the butanol fraction and that of morphine, but propranolol has no effect on the butanol but reversed the analgesic activity of morphine. The ability of prazosin to potentiate morphine analgesic has been reported (Ozdogan *et al*., 2003; Ozdogan *et al*., 2004). Also, both antagonists showed marked analgesic activities when individually administered. The analgesic potential of propranolol has been reported (Sadrabadi *et al*., 2011). The therapeutic uses of propranolol include; the management of migraine headache prophylaxis; and several clinical studies in adults have found that chronic therapy with propranolol reduces the frequency and severity of migraine in 60-80% of patients (Caruso *et al*., 2000; Sadrabadi *et al*., 2011).

Preliminary phytochemical screening gives a brief idea about the qualitative nature of active phytochemical constituents present in plant extracts/fractions, which will help the future investigators regarding the selection of the particular extract/fraction for further investigation or isolating the active principle (Mishra *et al.,* 2010). These constituents are known to be responsible for several pharmacological activities.

Phytochemical analysis of MEOS and its fractions revealed the presence of some phytochemicals such as alkaloids, tannins, flavonoids, cardiac glycosides, carbohydrates, saponins, steroid and triterpenes; this is in agreement with the work of Ayandele and Adebiyi (2006) and Ukwe *et al*. (2010) who reported similar phytochemicals in the stem and roots of *O. subscorpioidea* respectively. Wide ranges of phytochemicals have been reported to be responsible for analgesic and anti-inflammatory activities of medicinal plants; such phytochemicals include flavonoids, saponins, alkaloids and tannins (Wang *et al*., 2008; Anilkumar, 2010; Bellik *et al*., 2013). Phytochemicals have been shown to modulate various points in inflammatory processes. These modulations serve as controlling points where the amplification of the inflammatory processes can be disconnected and thereby reduce subsequent diseases risk (Yuva *et al*., 2013). Flavonoids were reported as inhibitors of prostaglandin synthase (Watanebe *et al.*, 2000) and inhibitors of COX-1 and COX-2 (Jang *et al*., 2002; Likhiwitayawuid *et al*., 2002). It has also been reported to reduce prostaglandin E_2 and nitric oxide (Takahashi *et al*., 2004). Saponins (saikosaponins) have been reported to inhibit inflammatory mediators (Bellik *et al*., 2013) and inhibit activation of nuclear factor-kappaβ (Haridas *et al*., 2001). Therefore, the observed pharmacological activities of MEOS and its fractions may be due to the presence of one or more of the reported phytochemicals. Also, it is observed that the residual aqueous and butanol fractions of MEOS exhibited great and comparable magnitude of analgesic and anti-inflammatory activities in all models tested; and this may be due to the fact that they have similar class of phytochemicals.

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1Summary

The aqueous-methanol extraction of the powdered leaf material of *Olax subscorpioidea* yielded 31.4% methanol extract. The methanol leaf extract further afforded hexane, ethyl-acetate, butanoland residual aqueous fractions. The methanol leaf extract, hexane, ethyl-acetate, butanol and residual aqueous fractions of *O. subscorpioidea* were found to possess several phytochemical constituents which include carbohydrates, cardiac glycosides, tannins, flavonoids, alkaloids, saponins, steroid and triterpenes. The oral acute toxicity studies showed that the methanol extract, residual aqueous and butanol fractions of *O. subscorpioidea* are relatively safe for oral human consumption. However, caution must be taken during long term administration. These studies also demonstrated that the methanol leaf extract as well as the residual aqueous, butanol and hexane fractions of *O. subscorpioidea* have significant analgesic and anti-inflammatory activity mediated via peripheral and central mechanisms and this therefore further support the ethno medical use of *O. subscorpioidea* in the management of pain and inflammatory conditions. The sub-acute inflammation studies showed that the residual aqueous and butanol leaf fractions of *O. subscorpioidea* are effective against acute and chronic inflammations. The

inflammatory cytokine studies demonstrated that the residual aqueous and butanol leaf fractions of *O. subscorpioidea* exhibit their anti-inflammatory activities via the inhibition of pro-inflammatory cytokines and/or stimulation of the synthesis of anti-inflammatory cytokines. The mechanistic studies show that the analgesic activity of *O. subscorpioidea* involves an interaction with serotonergic, and nitric oxide-l-arginine pathways.

6.2 Conclusion

The methanol leaf extract of *Olax subscorpioidea* possesses analgesic activity which is mediated through the central and peripheral mechanism andinvolves an interaction with serotonergic receptors and nitric oxide-l-arginine pathways; it also possesses anti-inflammatory activity whichinvolves inhibition of pro-inflammatory cytokines (such as IL-1, VEGF and EGF) and/or stimulation of anti-inflammatory cytokines (such as IL-5, IL-6 and IFN-γ).

6.3 Recommendations

1. Toxicity evaluation of *Olax subscorpioidea*extract and fractions should be carried out in evaluating the safety profile; and its effect on vital organs should also be observed.

2. The bioactive constituents responsible for the observed pharmacological activities of *Olax subscorpioidea* leaf should be isolated and characterized.

3. Pharmacokinetics studies should be undertaken so as to establish the pharmacological profile of the extract and fractions of *Olax subscorpioidea*.

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APPENDICES:

Appendix 1: Ethanobotanical/Pharmacological Information on *Olax subscorpioidea*

S/No	Traditional uses	Active part	Preparation	Reference
1	Anti-helminthes	Roots	Ethanol extract	Koné *et al*., 2012
2	Anti-malaria	Roots	Ethanol extract	Koné *et al*., 2012
3	Antioxidant	Roots		Konan *et al*., 2013
4	Anti-protease	Leaf	Isosaline and ethanol extract	Oyedapo*et al*., 1997
5	Anti-ulcer	Roots	Methanol extract	Ukwe *et al*., 2010
6	Arrhythmia	Leaf		Burkill, 1997
7	Cancer management	Roots, stem	Ethanol extract	Kayode *et al.*, 2008; Oloyede *et al*., 2012
8	Constipation	Roots,		Okoli*et al.*, 2007
9	Convulsions in children	Leaf		Oyedapo*et al*., 1997
10	Febrile symptoms	Leaf		Oyedapo*et al*., 1997
11	Guinea worm infestation	Roots		Okoli*et al.*, 2007
12	Intestinal worms	Roots	Ethanol extract	Koné *et al*. 2005.
13	Jaundice	Bark, leaf		Okoli*et al.*, 2007

14	Microbial diseases	Stem	Aqueous and ethanol extracts	Ayandele and Adebiyi, 2007
15	Reduction of pregnancy associated fat	Roots		Okoli*et al.*, 2007
16	Rheumatism	Leaf		Burkill, 1997
17	Venereal diseases	Bark, leaf		Okoli*et al.*, 2007
18	Yellow fever	Bark, leaf		Oyedapo*et al*., 1997; Okoli*et al.*, 2007

Appendix 2: Effect of Methanol Extract of *Olax subscorpioidea* on Acetic Acid Induced Writhing Test in Mice

Treatments (mg/kg)	**Mean no of Writhes ± SEM**	**Percentage Inhibition (%)**
Distilled water 10 ml/kg	34.40 ± 2.84	
MEOS 250	24.20 ± 1.44*	29.65
MEOS 500	16.60 ± 1.44**	51.75
MEOS 1,000	11.80 ± 1.43**	65.70
ASA 300	6.20 ± 1.83**	81.97

Values presented as Mean ± SEM, * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ versus control (one-way ANOVA followed by Dunnett's test), MEOS=Methanol leaf extract of *O. subscorpioidea*,ASA=acetylsalicylic acid, n=6.

Appendix 3: Effect of Methanol Extract of *Olax subscorpioidea* on Hot Plate Test in Mice

Treatments (mg/kg)	**0 min**	**60 min**	**90 min**	**120 min**	**150 min**
Distilled water 10 ml/kg	2.32 ± 0.15	1.38 ± 0.23	1.81 ± 0.26	1.80 ± 0.42	1.60 ± 0.20
MEOS 250	1.65 ± 0.11	3.04 ± 0.28 (7.57) *	2.74 ± 0.44 (5.94)	3.61 ± 0.75 (10.68)	4.92 ± 1.24 (17.82)
MEOS 500	1.25± 0.12	4.39 ± 1.20 (16.75) **	3.86 ± 0.83 (13.93)	7.14 ± 0.87 (31.41) **	10.32 ± 1.77 (48.37) **
MEOS 1,000	1.64 ± 0.14	7.28 ± 0.70 (30.72) **	11.19 ± 1.39 (52.01) **	11.47 ± 1.78 (53.54) **	14.41 ± 0.61 (69.55) **
Morphine 10	2.08 ± 0.29	8.07 ± 1.26 (33.42) **	11.84 ± 1.77 (54.46) **	15.11 ± 1.29 (72.71) **	15.34 ± 0.87 (74.00) ***

Values presented as Mean ± SEM, * $p<0.05$, ** $p<0.01$, *** $p<0.001$ versus control (repeated measures ANOVA followed by Bonferroni's test), MEOS=Methanol leaf extract of *O. subscorpioidea*,n=6.

Appendix 4: Effect of Methanol Extract of *Olax subscorpioidea* on Carrageenan-Induced Paw Oedema in Rats

Treatments(mg/kg)	Mean Increase in Rats Paw Diameter (mm)				
	1 hr	**2 hr**	**3 hr**	**4 hr**	**5 hr**
Distilled water 1 ml/kg	4.42 ± 0.30	5.98 ± 0.46	6.01 ± 0.29	6.20 ± 0.26	6.47 ± 0.32
MEOS 250	4.73 ± 0.13	5.80 ± 0.15	5.48 ± 0.31	5.62 ± 0.34	5.53 ± 0.19
MEOS 500	4.80± 0.15	5.39 ± 0.27	5.12 ± 0.18	5.02 ± 0.24*	4.75 ± 0.38**
MEOS 1,000	4.59 ± 0.31	5.29 ± 0.29	4.83 ± 0.29*	4.99 ± 0.25*	4.63 ± 0.14**
ASA 300	3.97 ± 0.30	4.50 ± 0.15**	4.06 ± 0.20**	3.77 ± 0.16**	3.78 ± 0.24**

Values presented as Mean ± SEM, * $p<0.05$, ** $p<0.01$ versus control (repeated measures ANOVA followed by Bonferroni's test), MEOS=Methanol leaf extract of *O. subscorpioidea*,ASA= acetylsalicylic acid, n=6.

Appendix 5: Effect of Residual Aqueous, Butanol and Hexane Fractions of *Olax subscorpioidea* on Acetic Acid Induced Writhing Test in Mice

Treatments (mg/kg)	Mean no of Writhes ± SEM	Percentage Inhibition (%)
Distilled water 10 ml/kg	34.40 ± 2.84	
AFOS 250	21.20 ± 2.80**	38.37
AFOS 500	16.60 ± 1.44**	51.74
AFOS 1,000	10.40 ± 1.03**	69.76
BFOS 250	23.40 ± 1.86*	31.97
BFOS 500	15.20 ± 1.24**	55.81
BFOS 1,000	11.40 ± 1.63**	66.86
HFOS 150	31.00 ± 1.58	9.88
HFOS 300	26.40 ± 2.20	23.26
HFOS 600	21.4 ± 1.03**	37.79
ASA 300	6.20 ± 1.83**	81.97

Values presented as Mean ± SEM,* $p<0.01$, ** $p<0.001$ versus control (one-way ANOVA followed by Bonferroni's test), AFOS=residualaqueous fraction, BFOS=butanol fraction, HFOS=hexane fraction, ASA=acetylsalicylic acid, n=6.

Appendix 6: Effect of Residual Aqueous, Butanol and Hexane Fractions of *Olax subscorpioidea* on Formalin-Induced Pain Test in Mice

Treatment (mg/kg)	**Duration of paw licking (sec)**		**Duration of paw licking (sec)**	
	0-5 min	**% inhibition**	**15-40 min**	**% inhibition**
Distilled water 10 ml/kg	64.00 ± 2.77		69.83 ± 2.8	
AFOS 250	43.67 ± 1.23**	31.77	44.83 ± 0.48**	35.80
AFOS 500	26.33 ± 0.87**	46.61	33.17 ± 0.65**	52.50
AFOS 1,000	26.33 ± 0.87**	58.86	26.00 ± 1.32**	62.77
BFOS 250	39.83 ± 0.31**	37.77	42.67 ± 0.95**	38.89
BFOS 500	29.17 ± 1.30**	54.42	34.83 ± 1.47**	50.12

BFOS 1,000	18.50 ± 0.99**	71.09	19.16 ± 2.10**	72.56
HFOS 150	60.50 ± 2.08	5.47	63.50 ± 1.36	9.06
HFOS 300	56.67 ± 1.15*	11.45	55.00 ± 0.63**	28.40
HFOS 600	41.33 ± 0.80**	35.42	41.17 ± 0.91**	41.04
Morphine 10	9.00 ± 0.73**	85.94	12.17 ± 1.25**	82.57

Values presented as Mean ± SEM,* $p<0.05$, ** $p<0.001$ versus control (one-way ANOVA followed by Bonferroni's test), AFOS=residualaqueous fraction, BFOS=butanol fraction, HFOS=hexane fraction, n=6.

Appendix 7: Effect of *Olax subscorpioidea* Fractions on Hot Plate Test in Mice

Treatments (mg/kg)	**Mean increase in latency (sec)**				
	0 min	**60 min**	**90 min**	**120 min**	**150 min**
Distilled water 10ml/kg	2.32 ± 0.15	1.38 ± 0.23	1.81 ± 0.26	1.80 ± 0.42	1.60 ± 0.20
AFOS 250	1.65 ± 0.15	2.02 ± 0.42	3.19 ± 0.56	4.01 ± 0.65	4.34 ± 1.34
AFOS 500	1.38 ± 0.09	2.21 ± 0.38	4.21 ± 0.37	6.76 ± 0.41*	7.21 ± 1.43*
AFOS 1,000	1.64 ± 0.24	2.19 ± 0.16	3.91 ± 0.33	6.98 ± 0.86**	8.98 ± 0.54**
BFOS 250	1.62 ± 0.14	1.98 ± 0.26	3.25 ± 0.26	3.35 ± 0.19	4.18 ± 0.77
BFOS 500	1.51 ± 0.14	2.63 ± 0.46	3.96 ± 0.50	4.88 ± 1.02	6.51 ± 1.24
BFOS 1,000	1.58 ± 0.21	2.63 ± 0.39	5.08 ± 0.84*	6.65 ± 1.51*	11.33 ± 2.23**

HFOS 150	1.39 ± 0.16	2.26 ± 0.23	2.38 ± 0.22	3.60 ± 0.43	3.36 ± 0.35
HFOS 300	1.88 ± 0.27	2.25 ± 0.24	2.66 ± 0.17	3.15 ± 0.24	3.43 ± 0.34
HFOS 600	1.62 ± 0.11	2.43 ± 0.08	2.88 ± 0.21	3.25 ± 0.18	3.62 ± 0.16
Morphine 10	2.08 ± 0.29	8.07 ± 1.26**	11.84 ± 1.77**	15.11 ± 1.29**	15.34 ± 0.87**

Values presented as Mean ± SEM, * $p<0.01$, ** $p<0.001$ versus control (repeated measures ANOVA followed by Bonferroni's post-hoc test), AFOS=residual aqueous fraction, BFOS=butanol fraction, HFOS=hexane fraction, n=6.

Appendix 8: Effect of Residual Aqueous and Butanol Fractions of *Olax subscorpioidea* on Pro-Inflammatory Cytokines

Treatments (mg/kg)	**Mean concentration of cytokines (pg/ml)**					
	IL-1α	**IL-1β**	**IL-2**	**VEGF**	**EGF**	**TNF-α**
Distilled water 1 ml/kg	6441.92 ± 524.96	232.49 ± 82.88	85.74 ± 10.24	228.90 ± 34.30	169.80 ± 33.60	6.00 ± 0.73
AFOS 1,000	4932.39 ± 809.48	592.95 ± 166.95*	101.20 ± 21.22	143.77 ± 17.49*	189.29 ± 23.60	6.82 ± 1.32
BFOS 1,000	3459.70 ± 378.09*	509.55 ± 169.42*	87.20 ± 19.89	113.10 ± 13.09**	81.74 ± 8.35*	6.32 ± 1.47
ASA 300	6166.07 ± 703.60	182.76 ± 92.94	69.24 ± 10.54	127.69 ± 11.94*	218.66 ± 30.31	5.66 ± 0.71

Values presented as Mean ± SEM, * $p<0.05$, ** $p<0.01$ versus control (one-way ANOVA followed by Dunnett's post hoc test), AFOS=residualaqueous fraction, BFOS=butanol

fraction, ASA=acetylsalicylic acid, IL=Interleukin, TNF=tumor necrosis factor, VEGF=Vascular Endothelial Growth Factor, EGF=Epidermal Growth Factor, n=6.

Appendix 9: Effect of Residual Aqueous and Butanol Fractions of *Olax subscorpioidea* on Anti-Inflammatory Cytokines

Treatments (mg/kg)	Mean concentration of cytokines (pg/ml)					
	IL-6	**IL-4**	**IL-5**	**IL-10**	**IL-13**	**IFN-γ**
Distilled water 1 ml/kg	2430.48 ± 511.42	8.45 ± 0.415	10.74 ± 4.85	48.73 ± 5.47	23.59 ± 1.84	66.39 ± 30.07
AFOS 1,000	4415.50 ± 691.95*	14.11 ± 5.29	33.53 ± 8.85**	44.96 ± 9.45	31.45 ± 6.45	142.90 ± 47.39*
BFOS 1,000	3453.10 ± 492.81	11.14 ± 3.05	24.38 ± 9.66*	53.25 ± 9.38	37.38 ± 11.07	168.74 ± 41.37*
ASA 300	3304.64 ± 971.06	7.57 ± 1.69	17.73 ± 4.59	43.61 ± 4.48	28.97 ± 3.51	120.35 ± 35.06

Values arepresented as Mean ± SEM, * $p<0.05$, ** $p<0.01$ versus control (one-way ANOVA followed by Dunnett's post hoc test), AFOS=residualaqueous fraction, BFOS=butanol fraction, ASA=acetylsalicylic acid, IL=Interleukin, IFN=Interferon, n=6.

Appendix 10: Effect of Naloxone on Analgesic Activity of Butanol Fraction of *Olax subscorpioidea* on AceticAcid-Induced Writhing Testin Mice

Pretreatment (mg/kg, *i.p*)	**Treatments (mg/kg, *p.o*)**	**No of writhes**	**Percentage inhibition (%)**
DW 10 ml/kg	DW 10 ml/kg	33.00 ± 2.31	
DW 10 ml/kg	NAL 2 (*i.p*)	29.67 ± 4.05 [a, 3]	10.09
DW 10 ml/kg	BFOS 1,000	13.50 ±1.18 [**]	59.09
NAL 2	BFOS 1,000	24.40 ± 2.54 [2]	26.06
DW 10 ml/kg	MOR 10	6.83 ± 2.43 [**]	79.30
NAL 2	MOR 10	20.00 ± 2.95 [* 1]	39.39

Values presentedas Mean±SEM; * $p<0.05$, ** $p<0.001$ versus control, [a]$p<0.01$, versus BFOS and [1] <0.05, [2] $p<0.01$, [3] $p<0.001$ versus MOR (one-way ANOVA followed by Bonferroni's test), DW=Distilled water, BFOS=Butanol fraction of *O. subscorpioidea*, NAL=Naloxone, MOR=Morphine, n=6.

Appendix 11: Effect of Prazosin on Analgesic Activity of Butanol Fraction of *Olax subscorpioidea* on AceticAcid-Induced Writhing Testin Mice

Pretreatment (mg/kg, *i.p*)	**Treatments (mg/kg, *p.o*)**	**No of writhes**	**Percentage inhibition (%)**
DW 10 ml/kg	DW 10 ml/kg	33.00 ± 2.31	
DW 10 ml/kg	PRA 1 (*i.p*)	14.67 ± 1.23	55.55
DW 10 ml/kg	BFOS 1,000	13.50 ±1.18 *	59.09
PRA 1	BFOS 1,000	9.17 ± 2.60	72.12
DW 10 ml/kg	MOR 10	6.83 ± 2.43 *	79.30
PRA 1	MOR 10	4.17 ± 1.05 * a	87.36

Valuespresented as Mean±SEM; * $p<0.001$ versus control and [a] $p<0.05$ versus BFOS (one-way ANOVA followed by Bonferroni's test), DW=Distilled water, BFOS=Butanol fraction of *O. subscorpioidea*, PRA=Prazosin, MOR=Morphine, n=6.

Appendix 12: Effect of Yohimbine on Analgesic Activity of Butanol Fraction of *Olax subscorpioidea* on AceticAcid-Induced Writhing Testin Mice

Pretreatment (mg/kg, *i.p*)	**Treatments (mg/kg, *p.o*)**	**No of writhes**	**Percentage inhibition (%)**
DW 10 ml/kg	DW 10 ml/kg	33.00 ± 2.31	
DW 10 ml/kg	YOH 1 (*i.p*)	26.33 ± 3.77 [a, 2]	20.21
DW 10 ml/kg	BFOS 1,000	13.50 ±1.18 [**]	59.09
YOH 1	BFOS 1,000	22.83 ± 2.72 [2]	30.82
DW 10 ml/kg	MOR 10	6.83 ± 2.43 [**]	79.30
YOH 1	MOR 10	18.33 ± 1.87 [* 1]	44.45

Valuespresented as Mean±SEM; * $p<0.01$, ** $p<0.001$ versus control, [a] $p<0.01$ versus BFOS and [1] $p<0.05$, [2] $p<0.001$ versus MOR (one-way ANOVA followed by Bonferroni's test),DW= Distilled water, BFOS=Butanol fraction of *O. subscorpioidea,* YOH=Yohimbine,MOR= Morphine, n=6.

Appendix 13:Effect of Propranolol on Analgesic Activity of Butanol Fraction of *Olax subscorpioidea* on AceticAcid-Induced Writhing Testin Mice

Pretreatment (mg/kg, *i.p*)	**Treatments (mg/kg, *p.o*)**	**No of writhes**	**Percentage inhibition (%)**
DW 10 ml/kg	DW 10 ml/kg	33.00 ± 2.31	
DW 10 ml/kg	PRO 20 (*i.p*)	9.83 ± 0.87 *	70.21
DW 10 ml/kg	BFOS 1,000	13.50 ±1.18 *	59.09
PRO 20	BFOS 1,000	16.00 ± 1.41 *[1]	51.52
DW 10 ml/kg	MOR 10	6.83 ± 2.43 *	79.30
PRO 20	MOR 10	13.83 ± 2.70 *	58.09

Values presented as Mean±SEM; * $p<0.001$ versus control, [1]$p<0.05$ versus MOR (one-way ANOVA followed by Bonferroni's test), DW=Distilled water, BFOS=Butanol fraction of *O. subscorpioidea*, PRO=Propranolol, MOR=Morphine, n=6.

Appendix 14: Effect of Metergoline on Analgesic Activity of Butanol Fraction of *Olax subscorpioidea* on AceticAcid-Induced Writhing Testin Mice

Pretreatment (mg/kg, *i.p*)	**Treatments (mg/kg, *p.o*)**	**No of writhes**	**Percentage inhibition (%)**
DW 10 ml/kg	DW 10 ml/kg	33.00 ± 2.31	
DW 10 ml/kg	MET 2 (*i.p*)	29.67 ± 3.66 [b, 2]	10.09
DW 10 ml/kg	BFOS 1,000	13.50 ±1.18 [**]	59.09
MET 2	BFOS 1,000	25.00 ± 1.23 [a, 2]	24.24
DW 10 ml/kg	MOR 10	6.83 ± 2.43 [**]	79.30
MET 2	MOR 10	21.00 ± 0.89 [* 1]	36.36

Values presented as Mean±SEM; * $p<0.01$, ** $p<0.001$ versus control, [a] $p<0.05$ [b] $p<0.001$ versus BFOS and [1] $p<0.01$, [2] $p<0.001$ versus MOR (one-way ANOVA followed by Bonferroni's test), DW=Distilled water, BFOS=Butanol fraction of *O. subscorpioidea,* MET=Metergoline, MOR=Morphine, n=6.

Appendix 15: Effect of Glibenclamide on Analgesic Activity of Butanol Fraction of *Olax subscorpioidea* on AceticAcid-Induced Writhing Testin Mice

Pretreatment (mg/kg, *i.p*)	**Treatments (mg/kg, *p.o*)**	**No of writhes**	**Percentage inhibition (%)**
DW 10 ml/kg	DW 10 ml/kg	33.00 ± 2.31	
DW 10 ml/kg	GLI 5 (*i.p*)	24.80 ± 4.16 [a, 1]	24.85
DW 10 ml/kg	BFOS 1,000	13.50 ±1.18 [*]	59.09
GLI 5	BFOS 1,000	23.40 ± 1.97 [1]	29.09
DW 10 ml/kg	MOR 10	6.83 ± 2.43 [*]	79.30
GLI 5	MOR 10	15.80 ± 1.24 [*]	52.12

Valuespresented as Mean±SEM; * $p<0.001$ versus control, [a]$p<0.05$, versus BFOS, [1] $p<0.001$ versus MOR (one-way ANOVA followed by Bonferroni's test), DW=Distilled water, BFOS=Butanol fraction of *O. subscorpioidea*, GLI=Glibenclamide, MOR=Morphine, n=6.

Appendix 16: Effect of L-arginine on Analgesic Activity of Butanol Fraction of *Olax subscorpioidea* on AceticAcid-Induced Writhing Testin Mice

Pretreatment (mg/kg, *i.p*)	**Treatments (mg/kg, *p.o*)**	**No of writhes**	**Percentage inhibition (%)**
DW 10 ml/kg	DW 10 ml/kg	33.00 ± 2.31	
DW 10 ml/kg	L-ARG 50 (*i.p*)	25.33 ± 2.70 [a, 3]	23.24
DW 10 ml/kg	BFOS 1,000	13.50 ±1.18 [**]	59.09
L-ARG 50	BFOS 1,000	28.50 ± 2.72 [b, 3]	13.63
DW 10 ml/kg	MOR 10	6.83 ± 2.43 [**]	79.30
L-ARG 50	MOR 10	18.33 ± 3.03 [* 1]	44.45
DW 10 ml/kg	L-NNA 50 (*i.p*)	12.67 ± 2.14 [**]	61.61

L-ARG 50	L-NNA 50 (*i.p*)	22.17 ± 2.33 [2]	32.82

Values presented as Mean±SEM; * $p<0.01$, ** $p<0.001$ versus control, [a] $p<0.05$, [b] $p<0.01$ versus BFOS, [1] $p<0.05$, [2] $p<0.01$, [3] $p<0.001$ versus MOR and [#] $p<0.05$ versus L-NNA (one-way ANOVA followed by Bonferroni's test), DW=Distilled water, BFOS=Butanol fraction of *O. subscorpioidea*, L-ARG=L-arginine, L-NNA=Nomega-nitro-l-arginine, MOR=Morphine, n=6.