CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF THE STUDY

Inflammation is an adaptive physiological response of the body to foreign agents such as infections, toxic chemicals, allergens, burns and other stimuli that could cause tissue injuries (Shrestha *et al.*, 2014). Unregulated level of inflammatory responses can result to inflammatory disorders such as rheumatoid arthritis, inflammatory bowel diseases, type-2 diabetes, cardiovascular diseases, among others (Deepa and Renuka, 2014; Yan *et al.*, 2015).

It has been established that one of the host defense mechanisms during inflammatory response is production of reactive oxygen species by phagocytic leukocytes which further results in chronic inflammatory disorders (Khatoon *et al.*, 2013; Pandey *et al.*, 2017).

Presently, synthetic anti-inflammatory drugs leave much to be desired due to their adverse effects such as gastrointestinal irritations, gastric ulcer, nephrotoxicity, hypertension among others (Rang *et al.*, 2003; Ezeja *et al.*, 2015). Thus, researches on alternative agents for the management of inflammatory disorders are being considered as an avenue in the discovery of new molecules with little or no side effects (Ezeja *et al.*, 2015).

Medicinal plants are the richest bio-resources for alternative systems of medicine and food supplements which promote better nutritional and health benefits to humanity (Obi *et al*., 2012). Presently, there is a revitalization of interest in the use of medicinal plants by over 80% of the world populace in both developing and developed countries (Ogbonnia *et al*., 2011).

For instance, a large proportion of African population depends on herbal medicines for their primary health care needs. This is because such remedies are affordable, acceptable and accessible to the populace (Ezeja *et al.,* 2015). Medicinal plants have beneficial effects due to their abilities to act on various targets, thereby eliciting desirable effects with minimal or no side effects (Okigbo *et al.,* 2009). Also, various existing conventional drugs have been derived from medicinal plants through pharmacological screening using established experimental models (Yadav and Agarwala, 2011).

Due to the availability of chemical diversity, natural products from medicinal plants provide limitless avenue for new drug leads in the management of inflammatory disorders (Robert and Ilse, 2014). As such, several medicinal plants are being screened and validated as potentials for anti-inflammatory agents from natural sources (Robert and Ilse, 2014).

It is also well known that anti-inflammatory regulatory process is complemented by anti-oxidants which aid in scavenging reactive oxygen metabolites thereby promoting healing process (Kumar *et al.,* 2014).

Safety concern of herbal medicines is a major issue. Most patronizers have the misconception that medicinal plants are safe because they are from natural sources (Uma *et al.,* 2013). Sometimes, pregnant women and breastfeeding mothers who patronize herbal products rarely seek advice from healthcare professionals concerning the safety of such herbal remedies. This has resulted to congenital malformation in infants (Costa *et al.,* 2012). A study conducted in Aminu Kano Teaching Hospital on the use of herbal medicines by pregnant women revealed that 31.4% of pregnant women used herbal medicines during pregnancy (Tamuno *et al.,* 2010). A socio-demographic study carried out amongst pregnant women at Imo State University Teaching

Hospital, Orlu, South-East Nigeria also showed that 36.8% of pregnant women used herbal medicines (Chukwuma *et al.*, 2016).

Dryopteris filix-mas (*D. filix-mas*) is one of the alternative herbal medicines commonly used among the populace in the Southern part of Nigeria in the treatment of rheumatoid arthritis and inflammation. However, there is no study to validate these ethnomedicinal claims as well as its toxicity profile. Therefore, it is important that the folkloric anti-inflammatory claim of *D. filix-mas* and its safety are assessed.

1.2 STATEMENT OF PROBLEM

Inflammation is implicated in the pathogenesis of several diseases such as rheumatoid arthritis, infections and other disease conditions. Prolonged use of the conventional anti-inflammatory drugs is associated with side effects such as gastrointestinal (G.I) ulceration and bleeding, hepatic and renal damage, hypertension among others.

Limitations posed by synthetic agents have necessitated the search for more effective anti-inflammatory drugs from natural sources which may pose little or no side effects.

D. filix-mas is a medicinal plant used by populace in Southern parts of Nigeria for the treatment of rheumatoid arthritis, wounds and inflammation, but this folkloric claim lacks scientific validation. Also, the use of *D. filix-mas* ethanol leaf extract in various disease conditions demands its toxicity evaluation.

1.3 JUSTIFICATION OF THE STUDY

Natural products from medicinal plants have played vital roles as alternative agents in the management of numerous inflammatory conditions, including rheumatoid arthritis (Mona *et al.*,

2014). Several medicinal plants have been screened and validated as potentials for natural anti-inflammatory agents (Robert and Ilse, 2014). Safety profiles of such medicinal plants have also been imperatively validated (Prasanth *et al.,* 2014).

In light of the foregoing, there is a paucity of scientific information about the anti-inflammatory properties and safety profile of the leaf extract of *D. filix-mass,* a medicinal plant traditionally used among the Southern Nigerian populace. This study is meant to fill up these gaps.

1.4 AIM OF THE STUDY

This study was designed to evaluate the anti-inflammatory properties and toxicological profile of the ethanol leaf extract of *D. filix-mas*.

1.5 OBJECTIVES OF THE STUDY

1. This study assessed the acute toxicity profile (LD_{50}) of *D. filix-mas*.
2. Phytochemical screening of the extract and fractions were carried out.
3. Anti-inflammatory and antioxidant activities of the extract and fractions were carried out using *in-vitro* and *in-vivo* models.
4. The most active fraction was purified using vacuum liquid chromatography (VLC) techniques and VLC fractions were subjected to anti-inflammatory and antioxidant assays.
5. The most activive VLC fraction was further purified by Sephadex Chromatography and the structure was elucidated using high performance liquid chromatography mass spectrometry (HPLC-MS) and proton nuclear magnetic resonance (NMR).

6. A two weeks, three months and six months repeated toxicity tests were carried out consecutively to determine the chronic effect of the extract on vital organs, haematological and biochemical parameters.
7. Teratogenic study was carried out to establish the safety of the leaf extract in pregnancy.

1.6 SIGNIFICANCE OF THE STUDY

It is of importance to search for more tolerable alternatives anti-inflammatory drugs from natural sources due to the side effects associated with synthetic counterparts. Also, safety evaluation of promising natural products (that most of the populace claimed to be safe) is necessary.

Consequently, this work would help to authenticate the anti-inflammatory potentials of *D. filix-mas* as well as isolate and characterize the bioactive component(s) responsible for its anti-inflammatory activity. It would also serve as an avenue for the discovery of potential anti-inflammatory agents from natural source.

The toxicological profile of *D. filix-mas* would aid in obtaining regulatory approval for its consumption and commercialization.

1.7 HYPOTHESIS

Based on the folkloric information gathered about this plant, it is hereby hypothesized that the ethanol leaf extract, fractions and bioactive constituent(s) of *Dryopteris filix-mas* possess anti-inflammatory properties using various *in-vitro* and *in-vivo* experimental models.

Also, the ethanol leaf extract possesses acute, long term and teratogenic toxic effects in animals.

CHAPTER TWO

LITERATURE REVIEW

2.1 *DRYOPTERIS FILIX-MAS*

2.1.1 DESCRIPTION

D. filix-mas, an evergreen fern growing up to 60-150 cm is native to Europe, Asia, and North America. Its growth is mostly favored by damp and shady environments (Bafor *et al.*, 2017). The leaves are bipinnated and consist of 20-35 pinnae on each side of the rachis. At both ends, the leaves taper with the basal pinnae about half the length of the middle pinnae. The pinules are rather blunt and equally lobed all around. The stalks are covered with orange-brown scales. Five to six sori develop in two rows on the abaxial surface of the mature blade (Sekendar *et al.*, 2012).

Its common names in ancient literature include, worm fern, wood fern and male fern (*filix mas*). Other names include; aspidium, bear's paw, knotty brake and shield fern (Bafor *et al.*, 2017). Homeopathically, it is called mother tincture (Uwumarongie *et al.*, 2016). It is locally recognized as Eraketa by the Urhobos, Akpaka or Akolor by the Igbos and Imu by people in Ondo State.

2.1.2 SCIENTIFIC CLASSIFICATION

Scientifically, it can be classified as follows; kingdom (plantea), division (pteridophyta), class (polypodiopsida/pteridopsida), order (polypodiales), family (Dryopteridaceae), genus (Dryopteris), specie (*D. filix-mas)* and binomial name (*D. filix-mas* (L.) Schott (Bafor *et al.*, 2017).

Figure 1: Picture of *D. filix-mas.*

2.1.3 ETHNO-MEDICINAL USES

D. filix-mas is known to be the ancient remedy against tapeworm infestation (Sekendar *et al.,* 2012). In Russia, the drug "Phylixan" (Filixanum) from the rhizome of *D. filix-mas* was used as anthelmintic remedy (Valentyna *et al.,* 2017).

In animal therapy, vegetarians use the extract of *D. filix-mas* against flatworms (*Dicrocoelium* and *Fasciola*) and tap worm infestations. It is also used in combination with carbon tetrachloride for the treatment of distomatosis in sheep. Its active component, filicin acts as a vermifuge and aids in the detachment of the scolex from the intestinal mucosa (Sekendar *et al.,* 2012; Laudato and Capasso, 2013).

D. filix-mas is one of the selected ferns having ethnomedicinal value as revealed by Dioscorides (50 AD) in his book (Amanika *et al.,* 2017). A historical source revealed that *D. filix-mas* is one of the medicinal plants used to cure splenomegaly in malaria infection, feverish condition as well as dropsy. Its decoction is applied topically in the treatment of mumps, carbuncles, abscesses, boils and sores provoked by severe burns (Tagarelli *et al.,* 2010). It is also used to stop bleeding (internal haemorrhage and uterine bleeding) due to its astringent properties (Tagarelli *et al.,* 2010). It is used among the rural dwellers in Lebanon in the treatment of neuralgia and rheumatic disorders (Marc *et al.,* 2008). An infusion of the rhizome is used externally as bath and application in rheumatism, septic wounds, hemorrhoids and ulcers in the Republic of Azerbaijan. The root infusion is also used as an anti-pyretic in Georgia (Batsatsashvili *et al.,* 2016). The extracts are used for the treatment of recurrent bloody nose, heavy menstrual bleeding, postpartum haemorrhage and wounds (Bafor *et al.,* 2017). It is also reported as one of the ferns with secondary metabolites useful in countering aging and chronic diseases (Valentyna *et al.,*

2017). The cooked young frond is eaten as an aid in losing weight. Adult dosage is about 5 to 8 grams per day. It is used as astringent, natural intestinal cleanser and revitalization of normal liver function. Its leaf infusion is used to heal cough and catarrh. Folkloric report revealed that the *D. filix-mas* could stimulate the uterus. It may induce diarrhea and colic in infants (Bafor *et al.,* 2017).

Its young coiled frond is used in various parts of Nigeria as a worm expeller. Report also showed that its leaves infusion is used as aphrodisiac (Nwosu *et al.,* 2002; Bafor *et al.,* 2017). It is used in various parts of Edo and Delta State, in the treatment of gastrointestinal disorders, boils, abscesses, sores and in management of rheumatoid arthritis. Local farmers also squeeze its leaves with water as an alternative to soap for bathing due to its foam.

2.1.4 REPORTED BIOLOGICAL AND PHARMACOLOGICAL ACTIVITIES OF *D. FILIX-MAS* AND OTHER RELATED SPECIES

Recent studies on the Pharmacognostic constituents of *D. filix-mas* revealed the presence of glycosides, tannins, flavonoids and steroids in its leaf extract (Uwumarongie *et al.,* 2016). Proximate analysis of the extract revealed the presence of protein (1.52 %), fibre (30.08 %), lipid (2.70 %), and carbohydrate (51.46 %). Other nutrients present include sodium (200 mg/kg), potassium (9700 mg/kg), calcium (6000 mg/kg), phosphorus (800 mg/kg), chloride (179.24 mg/kg), zinc (3.95 mg/kg) and manganese (0.85 mg/kg). However, lead, chromium, nickel and cadmium were absent (Uwumarongie *et al.,* 2016). Administration of the extract caused a dose-dependent decrease in gastrointestinal transit time as well as reduction in intestinal fluid volume (Uwumarongie *et al.,* 2016).

Flavaspidic acid and Filixic acid have also been isolated from *D. filix-mas* (Hwang *et al.,* 2013; Valentyna *et al.,* 2017).

Recent study by Bafor *et al* (2017) revealed that the leaves of *D. filix-mas* inhibited uterine contraction. These findings contradict its traditional use in postpartum haemorrhage and uterine stimulation.

The insecticidal activity of the ethanol extract of *D. filix-mas* against *Corcyra cephalonica* staint was also reported (Shukla and Tiwari, 2011). Antihelmintic activity (Urban *et al.,* 2014) and its broad antimicrobial activity against gram-positive and gram negative bacteria have been reported (Mandal and Mondal, 2014).

A study by Soare and co-workers on the antimicrobial and antioxidant properties of methanol extracts obtained from leaves of *Athyrium flix-femina* and *D- flix-mas* showed the best antimicrobial activity compared to the other extracts (Soare *et al.,* 2012).

Safety study on *Dryopteris crassirhizoma* (a related specie in the family of *Dryopteridicea*) using bacterial reverse mutation, chromosomal aberration and bone marrow micronucleus tests models of genotoxicity showed that *Dryopteris crassirhizoma* did not increase the number of the bacterial revertant and chromosomal aberration significantly. Also, it did not cause an increase in micronucleated polychromatic erythrocytes (MNPCE) in mouse bone marrow. The authors concluded that the oral LD_{50} of *Dryopteris crassirhizoma* was above 2000 mg/kg and it was non genotoxic (Hwang *et al.,* 2013). The anti-oxidative, anti-obesity, anti-bacterial, anti-viral, anthelmintic and anti-tumor properties of *Dryopteris crassirhizoma* have also been investigated (Chang *et al.,* 2006; Lu *et al.,* 2012; Hwang *et al.,* 2013).

Active components such as phloroglucinol derivatives (albaspidin, aspidin, flavaspidic acids, and dryocrassin), triterpenes (acylphloroglucinols), flavonoids (desmethoxymatteucinol, matteucinol, nerolidol, epicatechin) and phenolics (dryopteroside, caffeic acid, hexacosanoic acid) were found to be present in *Dryopteris crassirhizoma*. Phytochemical characterization (HPLC and GC/MS) of *Dryopteris crassirhizoma* aqueous extract revealed the presence of resveratrol, quercetin and kaempferol with retention times of 27.11, 29.14 and 32.73 min, respectively (Hwang *et al.,* 2013).

Flavonoid contents and radical scavenging activities of various parts of *Dryopteris erythrosora* (specie of Dryopteris) were also reported. The stem extract had the highest flavonoid content followed by root, rachis and leaf. Their DPPH radical scavenging abilities were also in the same pattern (Min *et al.,* 2012).

2.2 INFLAMMATION

2.2.1 DEFINITION AND CAUSES OF INFLAMMATION

Inflammation can be defined as an adaptive physiological response of the body to foreign agents such as infections, toxic chemicals, allergens, burns and other stimuli that could cause tissue injuries (Shrestha *et al.,* 2014). Whenever there is an attack of the body system by foreign insults, the immune system triggers complex series of responses leading to wide range of physiological and pathological morbidities (Shrestha *et al.,* 2014). The chief objective of inflammation is to localize and eliminate the causative agent and also repair the surrounding tissue from further injury (Shrestha *et al.,* 2014).

During inflammation, there is modification in the signaling pathways resulting to increase in the level of inflammatory cytokines, lipid peroxides as well as free radicals, thereby leading to the

development of several inflammatory diseases including; gastrointestinal diseases (inflammatory bowel disease and pancreatitis), diseases of other organs (neurodegenerative diseases, cardiovascular diseases, allergy, asthma, bronchitis, rheumatoid arthritis, chronic kidney diseases), metabolic diseases (diabetes, obesity), skin diseases (scleroderma and psoriasis) among others (Yan *et al.,* 2015).

Inflammatory disorders such as; rheumatoid arthritis, inflammatory bowel diseases, type-2 diabetes, cardiovascular diseases and aging are attributed to unregulated levels of pro-inflammatory cytokines. Inflammation is also involved in combating infections as well as in wound healing process (Yan *et al.,* 2015).

2.2.2 MEDIATORS OF INFLAMMATION

Mediators involved in the development of inflammatory disorders include neutrophil derived free radical, reactive oxygen species (ROS), nitric oxide (NO), prostaglandins and cytokines. Cyclooxygenases (constitutive COX-1 and inducible COX-2) and lipoxygenase (5-LOX) enzymes are responsible for the transformation of arachidonic acid into the potent biologically active lipid mediators which are associated with inflammation (Anoop and Bindu, 2015). The major player of inflammation is tissue mast cells, which are embedded with mediators of inflammation such as histamine and bradykinin, which induce the production of prostaglandins (PGs) and leukotrienes (LT) (Anoop and Bindu, 2015).

COX-1 mediates physiological response which is constitutively expressed as a house keeping enzyme in almost all tissues, thereby aiding as a cytoprotective agent in the stomach as well as facilitating platelet aggregation (Rekha *et al.,* 2014). Lipoxygenases (LOX), a family of non-heme iron-containing dioxygenases catalyzing the biosynthesis of leukotrienes, function as

initiators of inflammation (Anoop and Bindu, 2015). The first step in inflammatory process is by either the metabolism of arachidonic acid by cyclooxygenase pathway to prostaglandin and thromboxane A_2 or the metabolism of arachidonic acid by the lipooxygenase pathway to hydroperoxyeicosatetraenoic acid (HPETE) and leukotriene (LT_3), which serve as significant biologically active mediations of inflammatory and allergic reaction events. High level of leukotriene is associated with rhinitis, asthma, psoriasis, colitis ulcerosa as well as rheumatoid arthritis (Rekha *et al.,* 2014). Oedema, leukocyte infiltration and granuloma formation are the major components of inflammatory symptoms and tissue injury (Rekha *et al.,* 2014).

2.2.3 STAGES OF INFLAMMATION

Inflammation exists in two stages viz; acute and chronic inflammation. Acute inflammation is geared towards providing beneficial protection to the body system through the activation of the immune system, which acts for a short duration. In the process of acute inflammation, there is increased migration of plasma, white blood cells (neutrophils, monocytes- which become macrophages when they enter tissues from the blood, lymphocytes B and T lymphocytes for adaptive immunity) and natural killer cells from the blood to the site of injury as a result of interaction of toll-like receptors in mast cells invaders. This is followed by a flow of biochemical events which propagate and mature the inflammatory response (Rekha *et al.,* 2014).

The second stage of inflammation (chronic inflammation) comes into play when the acute stage of inflammation lasts for a longer time than its expected duration, thereby initiating several chronic diseases including obesity, cancer, metabolic disorders, diabetes, arthritis, pancreatitis, cardiovascular and neurodegenerative disorders. During chronic inflammation, inflammation and repair take place simultaneously (Yan *et al.,* 2015). Chronic inflammation may be caused by the

following; self-antigen which fails to induce autoimmune response, an irritant of low intensity and long persistence which does not stimulate a major acute inflammatory reaction (Shrestha *et al.*, 2014).

By comparison, acute inflammation is associated with the recruitment of several numbers of neutrophil leukocytes, while chronic inflammation has macrophages as its dominant infiltrating cells. The systemic effects of acute inflammation include fever, leukocytosis, while that of chronic inflammation include tiredness, sleepiness, weight loss and wasting (Shrestha *et al.*, 2014).

2.2.4 OXIDATIVE STRESS AND CHRONIC DISEASES

Oxidative stress is an imbalance between reactive oxygen species (free radicals and reactive metabolites) production and the antioxidant defense system. Among the reactive oxygen species (ROS), the hydroxyl radical appears to be the most harmful, leading to the activation of immune cells, which further liberate pro-inflammatory cytokines, reactive oxygen and nitrogen species. These damage biological molecules and also results to imbalances in physiological and pathological pathways (Yan *et al.*, 2015).

Free radicals (unpaired electrons) are constantly produced in all normal living cells. However, in the presence of exogenous and endogenous free radicals, the endogenous antioxidant system becomes inadequate, leading to oxidative damage to different molecules, such as lipids, proteins and nucleic acids thereby initiating degenerative diseases (Kumar *et al.*, 2014).

Continued oxidative stress and oxidative damage play a significant role in the pathophysiology of many chronic inflammatory disorders (Yan *et al.*, 2015).

Research has shown that oxidative stress and chronic diseases are closely related pathophysiologically (Sikora *et al,* 2010). Thus, oxidative stress can induce chronic diseases and vice versa. In several disease conditions, both of them are mostly involved. For instance, the propagation of oxidative stress from diseased organs to other parts of the body is mediated by inflammation (Reuter *et al.,* 2010).

2.2.5 ANTIOXIDANTS AND INFLAMMATORY DISORDERS

Antioxidants are agents that prevent free radicals from causing damage to the body system. Antioxidants donate electrons to stabilize and neutralize the harmful effects generated by free radicals, thereby protecting the body against degenerative diseases. They delay or inhibit the propagation of oxidizing chain reactions (Khatoon *et al.,* 2013).

Studies have shown that intake of antioxidant and anti-inflammatory compounds help in ameliorating oxidative stress and inflammation (Deepa *et al.,* 2014). Several medicinal plants and spices are known to exhibit good antioxidant properties by reducing oxidative stress-induced tissue injury. For instance, plant-derived polyphenols exhibit considerable free-radical scavenging activity by donating electrons and stabilizing the antioxidant system (Yan *et al.,* 2015).

Curcumin, a yellow coloring agent extracted from turmeric have been shown to alleviate oxidative stress, inflammation in chronic diseases and also regulates inflammatory and pro-inflammatory pathways associated with most chronic diseases (Sikora *et al.,* 2010; Yan *et al.,* 2015).

Conventional antioxidants have limitation side effects which has necessitated the search for natural antioxidants from medicinal plants that could alleviate oxidative stress and also regulate

inflammatory and pro-inflammatory pathways associated with chronic diseases (Sikora *et al.*, 2010).

2.2.6 MANAGEMENT OF INFLAMMATORY DISORDERS

The inflammatory process involves activation of several pathways. The body's inflammatory response possesses an on/off switch. However, during persistent inflammation when inflammatory reaction is beyond the body's self-protection, the need to control the inflammatory process with anti-inflammatory drugs comes into play (Rekha *et al.*, 2014; Ezeja *et al.*, 2015). Thus, agents that could suppress activation of such pathways could serve as useful anti-inflammatory candidates in drug development.

For instance, inhibition of COX-2-dependent prostaglandin (PG) synthesis accounts for the anti-inflammatory and analgesic effects of non-steroidal anti-inflammatory drugs (NSAIDs), while inhibition of lipoxygenase (LOX) pathway is considered to be partly responsible for the anti-inflammatory and anti-allergic activity due to the formation of the leukotrienes (LTs) (Anoop and Bindu, 2015).

2.2.6.1 SYNTHETIC ANTI-INFLAMMATORY DRUGS

Anti-inflammatory drugs can be classified as follows;

Non-selective COX inhibitors

These include; Salicylates (Aspirin), Propionic acid derivatives (Ibuprofen, Naproxen, Ketoprofen and Flurbiprofen), Anthranilic acid derivative (Mephenamic acid), Aryl-acetic acid derivatives (Diclofenac, Aceclofenac), Oxicam derivatives (Piroxicam, Tenoxicam), Indole derivative (Indomethacin) and Pyrazolone derivatives (Phenylbutazone, Oxyphenbutazone) (Day and Graham, 2013; Nicholas and Nicole, 2016).

COX-2 inhibitors

These include Nimesulide, Meloxicam and Nabumetone.

Selective COX-2 inhibitors

These include; Celecoxib, Etoricoxib, Parecoxib, analgesic-antipyretics with poor antiinflammatory action.

2.2.6.2 MECHANISMS OF ACTION OF NON-STEROIDAL ANTI-INFLAMMATORY DRUGS (NSAIDS)

It is established that prostaglandins and thromboxanes play crucial roles in the pathogenesis of pain, inflammation, fever and blood clotting. All NSAIDs elicit their activities by inhibiting cyclooxygenase which is responsible for the conversion of arachidonic acid to endoperoxide intermediate (PGG_2 and PGH_2) (Nicholas and Nicole, 2016).

2.2.6.3 INDICATIONS OF NSAIDS

The intended use of NSAIDs is in the treatment of acute and chronic inflammation and pains (especially nociceptive pains), neuropathic pain syndromes (when combined with other analgesics), symptomatic relief of rheumatoid arthritis, severe form of osteoarthritis, acute gout, ankylosing spondylitis, psoriatic arthritis, dysmenorrhoea (painful menstruation), postoperative pain, back pain, sciatica, sprains and dental pain among others (Rang *et al.,* 2003; Nicholas and Nicole, 2016).

2.2.6.4 CONTRAINDICATIONS AND PRECAUTIONS ON THE USE OF NSAIDS

NSAIDs are not recommended for use among patients that are allergic to Aspirin or any other NSAID. They are contraindicated among pregnant and breastfeeding mothers, patients below 16

years, people on anticoagulants (blood thinning agents) medications and those with active peptic ulcer (Rang *et al.,* 2003).

2.2.6.5 ADVERSE AFFECTS OF NSAIDS

The major side effects posed by NSAIDs are mainly gastrointestinal, hematological, renal, hepatic and central nervous system related.

Gastrointestinal side effects include gastric irritation, gastric ulcer, esophagitis, upper gastrointestinal bleeding, and death. In as much as the COX-1 pathway is associated with the maintenance of the integrity of the gastrointestinal (GI) lining by prostaglandin production, inhibition of COX -1 pathway by the use of NSAIDs would lead to gastric irritation and bleeding (Nicholas and Nicole, 2016).

Hepatic side effects include; Increase in transaminases level, hepatic failure. Renal side effects include; sodium and water retention, chronic renal failure, and interstitial nephritis. This occurs as a result of inhibition of the COX-1 pathway by NSAID and subsequent impairment of prostaglandin, which regulates blood flow, thereby causing acute renal failure and acute intestinal nephritis (Nicholas and Nicole, 2016).

Central nervous system (CNS) side effects include; mental confusion, vertigo, dizziness, headache, ataxia and seizure precipitation. These neurological side effects are as a result of prostaglandin synthesis inhibition by NSAIDs therapy (Auriel *et al.,* 2014).

Haematological side effects encompass increase bleeding rate (due to decreased platelet aggregability), thrombocytopenia, haemolytic anaemia and agranulocytosis. Other side effects include asthma, nasal polyposis and skin rashes (Nicholas and Nicole, 2016).

2.2.6.6 MEDICINAL PLANTS USED IN THE TREATMENT OF INFLAMMATION

Due to the side effects associated with conventional anti-inflammatory drugs, there is increase in reliance in alternative remedies. Most of these alternative remedies are derived from medicinal plants and some of them have been proven to elicit significant anti-inflammatory activities, thereby validating their ethno medicinal claims in the management of inflammation (Genesh *et al.,* 2013; Rheka *et al.,* 2014).

For example, the leaf extract of *Mimusops elegeni L* from the family of Sapotaceae has been reported to elicit good anti-inflammatory and antioxidant properties (Ganesh *et al.,* 2013), leaf extract from *Cissus aralioides* has been reported to posses significant anti-inflammatory and antioxidant potentials (Ezeja *et al.,* 2015).

Also, inhibition of COX- and LOX enzymes was suggested to be the possible anti-inflammatory mechanism(s) of *Abroma augusta* (from the family of Serculiaceae) and *Desmodium gangeticum* (from the family of Fabacea) (Rekha *et al.,* 2014).

Ethanol leaf extract of eight selected Zimbabwean plants including *Amaranthus spinosus* L (Amaranthaceae), *Cassia abbreviatta* Oliv (Leguminosae), *Parinari curatellifolia* Planch ex Benth (Chrysobalanaceae), *Brachystegia boehmii* Taub (Leguminosae), *Gymnosporia senegalensis* Loes (Combretaceae), *Combretum zeyheri* Sond (Combretaceae), Combretum molle (Combretaceae) and *Combretum platypetalum* Welw (Combretaceae) were also investigated for COX-1 and COX-2 enzyme inhibitory activities, erythrocyte membrane stabilization, albumin denaturation inhibition assays free-radical (1,1-diphenyl-2-picrylhydrazyl (DPPH) and tetramethoxy azobismethylene quinone (TMAMQ) scavenging assays. Among the samples screened, ethanol extracts of *P. curatellifolia* and *C. zeyheri* have potent antioxidant activity

while *C. platypetalum*, *C. molle* and *B. boehmii* extracts elicited significant *in vitro* anti-inflammatory activities (Elaine and Stanley, 2016).

In another study, experimental evaluation of *Albuca setosa* (Hyacinthaceae family) aqueous extract on membrane stabilization, protein denaturation and white blood cell migration during acute inflammation revealed that the extract possesses anti-inflammatory properties, thereby justifying its folkloric uses (Umapathy *et al.,* 2010).

A review article by Abdullatif and co-workers on the *in-vivo* and *in-vitro* anti-inflammatory activity of plant products from 2005 till 2016 revealed several anti-inflammatory compounds with various mechanisms of actions (Abdullatif *et al.,* 2016). Plant based food such as carotenoids, flavonoids, phenolic acids, monoterpenes and sulfides were reported to inhibit nuclear factor kappa B (NFκB), reduce C-reactive protein (CRP) and IL-6 levels (Abdullatif *et al,* 2016). Plant natural products such as polyphenols, capsaicin, curcumin, ascorbic acid, indol-3-carbinol, geraniol, sulphoraphane, gingerol, lycopene, deoxyelephantophin were reported to elicit profound anti-inflammatory activities by reduction of cytokine levels, inhibition of COX-2, iNOS, NFκB and STAT (signal transducers and activators of transcription) activities (Shah *et al,* 2011).

Oleanolic acid, polyphenols, coumarin, β-amyrin, ursolic acid, β-sitosterol from plant barks were reported to significantly inhibit COX and iNOS activity and also attenuate paw edema (Arya *et al.,* 2011). Sesquiterpenoids, diterpenoids, steroids, ceramide, cerebrosides, from marine natural products were reported to reduce cytokines, NO and PGs levels and also inhibit COX and iNOS activities (Lee *et al.*, 2013).

Other active compounds such as ambrosanolide, betulinic acid, ardisiaquinone G, polyphenols, alkaloids, glycosides, terpenoids, resins, essential oils, fatty acids, flavonoids, polysaccharides, phenolic compounds, steroids, cannabinoids, glycoprotein, essential oils, fatty acids, nyctanthic acid, phyllanthin, lignans, anthraquinones, saponins, isogarcinol, andrograpanin, hinokitiol, tectorigenin, α-iso-cubebene, schisantherin A, psoralidin, formosumone A, isofraxidin, maslinic acid, mangiferin, catethin, epicatechin among others from natural sources elicited profound anti-inflammatory activities (Kumar *et al.,* 2013; Abdullatif *et al.,* 2016).

Some isolated compounds such as Quercetin (from *Allium cepa, Camellia sinensis, Hypericum perforatum, Podophyllum peltatum*), Allicin (from *Allium* sativum), (-) - Myrtenol (from *Tanacetum vulgare* and *Aralia cachemirica*), various terpenes and polyphenols (from *Nepenthes mirabilis*), Ferulic acid (from *Solanum lycopersicum* L), 3-Hydroxyanthranilic acid (from *Hibiscus tilliaceus*) has been reported extensively to elicit profound anti-inflammatory activities (Abdullatif *et al.,* 2016).

2.2.7 SOME PLANT-DERIVED ANTI-INFLAMMATORY COMPOUNDS

In recent years, some prominent anti-inflammatory compounds have been derived from medicinal plants and tested in clinical trials. They include curcumin, colchicine, resveratrol, capsaicin, epigallocatechin-3-gallate (EGCG), and quercetin (Robert and Ilse, 2014).

Curcumin: Curcumin, the main ingredient of turmeric (*Curcuma longa*, Zingiberaceae), has been used for centuries in Ayurvedic medicine in the management of inflammatory disorders. It was reported to elicit potent anti-inflammatory, antioxidant, anticancer/proapoptotic, and antibacterial activities as a result of its several mechanisms of actions. Curcumin has been reported to display anti-inflammatory activities by inhibiting important pro-inflammatory

signaling cascades, such as the nuclear factor kappa B (NFkB), cyclooxygenase and lipooxygenase pathways, as well as down-regulating the secretion of essential cytokines such as tumour necrosis factor alpha (TNFa), interleukin 1B (IL-IB) and interleukin 6 (IL-6). From clinical studies, Curcumin has been shown to have a good safety profile (Robert and Ilse, 2014).

Colchicine: Colchicine, a major alkaloid from *Colchicum autumnale* (Colchicaceae), is well known in the management of gout attack. Several clinical trials have been carried out on Colchicine against inflammation mediated disorders relating to cardiology (myocardial infarction) and nephrology (diabetic nephropathy). Since the number of diseases with inflammatory components are many, it might be speculated that colchicine will further stay an interesting, not yet fully exploited drug (Imazio *et al.,* 2013).

Resveratrol: Resveratrol, a stilbene derivative and phytoalexin have been derived from several plants as well as dietary products such as grape, red wine and peanuts. It has profound anti-inflammatory, antioxidant, anticancer/proapoptotic, chemopreventive, and antimicrobial properties. Due to its poor bioavailability, more researches are being done to improve on its bioavailability. Its mechanisms of actions have been proposed to be via inhibition of NFkB, and COX-2 pathway. Over 40 clinical trials have been conducted on resveratrol in the area of obesity, cancer, coronary artery disease as well as in diabetes (Fulda, 2012; Robert and Ilse, 2014).

Capsaicin: Capsaicin, a hydrophobic alkaloid derived from chili peppers (*Capsicum* species; Solanaceae), has been traditionally used to relieve arthritic and rheumatic disorders (O'Neill, 2012). Researches have shown that capsaicin acts via the transient receptor potential channel vanilloid subfamily member 1 (TRPV1). It has been reported to inhibit; COX-2 activity, iNOS

expression, NFkB, paw inflammation in arthritic rats as well as ethanol-induced inflammation of the gastric mucosa in rats. Topical application of capsaicin has been reported to be effective against osteoarthritis. However, there are no advance clinical trials on the use of capsaicin in the treatment of inflammation (Robert and Ilse, 2014).

Epigallocatechin-3-gallate (EGCG): This is a compound derived from green tea, *Camellia sinensis* (Teaceae). Researchers have found EGCG to exert significant anti-inflammatory, anti-oxidant, anti-infective, anti-cancer, anti-angiogenetic and chemopreventive effects (Riegsecker *et al.*, 2013). Only few clinical trial data are available on this agent (Robert and Ilse, 2014).

Quercetin: This is a flavonol (a class of flavonoids) mainly found in various foods, including grapevine, apple, red onions, broccoli and tea as well as other plant parts. It has been reported to exhibit anti-inflammatory, anti-oxidant, anti-cancer, neuroprotective, anti-hypertensive and blood glucose lowering activities (Chirumbolo, 2010). Clinical studies are ongoing to use pure quercetin as a pharmacological agent. In the area of inflammatory disorders, two phases (1 and 2) have been planned for its safety and efficacy in chronic obstructive pulmonary diseases (COPD). In the area of diabetes, it has been proposed to be administered to type 2 obesed and non-obesed patients to evaluate its ability on such patients to tolerate glucose. In the field of cancer, it will be screened against prostate-specific antigen (PSA) to know whether it can prevent prostate cancer (Robert and Ilse, 2014).

2.2.8 SOME MODELS OF SCREENING ANTI-INFLAMMATORY DRUGS

The search for alternative anti-inflammatory agents is not complete until such agents are subjected to various experimental models. In these screening models, the potencies of anti-inflammatory agents are measured by inducing inflammation *in-vivo* using experimental animals

such as rats and mice or *in-vitro* using red blood cell, bovine serum albumin among others (Mitul and Shivalinge, 2012).

Based on the symptoms observed during inflammation, *in-vivo* screening models can be characterized into two phases, including the acute phase (where vasodilation and increased capillary permeability are observed, leucocytes and phagocytes infiltrate into the blood) and chronic inflammatory phase (which involves the formation of granuloma) (Mitul and Shivalinge, 2012).

Acute phase: Models of acute inflammation include the following; Carrageenan induced paw edema, Croton-oil induced ear edema, xylene induced topical ear edema, Oxazolone induced ear edema, pleurisy in rats, vascular permeability and leukocyte migration tests (Mitul and Shivalinge, 2012).

Chronic phase: The models that fall under this category include: formaldehyde induced arthritis, Cotton wool granuloma, glass rod granuloma and sponge implantation models (Mitul and Shivalinge, 2012).

Acetic acid-induced vascular permeability test:

This test is usually carried out to assess the inhibitory ability of test drugs against vascular permeability which is usually associated with inflammatory conditions. Release of inflammatory mediators such as histamine, prostaglandins and leucotrienes by mast cells cause dilation of arterioles and venules, thereby resulting to the extravasation of fluid and plasma proteins. Injection of acetic acid induces vascular permeability, thereby releasing inflammatory mediators

while injection of Evans blue dye serves as a means of identifying the level of permeability due to infiltration of inflammatory mediators (Mohini *et al.,* 2012).

Paw edema model:

This test evaluates the ability of an anti-inflammatory agent to prevent edema produced in the hind paw by phlogistic (inflammatory) agents such as formaldehyde, egg albumin, sulfated polysaccharides (such as Carrageenan), kaolin and brewer's yeast among others. Animals (rats or mice) are starved overnight and randomnized into groups (control, reference and tests). Thirty minutes to one hour after treatment, animals are challenged with 0.1 ml of 1% of the phlogistic agent. Paw volume is usually measured before application of irritant (to serve as a baseline) and then at thirty minutes, 1^{st} through 5^{th} or 6^{th} hour after the application of phlogistic agents. Paw volume can be measured using any of the following methods; water displacement method, micrometer screw gauge, plethysmometer or by wrapping a piece of cotton thread around the paw and measuring the circumference with a meter rule (Mitul and Shivalinge, 2012).

Three phases of inflammatory responses are associated with paw edema induced by carrageenan and other phlogistic agents. In the first phase (which lasts for one hour), there is mast cell degranulation and release of histamine and serotonin. The second phase (which last between 60 and 150 min) is associated with pain and bradykinin release while the last phase (which last 3 - 4 hr) is associated with eicosanoid production (Ezeja *et al.,* 2015).

Leukocyte migration model:

This test is based on the fact that leukocyte travel from their place of rest to site of inflammation. Thus, inhibition of leukocyte migration to the site of inflammation could be linked to the

mechanism of anti-inflammatory agents. Animals (mice or rats) are randomized into groups (control, reference and tests). One hour after treatment, animals are administered with 10 ml/kg of 1-3 % carrageenan intraperitoneally. After 3-4 hr, animals are anesthetized and sacrificed. After gentle massage, the peritoneal cavity is cut open and 5 ml of phosphate buffer saline or normal saline is used to wash the intraperitoneal cavity into a petri dish and the peritoneal exudates is used for the determination of leukocyte count (Mitul and Shivalinge, 2012; Ajaghaku *et al.,* 2013).

Xylene induced topical edema model:

This is another acute model of screening anti-inflammatory agents. Animals (mice or rats) are randomized into groups (control, reference and tests). Treatment (dissolved in 50 µL of a soluble solvent, usually methanol) is applied to the outer part of the right ear while 50 µL of xylene is applied to the inner surface of the right ear. The left ear serves as control. Two hours after treatment, animals are sacrificed by cervical dislocation and both ears are removed with the aid of cork borer and weighed. Increase in ear weight as a result of edema produced by the irritant is obtained by subtracting the weight of the treated ear (right ear) from untreated ear (left ear) and the inhibition of paw edema is calculated relative to control group (Mitul and Shivalinge, 2012; Nworu *et al.,*2012; Ajaghaku *et al.,* 2013).

Formaldehyde induced arthritis model:

Formaldehyde is useful in an arthritic model of inflammation. It produces edema leading to release of several mediators of inflammation. Animals are assigned into control, reference and test groups. Inflammation is initiated by the injection of 0.1 ml of 2-2.5% formaldehyde into the right hind paw on the 1^{st} and 3^{rd} day in other to maintain the level of inflammation. Treatment of

animals and measurement of paw volume is done daily for ten consecutive days. The percentage inhibition in paw edema is calculated from the area under the curve (Mitul and Shivalinge, 2012; Nworu *et al.,* 2012).

Human red blood cell membrane stabilization test:

This test evaluates the ability of a drug to stabilize human red cells that are predisposed to hypotonic solution or heat. This is because viable cells depend on the integrity of their membrane, which can be damaged when they are exposed to foreign insults. Since the erythrocyte membrane has similarity with the lysosomal membrane, erythrocyte stabilizing agents could also be related to lysosomal stabilizing agents. Drugs with membrane stabilizing ability are useful against the early phase of inflammation (Islam *et al.,* 2015).

In this assay, whole human blood from a healthy volunteer is placed into heparinized tube and centrifuged. The supernatant is discarded and the whole blood is washed with isotonic buffered solution (normal saline) and then centrifuged. This process is repeated until the supernatant appears clear. The clear supernatant is discarded and the red cells are reconstituted using phosphate buffer saline for hypotonic and heat induced hemolysis tests (Ajaghaku *et al.,* 2013; Hema, 2014).

Ulcerogenic effects:

It is well known that the mechanism of action of most NSAIDs is by irritation or ulceration of the gastric mucosa due to inhibition of prostaglandins, a key player in inflammation pathogenesis. Thus, agents that inhibit prostaglandin in the gastric mucosal could play significant roles in the management of inflammatory disorders. However, gastric irritation caused by anti-

inflammatory drugs have constituted a major setback in the management of inflammatory conditions (Ajaghaku *et al.,* 2013; Mezui *et al.,* 2017).

In this test, animals (rats or mice) are randomized into groups and fasted between 18 and 24 hours. They are administered with various doses of the test drug, while indomethacin or diclofenac serves as a reference anti-inflammatory drug (ulcerogenic agent). Animals are sacrificed 3- 4 hr after treatment and the stomachs are removed and cut along the greater curvature and, the mucosa is exposed and washed with normal saline or distilled water for ulcer scoring (Moke *et al.,* 2015).

Cyclooxygenase (COX) and Lipoxygenase (LOX) inhibition:

Cyclooxygenase and Lipoxygenase play significant role in the mediation of inflammatory response by transforming arachidonic acid into biologically active lipid inflammatory mediators. Inhibition of COX-2 accounts for the anti-inflammatory mechanism of NSAIDs, while suppression of COX-1 can lead to several unwanted gastrointestinal effects. Lipoxygenase, which catalyzes the biosynthesis of leukotriene also plays an important role in initiating inflammation (Rehka *et al.,* 2014). Thus, inhibition of these enzymes serves as a bench mark in screening anti-inflammatory drugs.

The ability of a test compound to inhibit COX-1 and COX-2 is usually carried out using enzyme immunoassay kits following manufacturer's instructions. In this assay, selected concentrations of test compounds and reference drug are interacted with suitable reagents and enzymes. The mixtures are incubated and absorbances are recorded using microplate titre plate reader at particular wavelenght. In most lipoxygenase assays, quercitin serves as a reference drug, while in

most COX assays, indomethacin or diclofenac serve as reference drugs. Reactions are carried out in triplicates (Rekha *et al.,* 2014; George *et al.,* 2015).

2.2.9 SOME MODELS OF SCREENING ANTI-OXIDANTS

DPPH (1, 1-diphenyl-2-picryl hydrazyl) model:

DPPH assay is a common, fast, reliable and reproducible test used in evaluating free radical scavenging activity of both natural and synthetic compounds. DPPH is a free radical generator which usually receives electrons to attain stability. It has been used in the screening for agents that have the capacity to scavenge free radicals (Patel *et al.,* 2010). Whenever such agents react with DPPH (which appears as deep violet color), discoloration takes place by forming yellow color (α, α-diphenyl-β-picryl hydrazyl). The antioxidant activity of the screened compound depends on the intensity of discoloration produced from deep violet to yellow (Deep *et al.,* 2013).

Total phenolic content model:

Presence of hydroxyl group in phenolic compounds plays a major role in scavenging free radicals. They terminate lipid peroxidation chain reactions by donating hydrogen to free radicals. Higher phenolic contents of some tested medicinal plants are correlated with higher antioxidant activities (Milan, 2011). Literatures have also revealed that most phenolic compounds have anti-inflammatory, anti-oxidants, anti-diabetic, antimutagenic and anti-carcinogenic activities (Deep *et al.,* 2013; Genesh *et al.,* 2013).

Reducing power:

The reducing ability of a compound generally depends on the presence of reductants which exhibit antioxidative potential by breaking free radical chains and donating hydrogen atoms. For

instance, the ability of a compound to reduce iron III to iron II confirms its reducing ability of the test agent (Deep *et al.,* 2013). In reducing power assay, the intensity of the reducing power of the test samples depend on their ability to convert the ferricyanide complex (Fe^{3+}) to ferrous form (Fe^{2+}), which can be measured by monitoring the formation of pearls Prussian blue, where higher absorbance is an indication of higher reducing power (Habibur *et al.,* 2013).

H_2O_2 scavenging activity:

Hydrogen peroxide plays an important role as a weak oxidizing agent in several diseases. Reaction of hydrogen peroxide with iron -2 (Fe^{2+}) and copper ion (Cu^{2+}) results in the formation of hydroxyl radical which may be toxic to living organisms. This assay is carried out by reacting 1 ml of various concentrations of test compound with 2 ml of 20 Mm hydrogen peroxide. After 10 min of incubation at room temperature, the absorbance is measured at 230 nm against blank. The percentage scavenging activity of the tested sample is calculated relative to control (Deep *et al.,* 2013).

Nitric oxide (NO) scavenging activity:

Nitric oxide, an important chemical mediator generated by endothelial cells, macrophages, neurons among other tissues is known to play significant role in inflammation and other physiological processes. When it is produced in excess, it results to the generation of several diseases. Upon the reaction of sodium nitropruside with oxygen, nitric oxide produced will in turn form nitrite and peroxynitrite anions, which act as free radicals.

The assay is conducted based on Greiss reagent method. Various concentrations of the test sample (0.5 ml) are reacted with 2 ml of 10 Mm sodium nitroprusside and the mixtures are incubated at room temperature for 2.5 hr. From the incubated solution, 1 ml is added to 1 ml of

Greiss reagent (1% sulphanilamide and 3% phosphoric acid) and the mixture is further incubated at room temperature for 30 min. This will lead to the formation of a pink chromophore due to the diazotization of nitrite with alpha-napthylene. The absorbance of the chromophore is measured at 540 nm and percentage inhibition is calculated relative to control (Habibur *et al.,* 2013; Ezeja *et al.,* 2015).

2.3 CHROMATOGRAPHY

Chromatography (a Greek word, meaning color) is a laboratory technique used in the separation of mixtures. Upon dissolution of the mixture (sample) in a fluid called the mobile phase, it is carried through the stationary phase. This causes the various constituents in the mixture to travel at various speeds, thereby causing them to separate, based on different partitioning between the mobile phase and the stationary phase. It is based on the concept of partitioning coefficient where solute partitions between two solvents that are immiscible. In forward phase chromatography, the immobile phase (solid support matrix, e.g silica gel) is polar while in reverse phase chromatography, the matrix support (C-18, octadecylsiyl) is non polar (Krishnananda *et al.,* 2017). In natural product chemistry, chromatographic techniques play significant roles in the discovery of novel compounds of pharmaceutical and biological interests (Vivek *et al.,* 2016).

2.3.1 SOME TERMS USED IN CHROMATOGRAPHY

A chromatograph is an instrument that causes separation to take place. A chromatogram is a visual display or output of a chromatograph. An eluent is the solvent that carries the analyte (sample to be separated). An eluate is the mobile phase that is leaving the column. The mobile phase (a sample that is being separated and the solvent which moves it) is that which moves at a

definite direction by interacting with the stationary phase thereby causing separation to occur. Retention time is the time taken for an analyte to pass through the system (from column to detector). Stationary phase (e.g silica gel) is a substance that is fixed in place for a chromatographic procedure. A detector is an instrument for qualitative and quantitative detection of an analyte after a separation has occurred. A preparative chromatography is useful for the purification of samples for further analyses.

2.3.2 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

HPLC is an analytical technique for separation and determination of organic and inorganic solutes in samples. In liquid chromatography technique, the mobile phase is liquid. However, in the HPLC, the sample is forced through a small parking particle by a liquid (mobile phase) using high pressure. The stationary phase of HPLC is composed of spherically shaped particle (porous membrane). In modern HPLC, non-polar solid phase (C18) and a polar liquid phase (mixture of water and other solvents) are used. With the coupling of Diode array detector (DAD) to HPLC, the absorption spectra of analyte can be measured for easy detection (Vivek *et al.,* 2016). Analytical HPLC coupled to a photo diode array (PDA) detector is useful for analyzing natural products with chromophores. Individual peaks can be analyzed and their complete UV spectrum is detected. Characteristics of certain compound(s) can be obtained from HPLC retention time and UV spectrum of each individual component. Semi-preparative HPLC is a versatile and rapid technique which is used for the isolation of pure compounds from complex fractions that has been previously cleaned by using conventional column chromatography. Analytical HPLC is used to identify various peaks from samples. Various components in the mixture pass through the HPLC column at different rates due to differences in their partitioning characteristics between the mobile and stationary phase.

2.3.3 VACUUM LIQUID CHROMATOGRAPHY (VLC)

This is a technique used for purification of organic and inorganic compounds from a mixture based on order of polarity. The components include a cylinder shaped glass column containing a stationary phase (silica gel) and a mobile phase (solvents). The sample is mixed with silica gel properly and allowed to dry. After loading of the sample in the column, the mobile phase (solvents of increasing polarities) is allowed to flow down under gravity with the aid of vacuum pump. The mobile phases then interact with the sample and separate them into various components (fractions) (Sheeja *et al.,* 2010; Vivek *et al.,* 2016).

2.3.4 SIZE EXCLUSION CHROMATOGRAPHY (SEC), SEPHADEX

Also known as gel permeation chromatography or gel filtration chromatography, size exclusion chromatography separates substances based on their molecular sizes, but not based on chemical interaction or attraction. Thus, the pores would allow the intake of smaller molecules than bigger molecules and such smaller molecules are removed from the flow of the mobile phase. Molecules larger than the pore size are first excluded from the gel. SEC is often reserved for a final stage of purification of mixture (Krishnananda *et al.,* 2017).

2.3.5 MASS SPECTROMETRY (MS)

HPLC-MS involves the coupling of an HPLC with a mass spectrometer (MS), which combines the chemical separating power of LC with the ability of a mass spectrometer to selectively detect and confirm molecular identity. This is a sophisticated technique useful in identification of unknown compounds, quantification of known compounds as well as elucidation of chemical properties of a molecule. It is also useful in molecular weight determination of compounds (Doughari, 2012; Vivek *et al.,* 2016).

2.3.6 NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY (NMR)

This is the most efficient method of studying the molecular structure of isolated compounds. It operates based on the fact that the nuclei of atoms have magnetic properties that can be used to generate chemical information of a compound. Shielding and deshielding effects occurs in various parts of the resulting NMR spectrum, depending on the electron density around each proton, which presents information about the chemical environment of the proton. The resulting frequency, where the nuclei resonate (chemical shift) is presented in ppm and the coupling constants between adjacent nuclei in Hertz. NMR experiment can be done in one or two dimensional condition.

It gives the physical and chemical properties of substances to be identified. Although, one dimension technique is routinely used, complicated structures of molecules can be detected with the aid of two dimension NMR technique. For the determination of molecular structure of solids, a solid NMR technique is used. For the identification of the type of carbon present, a carbon NMR technique is applied, while H-NMR is useful for the type of hydrogen, carbon present in a compound, to know how they are related (Doughari, 2012; Vivek *et al.*, 2016).

2.4 TOXICITY SCREENING

Toxicity testing deals with the evaluation of deleterious effect(s) of substances. Preclinical safety assessment of substances such as pharmaceuticals, cosmetics, food and food ingredients, medicinal plants among others is necessary before their approval for human uses. Such tests help avert dangers of chemicals that may be toxic to human and the environment (Parasuraman, 2011).

The use of experimental animals in toxicity testing which started with John William Trevan in 1920 was based on the postulation that animals respond to drugs the same way that humans respond to drugs when the test substance is administered using a similar route. For example, asbestos causes lung cancer while plastic solvent causes liver cancer in both human and animal species (Saganuwan, 2016).

Historically, toxicity testing started with Paracelsus, the father of toxicology after discovering that some chemical substances are responsible for toxicity in both plants and animals. He is attributed to the proclamation that "All substances are poisons; there is none which is not a poison, hence the right dose differentiates a poison and a remedy". In a nutshell, toxicity effects of toxicants and therapeutic agents are dose dependent (Saganuwan, 2016).

2.4.1 ACUTE TOXICITY (LD_{50}) TEST

Acute toxicity test (short term toxicity study) is a test carried out to evaluate the deleterious effects of a substance following its single or multiple exposures to experimental animals by a given route within 24 hr (Enegide *et al.,* 2013; Saganuwan, 2016). A maximum of two weeks is required for observation of delayed toxicity after a single dose exposure. This test aids in assessing the short term toxic potential of substances that might be taken (ingested or absorbed) accidentally or deliberately. The conduct of acute toxicity tests usually involves two species of laboratory animals (mice and rats) (Enegide *et al.,* 2013).

The various methods of assessing LD_{50} include; Arithmetical Method of Karber, Lorke's Method, Miller and Tainter methods, the OECD test guidelines which include: Fixed Dose Procedure (OECD TG 420), Acute Toxic Class Method (OECD TG 423) and Up-and down procedures (OECD TG 425) (Enegide *et al.,* 2013).

2.4.2 SUB-ACUTE/REPEATED DOSE TOXICITY TEST

This involves the administration of the test substance by a known route for a period of two to four weeks (Mondal *et al.,* 2017). The outcome of 14-day sub-acute toxicity test is required for establishing doses for sub-chronic toxicity test. The experimental animals are subjected to at least three graded doses of the test substance. Baseline parameters including body weight, hematological, biochemical, and cardiovascular are assessed before dosing animals with the test substance. At the end of the study, final body weights are measured and samples are collected for biochemical, haematological and histopathological evaluations (Ikonone *et al.,* 2017; Munawaroh *et al.,* 2017). This study helps to evaluate systemic side effects of substances on vital organs. The results of this study serve as a basis for classification and labeling. It provides information on the mode of toxic action of a substance. It also provides a guideline in selecting doses for studies involving longer duration of exposure (Parasuraman, 2011).

2.4.3 SUB-CHRONIC TOXICITY TEST

This test is carried out to determine the toxicity that may arise due to repeated exposures of animals to a test substance over a period of time. The test can last for different periods of time but 90 days is the most common. Monthly evaluation of various parameters including body weight, haematological, biochemical and histopathological parameters are carried out (Parasuraman, 2011).

The principal goal of sub-chronic toxicity test is to establish no observed adverse effect level (NOAEL) to further identify and characterize specific organs affected by the test compound after repeated administration. Also, it establishes the potential of the test compound to accumulate in the test organism. At least three doses are employed: a high dose that produces toxicity but does

not cause more than 10% fatalities, a low dose that produces no apparent toxic effect and an intermediate dose (Madhavi *et al.,* 1995).

Sub-chronic toxicity test is useful in predicting appropriate and reasonable doses for chronic toxicity tests (Parasuraman, 2011).

2.4.4 CHRONIC TOXICITY TEST

This study provides inferences about the long-term effect of a test substance to experimental animals. This test involves the exposure of the maximum tolerable dose (MTD) and its fractions to animals for a long duration of time, usually between six months – two years in rodents (Goodman *et al.,* 2006; Jacobs and Hatfield, 2012). The MTD serves as the highest dose in chronic toxicity test (Jacobs and Hatfield, 2012). It evaluates the cumulative effect of poisons (Saganuwan, 2016). A group is usually included to monitor reversibility in toxicity for a period of four weeks (OECD (2008). The results from chronic toxicity test are useful in the establishment of safety criteria for human exposure to new drugs undergoing clinical trials (Jaijoy *et al.*, 2010). Chronic toxicity testing is applicable to drugs used in the management of terminal diseases such as diabetes, hypertension and rheumatoid arthritis among others. The major difference between chronic toxicity and sub-acute or sub-chronic tests is the duration of exposure (Parasuraman, 2011).

2.4.5 MUTAGENICITY AND CARCINOGENICITY TESTS

Mutagenicity tests investigate the toxic potential of substances such as pharmaceuticals, herbal products, industrial chemicals, and consumer products on the base sequence of DNA. Animals are usually exposed to the test chemical for up to a minimum of two years, depending on the lifespan of the specie (Saganuwan, 2016). Animals are observed for signs of toxicity and

development of tumors. At the end of the study, animals are sacrificed and observed for gross and histopathological changes. Since mice and rats have relatively short lifespan, they are preferred for these studies (Goodman *et al.*, 2006; Saganuwan, 2016).

2.4.6 REPRODUCTIVE AND DEVELOPMENTAL TOXICITY

This evaluates the effect of a chemical on the reproductive function as well as offspring development. These tests came to limelight after the thalidomide disaster (Wu, 2010).

In this test, animals are dosed repeatedly with graded doses of the test substance before mating, during gestation or after delivery up to the maturity period of the offspring, to identify effects of the test substance on reproductive function performance and on the developing offspring. Endpoints of these toxicological screening include premature delivery, delayed delivery, birth defects and spontaneous abortion. Others include alteration in reproductive hormones and semen quality (OECD, 2012).

Effects of substances on reproductive functions for males and females may be assessed during repeated-dose studies by evaluating reproductive endpoints such as semen analyses and testicular histopathology, for males as well as oestrous cycles, follicle counts/oocyte maturation and ovarian integrity histopathology, for females (OECD, 2012; Saganuwan, 2016).

2.4.7 TERATOGENIC TEST

This evaluates the potential of a test substance to cause birth defects during development between conception and birth (Saganuwan, 2016). Teratogens interfere with normal prenatal development, thereby causing embryo or fetus deformities. During the period of fertilization and post-implantation (embryogenesis), the embryos are more susceptible to teratogenic agents. Teratogenic studies are carried out by administering the test substance to pregnant rats, mice or

rabbits during the period of organogenesis (Goodman *et al.*, 2006). This is the actual embryonic stage where the organ systems differentiate at great rate (Hill, 2018). In rodents (usually mice and rats), organogenesis occurs between 6 and 15th days of conception (Oyedeji and Bolarinwa, 2013; Saganuwan, 2016).

It has been reported that 65 to 75% cases of congenital malformations are from unknown cause, while 15 to 25 % is due to inherited genetic disorders while 10% is due to exposure of mothers to infections, mechanical injury, radiation, chemicals and drugs (Herrer *et al.*, 2011).

2.4.7.1 SOURCES OF TERATOGENS

Teratogen can arise from various sources including; pollution, infections, maternal diseases, alcohol, drugs and malnutrition. Pollution encompasses physical (e.g, atomic and nuclear explosion) and chemical (e.g, carbon monoxide from cigarrete, car exhaust and incomplete combustion of coal which can lead to hypoxia, spontaneous abortion, still birth and growth retardation). Lead from car exhaust and water pipes can causes spontaneous abortion, still birth. Vinyl chloride can cause disorders in spermatogenesis. Mercury can cause mental retardation.

Infections arising from rubella can cause deafness, blindness, cataract, retinopathy, microcephaly and mental retardation. Hepatitis, small pox, chicken pox may cause abortion and stillbirth. Bacterial infections such as syphilis can cause mental retardation, deafness, meningitis, tooth malformation and CNS disorders. Protozoal infection (toxoplasmosis, arising from exposure of pregnant women to feces of domestic animals) can lead to hydrocephalus, CNS disturbance among others.

Maternal disease such as uncontrolled diabetes mellitus, thyrotoxicosis can cause teratogenicity. Exposure of pregnancy to alcohol can cause delayed development, mental retardation, and defect

in eye, face (cleft palate), congenital abnormalities in the heart, kidney and skin of fetuses (Van Gelder *et al.,* 2010).

Drugs such as thalidomide, warfarin, corticosteroids, anticancer are highly teratogenic. Others include; androgen and progesterone (causes masculization of the fetus), estrogen (causes feminization and abnormal spermatogenesis of male fetus), diethylstibesterol (causes adrenocarcinoma of the vagina).

Deficiency in some vital nutrients due to malnutrition can result in some congenital abnormalities. For instance, deficiency of Vitamin A causes anophthalmia, deficiency of Vitamin D causes teeth and bone malformation, deficiency of folic acid causes abnormal development of brain neurons and mental retardation. Deficiency of iron, calcium and potassium can cause anemia, calcium and pre-term labor respectively (Van Gelder *et al.,* 2010).

2.4.7.2 STAGES OF DEVELOPMENT AND SUSCEPTIBILITY TO TERATOGENS

During embryogenesis, teratogens pose attack at certain stages.

Pre-implantation stage (from fertilization to implantation): At this stage, embryos are not susceptible to teratogens (Oyedeji and Bolarinwa, 2013).

Embryogenesis (stage of organogenesis)

Six to seven days after gestation, implantation occurs, followed by gastrulation (the formation of ectoderm, mesoderm and endoderm) which is characterized by differentiation and organization. At this period, the embryo produces major morphological changes (especially face) and it is highly susceptible to teratogens (Golalipour *et al.,* 2011; Oyedeji and Bolarinwa, 2013).

Fetogenesis (histogenesis)

This is the period of growth and functional maturation. Morphological changes are minimal, except structural changes. During the period of histogenesis, growth and functional aspect, such as intelligence and reproduction may be affected by a teratogen (Oyedeji and Bolarinwa, 2013).

2.4.7.3 SCREENING FOR TERATOGENIC POTENTIAL OF SUBSTANCES

It has been reported that infant mortality as a result of congenital malformation due to exposure of pregnant women to toxic chemicals from the environment, cosmetic, food, medicinal plants and synthetic drugs ranks as the second cause of mortality in developing countries (Saleem *et al.*, 2014; Madu, 2015).

Mice and rats are preferred in this test because they have short gestation periods, 20 - 21 days. In this test, matured male and female animals (mice or rats) are selected and mated. Successful pregnant female animals are separated from the males and are dosed with the test substance between the day 6^{th} and 15^{th} day of pregnancy (period of organogenesis) by a selected route. Animals are allowed to give birth or a caesarean section is carried out on day 21 of gestation, and numbers of dead and live pulps as well as pulps with deformities are recorded. The live fetuses or pulps are weighed and their organs are harvested for histopathological analyses (Oyedeji and Bolarinwa, 2013; Saganuwan, 2016).

2.4.7.4 TERATOGENIC STUDIES ON SOME MEDICINAL PLANTS AND OTHER AGENTS

Study on teratogenic potential of latex and the leaves of *Euphorbia helioscopia* (100, 300, 500, 700, 1000, 1500 and 2000 mg/kg body weight revealed no teratogenic effect on mice (Saleem *et*

al., 2014). Cypermethrin, a synthetic pyrethroid insecticide used in the West African sub-region has been reported to be neurotoxic, because it crosses the placental barrier thereby causing physiological malfunction (Brender *et al.,* 2010).

A recent study revealed that Cypermethrin administration caused a dose dependent significant reduction in mean fetal body weight of pulp, non-prominent head size, bilateral eye bulges, tails, face, muzzles and other external appendages when compared to control group. There was no defect in organ of fetuses whose mothers were treated with Cypermethrin. Their neural tissues were normal and bones were in the cartilaginous stage of development (Madu, 2015).

In another study, administration of 75 mg/kg dose of aqueous and methanol extract of *Portulaca oleracea* (*Portulacaceae*) to rats did not cause gross morphological changes as well as significant change in litter size and weights when compared to control group (Oyedeji and Bolarinwa, 2013). Administration of high dose of ethanol bark and root extracts of *Rauwolfia vomitoria* (Apocynaceae) to pregnant rat revealed numerous osteoblast and osteoclast, hypertrophy, and hyperplasia of bone cells of fetus when compared with the control group (Mokutima *et al.,* 2013). Another study on the embryotoxicity of hydroalcoholic extract of *Mentha piperita* (600 and 1200 mg/kg doses) during organogenesis in mice showed no teratogenic effect in fetuses (Golalipour *et al.,* 2011).

A study on the embryotoxic and teratogenic effect of a phytodrug (RICOM 13-J) on pregnant female Wistar rats revealed no evidence of teratogenicity in pulps at 5 and 20 mg/kg. However, 2 and 20 g/kg were found to be embryotoxic on animals (Ekwere *et al.,* 2011).

A study by Eluwa and co-workers on comparative teratogenic potentials of crude ethanol root bark and leaf extracts of *Rauwolfia vomitoria* (apocynaceae) (250 and 500 mg/kg) revealed no gross malformation. However, histological study on the fetal heart showed obvious distortion of the cardiac muscle nuclei and myocardial fibers in the treated groups, particularly those whose mothers received 250 mg/kg of the extracts (Eluwa *et al.*, 2011). In another study, administration of Silymarin (from *Silybum marianum*) at 50, 100 and 200 mg/kg caused reduction in mean fetal body weights and other fetal abnormalities including limb, vertebral column and craniofacial malformations. The most common abnormality observed was craniofacial malformations (Gholami *et al.,* 2016).

2.4.8 SOME BIOMARKERS OF TOXICITY

2.4.8.1 BIOCHEMICAL PARAMETERS

2.4.8.1.1 LIPID PROFILE

Lipids are fat-like substances which are important source of energy for cell functions. Cholesterol and triglyceride are two major lipids that are transported into the blood by lipoprotein particles (Stone *et al.,* 2014). Lipid profile is used to describe the outlook of the lipid level of the body system. It encompasses fatty acids, cholesterol esters, cholesterol, triglyceride as well as phospholipids. Consumption of foods that are rich in saturated and trans-saturated fatty acids can increase cholesterol level in the blood. Excess cholesterol can be deposited as plaque in the walls of blood vessels leading to narrowing of the arteries, blockage of blood vessels, and eventually hardening of the arteries (atherosclerosis, heart diseases and stroke precipitation) (Stone *et al.,* 2014). Lipid profile assessment (lipid profile test) aids in knowing the state of wellbeing of an individual, whether they are predisposed to cardiovascular and coronary heart diseases (Ogbonnia *et al.,* 2011).

Cholesterol: This is an organic substance that plays a useful role in mammalian cell membrane. It is useful in the manufacture of bile acids, steroid hormones and vitamin D. However, excess of cholesterol in the blood could cause artherosclerosis thereby contributing to cardiovascular diseases (Obel *et al.,* 2016).

Triglycerides: This is also known as triacylglycerol or TAG, it is an ester derived from glycerol and three fatty acids. Triglyceride could be mobilized during starvation, for calorie consumption. It is available in vegetable oil (usually unsaturated) and animal fats (usually saturated). Consumption of fatty diets could increase triglyceride level in the blood which could lead to atherosclerosis, coronary heart disease and stroke (Obel *et al.*, 2016).

Disorders in lipid metabolism could lead to hypercholesterolemia (high cholesterol level) as well as hypertriglyceridemia (high triglyceride level), which are connected with heart diseases (Arowora *et al.,* 2016).

High density lipoprotein (HDL) cholesterol: Also called "good cholesterol", it plays useful role in transporting excess cholesterol from wall of the arteries to the liver for elimination. HDL-cholesterol contains about 30% of blood cholesterol. High levels of HDL-cholesterol and low level of blood cholesterol plays a favorable role in preventing atherosclerosis and ischemic heart diseases (Kayode *et al.,* 2016).

Low density lipoprotein (LDL) cholesterol: This type of cholesterol, produced in the liver transports cholesterol to the body cells and tissues. It is called "bad cholesterol" and it is deposited in the wall of the arteries and other blood vessels, thereby increasing the chance of heart diseases and stroke (Kayode *et al.*, 2016).

Very Low density lipoprotein (VLDL) cholesterol: VLDL cholesterol is also produced in the liver when synthesis of fatty acids increases. It also transports triglycerides, phospholipids, cholesterol as well as cholesteryl esters (Kayode *et al.*, 2016).

2.4.8.1.2 LIVER FUNCTION

The liver, the body's chemical factory is known to be the largest internal organ involved in detoxification and elimination of foreign substances. Anatomically, it is soft dark reddish brown and triangular with four unequal lobes with an estimated weight of about 1.5 kg in adult. It is positioned at the upper right side of the stomach, below the rib cage, overlying the gall bladder. It has two large vessels, hepatic artery (which transports blood from the aorta) and portal vein (which transports digested nutrients from the gastrointestinal tract, pancreas and spleen back to the liver for metabolism). Histomorphologically, blood vessels in the liver form capillaries and lobules, which encompasse millions of hepatic cells (which play metabolic roles) (Adi and Alturkmani, 2013).

Physiologically, the liver plays the following roles; production and secretion of bile salts for digestion, regulation of stored sugars and release of glucose for energy, protein and fat metabolism, regulation of cholesterol production, production of urea (a waste product), production of clotting factors, synthesis of plasma protein, and production of immunity against infections. The liver handles all drugs as foreign substances (xenobiotics) and it subjects them to metabolism so as to be suitable for excretion by the kidney. After the breakdown of toxic agents by the liver, such agents are excreted into the blood or bile. Bile products are eliminated in the feces and in the form of urine via the kidneys (Adi and Alturkmani, 2013).

During liver damage, cellular necrosis, fibrosis as well as lipid peroxidation and reduction in glutathione level occurs. Thus most hepatotoxic agents induce lipid peroxidation and oxidative damage to the liver. In determining the function of the liver, liver function tests are performed to give an idea of the liver integrity (Debelo *et al.,* 2016).

In toxicity screening, liver function assessment is very vital. This is achieved by measuring the concentration of liver biomarkers, using clinical, commercial or compounded reagents as well as morphological and histopathological analysis of excised liver samples (Debelo *et al.*, 2016).

Serum tansaminases such as aspartate aminotransferase (AST, formerly serum glutamic-oxaloacetic transaminase, SGOT), alanine aminotransferase (ALT, formerly serum glutamate-pyruvate transaminase, SGPT) are biomarkers in the assessment of hepatic injury. During liver injury, there is damage to the hepatocyte membrane thereby causing elevation in the level of these enzymes in the circulation. ALT and AST are products of amino acid metabolism. While ALT is liver specific (cytosolic, present in cytoplasm of the hepatocyte at high concentration), AST possesses both cytosolic and mitochondrial properties present in several tissues such as heart, skeletal muscle and brain tissues. Thus, damage to these organs can cause elevation in blood AST level. In some liver disease ALT level may exceed AST level, because ALT is located only in the cytoplasm and its serum levels tend to be relatively higher than AST, as a result of membrane leakage from the hepatocyte. Factors that could increase ALT level include liver tumor, obstructive jaundice, pancreatitis, heart attack among others (Debelo *et al.*, 2016).

Serum alkaline phosphatase (ALP), gamma glutamyl transpeptidase (GGT), bilirubin and bile acids are biomarkers of hepatobiliary injury such as cholestasis. ALP level is elevated during intrahepatic abnormalities and extrahepatic biliary obstruction, inflammation of the hepatocytes,

pancreatitis and duodenitis. Total protein and albumin are useful in the evaluation of protein synthesis (Debelo *et al.,* 2016).

Recent report has revealed that the liver is a stable organ with a high continuous regeneration capacity that can resist exposure to some toxicants (Suzery *et al*., 2017). Regeneration of the liver is usually activated spontaneously after injury and can be further stimulated by cell therapy with hepatocytes, hematopoietic stem cells, or mesenchymal stem cells. Reports have also revealed that three liver lobes (out of five lobes contained in the liver) can be easily removed without significant damage to the other two, which can increase their size and restore the organ mass (Suzery *et al*., 2017). This repair may take up to 5–7 days in mice and rats (Irina and Konstantin, 2017). It should also be noted that the liver, unlike other quiescent or stable tissues (such as kidney and pancreas, mesenchymal cells, such as fibroblasts and smooth muscle cells, endothelial cells and lymphocytes) has a relatively robust proliferative capacity (Kristine, 2010). Although repair of damage done to the parenchymal cells of continuously dividing or quiescent tissues is possible, repair of severe injury done to dividing parenchymal cells alongside with the underlying stromal framework of the tissue or injury done to the non-dividing tissues may not be possible due to exposure to most highly toxic agents (Kumar *et al.,* 2010).

2.4.8.1.3 KIDNEY FUNCTION

The kidney is a vital organ (bean shaped) that plays a major role in excretion of waste products from the body. Anatomically, it consists of the medulla, cortex and renal pelvis. The medulla aids in concentrating urine, the cortex aids in reabsorption of filtered material, while the renal pelvis serves as a collection center for urine elimination (Wahyuni *et al.,* 2017).

Physiological roles of the kidney include retention of nutrient and excretion of nitrogenous waste products and toxins, regulation of water, ions and pH level as well as maintenances of plasma volume (Wahyuni *et al.,* 2017).

The kidney could be predisposed to toxicant from various sources such as conventional drugs, poisonous plants, industrial chemicals, environmental pollutants, microorganism among others, thereby leading to alteration in its normal function (Wahyuni *et al.,* 2017). In the assessment of kidney toxicity, certain biomarkers such as urea, creatinine, electrolytes as well as kidney histopathology are evaluated. Urea, a major nitrogenous end product of protein and amino acid catabolism produced by the liver is distributed in the intracellular and extracellular fluid. Urea is filtered out of the blood via the glomeruli, while some part of it is partially reabsorbed along side with water. Whenever there is impairment in glomerular filtration, elimination of urea becomes reduced, thereby leading to its elevation in the blood. This is common with kidney diseases attributed to renal failure, blockage of the urinary tract, kidney stone, dehydration, congestive heart failure among others. Also, low level of urea is common with the use of opioids and anabolic steroids and malnutrition (Musila *et al.,* 2017).

Creatinine, a breakdown product of creatine phosphate in muscle, does not play any vital role to body function, thus it is excreted unchanged (as it is produced) and no part of it is reabsorbed into the blood circulation. It is used as a biomarker of kidney function and its elevation above the normal range is an indication of kidney disease. Elevated level of creatinine is found in leukemia, hyperthyroidism, anemia and muscle dystrophy. Its reduced level is common with glumerulonephritis, tubular necrosis, dehydration, shock and congestive heart failure (Shivarai *et al.,* 2010).

Electrolytes such as sodium, potassium, chloride and bicarbonates play significant roles in the maintenance of heartbeat, muscle contraction, nerve transmission, pH and fluid acid/base equilibrium in both intra-cellular and extra-cellular compartments (Odey *et al.,* 2012**).** Alteration in electrolyte balance may result to nervous disorders such as, muscle spasms, weakness, convulsion, and changes in blood pressure. Factors that could decrease chloride level include; intestinal obstruction, nephritis, excessive vomiting, extensive burns and metabolic acidosis. Chloride level can be elevated during dehydration, urinary obstruction, congested heart valve among others (Onyebuagu *et al.,* 2013). When there is a reduction in blood sodium level (hyponatremia), low plasma osmolarity inhibits anti-diuretic hormone, thereby leading to the excretion of diluted urine as a means to correct hyponatremia. This may cause reduction in hematocrit level. Some factors responsible for hyponatremia include; polydipsia, anti-inflammatory drugs among others. Carbon dioxide (CO_2) in serum or plasma usually occurs as dissolved CO_2 and bicarbonate anion (HCO_3^-). Factors that could increase carbon dioxide level in the blood include metabolic alkalosis and respiratory acidosis while factors that could decrease its level include metabolic acidosis and respiratory alkalosis (Onyebuagu *et al.,* 2013).

Another important electrolyte that regulates nerve and muscle function is potassium. Its extracellular functions relate to its influence on muscle activity, while its intracellular function relates to cellular function, namely influencing acid-base balance and osmotic pressure, including water retention. It repolarizes the membrane to its resting state after an action potential has been generated. Excess potassium in the blood (hyperkalemia) may result from its reduced elimination or its high intake. Heart failure, dehydratrion and shock can elevate potassium level. Medications such as ACE inhibitors, angiotensin receptor blockers, potassium sparing diuretics as well as NSAIDS that interfere with urine elimination can increase potassium level.

Hypokalemia, resulting from inadequate potassium intake, starvation, diarrhea, vomiting, and the use of diuretics such as thaizide, hydrochlothiazide and furosemide can lead to muscle weakness (Onyebuagu *et al.,* 2013; Debelo *et al.,* 2016; Sudip *et al.,* 2017).

In the body system, over 99% of bones and teeth are made up of calcium, while the remaining 1% is present in the blood and other soft tissues and it serves as cofactor in blood coagulation, metabolism as well as neuromuscular transmission. Although calcium exists in three different forms (45% bound by serum protein, 5% in non-ionized form and 50% serum calcium in free state), it is the free state calcium that plays significant role in biological function. Conditions that could increase calcium level (hypercalcemia) include hyperparathyroidism, hypervitaminosis, myeloma and some certain cancers associated with the bone. Conditions that could decrease calcium level include hypoparathyroidism, rickets, nephritis, nephrosis, steatorrhea as well as pancreatitis. There is direct relationship between serum calcium level and serum total protein level. Thus increase serum calcium level may lead to increase in serum protein level (Igbe *et al.,* 2015).

Serum protein plays significant role in the regulation of water distribution between blood and tissues by osmotic pressure. Factors that cause low protein level include fluid and blood loss, malnutrition and excessive protein breakdown. Protein level is elevated during dehydration. Protein level may be depleted due to decrease in protein synthesis as well as increase in protein catabolism in the liver. It is also useful in the diagnosis of liver and kidney diseases as well as other disorders. High total protein levels may also be associated with chronic inflammation or liver infections (Chivapat *et al.,* 2011; Igbe *et al.,* 2015).

Albumin is the most prevalent protein in serum. It serves in the maintenance of proper osmotic pressure as well as a carriage for several substances, including bilirubin, fatty acids, uric acid and other drugs, including antibiotics. It is useful in describing the functionality of the liver. In some situations, albumin level can be elevated during dehydration, while its level can be reduced during liver disease, malnutrition, kidney disease and rheumatoid arthritis (Igbe *et al.,* 2015).

2.4.8.1.4 HAEMATOLOGICAL PARAMETERS

Haematology deals with the study of blood. Assessment of hematological indices, (packed cell volume (hematocrit), hemoglobin, white blood cells, red blood cells and platelets) is useful in drug safety assessment to know whether bone marrow activity and hemopoiesis are disrupted by foreign substances (Bashir *et al.,* 2015).

Packed cell volume (hematocrit), the ratio of red blood cell to plasma as well as hemoglobin and red blood cell indices are markers of anemia that could be caused by bleeding, reduction in red blood cell production or increase in red blood cell destruction due to bone marrow failure. Increase in red blood cell, hematocrit and hemoglobin could result from dehydration, hypoxia, polycythemia vera and renal cell carcinoma. Red blood cell indices (mean corpuscular volume, MCV, mean corpuscular hemoglobin, MCH and mean corpuscular hemoglobin concentration, MCHC) are biomarkers for early diagnosis of anemia. Red blood cells are the most numerous blood cells that form 40% of total blood volume. Hemoglobin is a protein that transports oxygen from the lung to the tissues (Musila *et al.,* 2017).

White blood cells serve as a biomarker of immunotoxicity. High level of white blood cells (leukocytosis) is common with inflammation, stress disorders, tissue injury, leukemia and infections, while low level of WBC (leucopenia) is associated with immune suppression, viral

infection and bone marrow failure. White blood cells (WBCs) contain two major sub-categories which include the mononuclear cells and the granulocytic cells. The mononuclear cells encompass, lymphocytes and monocytes, while granulocytes encompass neutrophils, eosinophils, and basophils (Debelo *et al.,* 2016). Monocytes are phagocytic cells, which prepare and present antigens to lymphocytes for activation. Although lymphocytes (which originate in the lymphoid tissues) are not phagocytic, they are responsible for initiating and regulating the immune response by the production of antibodies and cytokines. Basophils are meant to mediate allergic response by releasing histamine. During allergic and parasitic infections, eosinophils are increased. Neutrophils speed up the elimination of bacteria by phagocytosis and help in destroying bacteria and other ingested cells. MID (mid-range absolute count) generally include monocytes, eosinophils and basophils. They are also called mid-range because these cells are between the sizes of neutrophils and lymphocytes (Debelo *et al.,* 2016).

Platelets (thrombocytes) are blood components that aid in coagulation (hemostasis). They are derived from megakaryocyte of the bone marrow. Increased destruction or decreased production of platelet (thrombocytopenia) may result from exposure to agents that may stimulate platelet antibodies and platelet destruction. Symptoms of thrombocytopenia include easy bruising, frequent bleeding from the gum, nose and gastrointestinal tract. Conditions that can cause decrease in platelet include exposure to poisonous substances like pesticides, benzene, arsenic, excess alcohol, infections, medications such as Acetaminophene, Ibuprofen and Naproxone. On the other hand, thrombocytosis (abnormal elevation in platelet count) may also result from excess or unregulated production of platelets (Berinyuy *et al.,* 2015; Musila *et al.,* 2017).

2.4.8.1.5 BODY WEIGHTS AND ORGANS WEIGHT

Measurement of body weight is useful in drug safety assessment. Alteration (increase or decrease) in body weight could be linked to the accumulation of fats or physiological adaptation to foreign substances. Some toxic agents can decrease appetite leading to lower caloric intake and decrease in body weight and organ weights (Arsad *et al.,* 2013). Agents that have nutritive components can promote weight gain. However, anti-nutritive components such as tannins, saponins, oxalates and phyrates can chelate minerals, decrease nutrient absorption thereby leading to weight reduction. Tannins have been reported to decrease absorption of protein (Jigam *et al.,* 2011).

Organ weight assessment in toxicology is an important endpoint for identification of potentially harmful effects of chemicals on test species. In order to avoid the confusion of differences in body weight between treated and control groups, relative organ weight is applied. Change in relative weight of the vital organs such as liver, kidney, heart, lung, spleen, pancreas and others may also be an indication of toxic effect of test substance (Muhammad *et al.,* 2015). Increase in organ weight and size could be due to hypertrophy resulting from an increase in the size of cells. On the other hand, hyperplasia is characterized by an increase in cell number. Hypertrophy and hyperplasia can also occur together, resulting in an enlarged organ (Khalida *et al.,* 2012). Increased heart weight could be an evidence of myocardial hypertrophy. Renal toxicity, tubular hypertrophy and chronic progressive nephropathy may be associated with changes in kidney weights. Treatment related effects, including hepatocellular hypertrophy (e.g., enzyme induction or peroxisome proliferation) are associated with change in liver weight (Khalida *et al.,* 2012).

2.4.8.1.6 HISTOPATHOLOGY

This is a branch of pathology that deals with the study of diseases in tissue sections. Organs and tissues isolated from animals are fixed in the right fixative to arrest metabolic activities in tissues as well as to avoid autolysis and protein precipitation so as to prevent enzymatic digestion of dead tissues (Bancroft and Gamble, 2002). Tissues undergo a series of processing before they can be observed and interpreted by a histopathologist using a microscope (Ugbogu *et al.,* 2016). The histological examination is the gold standard for evaluating treatment related pathological changes in tissues and organs. In toxicity testing, histological analysis is necessary to confirm alteration in cell structure of organs (Subramanion *et al.,* 2011).

CHAPTER THREE

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 EQUIPMENT

Equipment used in this study include Analytical High Pressure Liquid Chromatography (HPLC) machine containing the following components- pump: P580A LPG (Dionex); Column oven; STH 585 (Dionex); Column Eurosphere 100-C18, (5 µm; 125mm × 4mm) (Knauer); Pre-column: vertex column, Eurosphere 100-5 C18 (5-4 mm) (Knauer); Software: Chromeleon (v.6.30). HPLC-MS; Analytical HPLC: Agilent 1100 series (photodiode array detector) (Agilent); MS: Finigan LCQ- DECA (Thermoquest), Ionizer: ESI and APCI (Thermoquest); Vacuum pump (Edwards 30 BOC); Column: Eurospher 100-C18, [5 µm; 227mm×2 mm] (Knauer); pre-column: Vertex column, Eurosphere 100-5 C18, [5-4 mm] (Knauer); Software: Chromrleon 6.30.

Other equipment used include Visible Spectrophotometer (721G, Zhejiang Top Cloud-Agri Technology Co., Ltd., China), Abacus Junior Haematology Analyzer (Diatron Abacus 380, Hungary), Thermostatic water bath (Equitron Mumbai India, 400013), Analytical weighing balance (Ohaus Corp. Pine Brook, NJ USA., China), Top electronic weighing balance (Scout Pro SPU401, S/N: 7126090808, Ohaus Corp. Pine Brook, NJ USA. China). TECAN Safira 2 microplate reader (Vector Scientific, NI, UK).

3.1.2 APPARATUS

Apparatus usued include Dissecting kit, Beakers, Sample bottles, Plain tubes, Syringes, Beakers, Measuring cylinder, Micropipette, Micropipette tips, Test tubes, Cuvettes, Timer, Microscope

slides, Cover glass, Oral gavage tube, Heparinized capillary tubes, Sephadex column (Rotaflo, TF2/18, England), Paper tape, Separating funnel (Pyrex), Examination gloves, Dissecting board, Tissue basket, Wax bath, Wooden block and Paraffin block.

3.1.3 CHEMICALS

These include Carrageenan (Sigma Aldrich, Lot: #SLBD1934V, CAS: 900-07-1), Sodium chloride (Cartivalues, Cat No: 34.410, Lot No: 1984), Sodium hydroxide pellets (Avondale Laboratories supplies and Services, England, UN1823, 940290), Xylene (BDH Chemicals Ltd. Poole England, 0311030), Acetic acid (BDH Chemicals Ltd. Poole England, 27013), Ethanol, Methanol, Ethylacetate, Butanol and Dichloromethane (from JHD, Guangdong Guanghua Schi-Tech. Ltd China), Formaldehyde 40 %w/v (May and Baker Ltd, Dagenham England, MUO124), Silica Gel (60-200 Mesh size, Kem Light Laboratories PVT. Ltd. CAS No: (112926-00-8), Methanol, HPLC grade (Sigma Aldrich), 2,2-Diphennyl-1-picrylhydrazyl, DPPH (Sigma Aldrich, Lot: S44112-518, Cat: D913-2), Cyclooxygenase assay kit (Cayman Ann Arbor, MI, USA, Catalog number 760111), Sephadex LH-20 (Lot no: 10079827, Pack: 254069, Label no: 51-2180-DO-EF, GE Healthcare Bio-science AB, Sweden), Quercetin (Institute of Pharmaceutical Biology and Biotechnology, Heinerich-Heine University, Dusselforf, Germany), Ascorbic acid (Sigma- Aldrich). Evans blue (Qualikems Laboratories, Batch no: E021607, Product no: CFQ231109). Indomethacin (Jiangsu Ruinian Qianjin Pharmaceutical Co Ltd, China) and Diclofenac sodium (Sidom Pharmaceutical Industries Limited, Enugu, Nigeria).

3.1.4 REAGENT KITS

Folin and Ciocalteu's phenol reagent (Loba Chemie Pvt Ltd 107, UN No: 3264, mumbia, India), Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Triglyceride, Total

cholesterol, High density lipoprotein cholesterol (HDL-c), were all procured from Randox Laboratories Limited, Country Atrium, United Kingdom. Other kits include; Sodium, Chloride, Potassium, Urea, Creatinine, Total protein, Albumin and Alkaline phosphatase (ALP), were all procured from Teco diagnostics, California U.S.A.

3.1.5 OTHER MATERIALS

Animal feed (Pelletised grower feed, Vital feed Ltd, Jos, Nigeria), tap water, saw dust.

3.1.6 PLANT MATERIAL

Fresh leaves of *D. filix-mas* were collected from a swampy location beside the Horticulture botanical garden Amawbia, Awka South L.G.A, Anambra State between 6:30 and 8:00 am in the month of March, 2016. Plant specimen was validated by Dr. H.A. Akinnibosun, a plant taxonomist in the Department of Plant Biology and Biotechnology, Faculty of Life Sciences, University of Benin, Benin City, Nigeria. A voucher specimen, with a reference number UBH_d285A was deposited in the herbarium of the Department.

3.1.7 EXPERIMENTAL ANIMALS

Albino rats and mice of either sex used in the various animal studies were procured from the animal facility, Faculty of Veterinary Medicine, University of Nigeria, Nsukka. They were housed in the animal house, Department of Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Agulu. They were given access to feed and water *ad libitum.* Animals were handled in compliance with the National Institute of Health Guidelines for the care and use of laboratory animals for research purposes (Pub No. 85-23, revised 1985).

3.2 METHOD

3.2.1 PLANT EXTRACTION

Freshly collected leaves of *D. filix-mas* were washed with tap water and dried at room temperature for one week. Crisply dried leaves were pulverized using a mechanical grinder and a quantity (4.7 kg) was cold-macerated using 14.1 litres of 80 % aqueous ethanol (ratio 1:3) in batches for a period of 48 h, with occasional agitation. The filtrate was recovered with the aid of a muslin cloth (Anosike and Obidoa, 2010). The marc was macerated in fresh 80% ethanol for another 48 h on two occasions. Final filtrate recovered was concentrated using a water bath set at 40^0C to form a greenish paste. Percentage yield was calculated using the formula below (Azubike *et al.,* 2015).

Percentage yield (% yield) = $\frac{\text{Weight of extract}}{\text{Weight of plant material used}} \times \frac{100}{1}$

3.2.2 FRACTIONATION

Liquid-liquid partitioning of the extract with n-hexane, ethyl acetate, and butanol was carried out using the method described by Ajaghaku *et al* (2017). The extract (70 g) was dispersed in 250 ml of distilled water. The solution was poured into 1000 ml separating funnel and was mixed with n-hexane (500 ml) properly by inversion and allowed to stand until two phase separation was achieved. The upper layer (n-hexane partition) was separated and the lower portion was subjected to fresh n-hexane solvent until the upper layer was clear. After n-hexane phase, the remaining portion was subjected to ethyl acetate followed by butanol successively. The left over served as the water fraction. The procedure was repeated with new sets of extract to obtain reasonable quantities of the various fractions. All the fractions obtained were filtered and concentrated to dryness using a water bath set at 40^oC.

3.2.3 PHYTOCHEMICAL SCREENING

3.2.3.1 QUALITATIVE PHYTOCHEMICAL SCREENING

The extract and fractions were screened for the presence of bioactive compounds (proteins, reducing sugar, tannins, flavonoids, saponins, cardiac glycosides, steroids, terpenoids, anthraquinolones and alkaloids) using methods of Sofowora (1993), Evans and Trease (1989), and Harborne (1973) as described by Yadav and Agarwala (2011).

3.2.3.2 QUANTITATIVE PHYTOCHEMICAL SCREENING

Determination of total phenolic content:

Folin-Ciocalteu reagent method described by Yadav and Agarwala (2011) was used to determine the amount of phenol present in the samples. The reaction mixture contained 0.1 ml of Folin-Ciocalteu reagent, 0.5 ml of 7.5 % solution of Na_2CO_3 solution and 0.2 ml of sample (1 mg/ml) and 0.4 ml of distilled water. The mixture was heated for 30 min at 40^0C in water bath and was allowed to cool. The absorbances of the samples were measured against blank at 760 nm using Spectrophotometer. All the tests were performed in duplicate and results were determined from gallic acid calibration curve and expressed as gallic acid equivalent (GAEmg/g).

Total flavonoid content:

Flavonoid assay was carried out using aluminium trichloride method described by Ahlem *et al* (2015). Volumes of 0.125 ml of 1 mg/ml of samples were added to 0.075 ml of 5% sodium nitrate ($NaNO_2$) solution. The mixture was allowed to stand for 6 min at room temperature. A volume of 0.15 ml of 10% aluminium trichloride was added and incubated for 5 minutes at room temperature. Then 0.75 ml of sodium hydroxide (1M) was added and the final volume was adjusted to 2 ml by adding 0.9 ml of distilled water. Final mixture was incubated for 15 min at

room temperature and the absorbance of the mixture was measured at 510 nm against blank. The total flavonoid content was calculated from Quercetin calibration curve, and the result was expressed as mg Quercetin equivalent per g dry weight (mgQEq/g).

3.2.4 ACUTE TOXICITY TEST

Acute toxicity test was carried out using the method of Miller and Tainter as described by Randhawa. Two species of animals (60 mice, 23.47 ± 0.35 g and 60 rats, 129.86 ± 0.98 g) of either sex were fasted overnight, weighed, labeled and randomized into six groups of 10 animals each (5 males and 5 females respectively) as follows; Control, 100, 1000, 2000, 3000 and 5000 mg/kg of extract. The control group received 10 ml/kg of distilled water. After administration, animals were observed for signs of toxicity or death during the first 4 hr and after 24 hr. Animals were further observed for two weeks for signs of delayed toxicity and death (Randhawa, 2009).

3.2.5 ANTI-INFLAMMATORY ASSAY

3.2.5.1 PRELIMINARY ANTI-INFLAMMATORY SCREENING AND ESTABLISHMENT OF ED_{50} OF EXTRACT

This test was carried out using the methods described by Anosike and Obidoa, (2010) and Nworu *et al.,* (2012). A total of thirty rats of either sex, 134.93 ± 0.89 g were randomly divided into six groups of five each as described below; control (distilled water, 10 ml/kg), 50, 100, 200, 400 and 800 mg/kg of the extract. After 1 hr of administration, initial paw diameter (0 hr) was measured with the aid of cotton thread wrapped around the perimeter of the left hind paw and placed on a meter rule (Bamgbose and Noamesi, 1981). Then, 0.1 ml of fresh egg-albumin was injected into the sub-planter region of the left hind paw of animals and subsequent paw diameters were measured and recorded at 1^{st}, 2^{nd}, 3^{rd}, 4^{th}, 5^{th} and 6^{th} hr respectively after the injection of egg-

albumin. Paw edema was estimated as the difference between the paw diameter at zero hour (Vo) and the paw diameter at other time intervals (Vt) after the administration of the fresh egg-albumin. Percentage inhibition of paw edema was calculated relative to paw edema in control groups at various time intervals.

ED_{50} (dose that elicits 50% of maximal anti-inflammatory activity) of the extract was calculated from the graph of percentage response against log dose. The ED_{50} was selected for further anti-inflammatory tests.

3.2.5.2 EFFECTS OF EXTRACT AND FRACTIONS ON EGG-ALBUMIN INDUCED PAW EDEMA

From the preliminary anti-inflammatory studies on the extract, the ED_{50} (400 mg/kg) and half (1/2) ED_{50} (200 mg/kg) were selected for comparative assessment of anti-inflammatory activities of various fractions (n-hexane, ethyl acetate, butanol, water fraction) alongside with the extract.

A total of sixty rats of either sex (134.93 ± 0.89 g) were randomly divided into twelve groups of five animals each as described below; Control (distilled water, 10 ml/kg), Indomethacin (10 mg/kg), extract (200 and 400 mg/kg), n-hexane fraction (200 and 400 mg/kg), ethyl acetate fraction (200 and 400 mg/kg), butanol fraction (200 and 400 mg/kg) and water fraction (200 and 400 mg/kg). Extract and fractions were tested using a similar method and procedure described in section 3.3.5.1 above.

3.2.5.3 EFFECTS OF EXTRACT AND FRACTIONS ON XYLENE INDUCED TOPICAL EAR EDEMA

This test was carried out following the method described by Nworu *et al*, (2012) and Ajaghaku *et al.*, (2013). A total of sixty five mice of either sex (23.25 ± 0.34 g) were radomized into thirteen (13) groups of five mice each as follows: Group 1 (control, 50 µL/ear of methanol), groups 2 and 3 (Indomethacin 50 and 100 µg/ear), groups 4 -13 (50 and 100 µg/ear of extract, n-hexane, ethyl acetate, butanol and water fractions).

After grouping, 50 µL of various treatments were applied to the anterior surface of the right ear, while 50 µL of xylene was applied to the posterior surface of the same ear. Two hours later, mice were sacrificed by cervical dislocation and both (right and left) ears were removed with the aid of 4 mm diameter cork borer and weighed using analytical weighing balance.

Edema was quantified as the weight difference between right and left ear plug of animals. Percentage inhibitions of edema by extract and fractions were calculated relative to edema in the control group.

The VLC fractions that elicited significant membrane stabilizing activities were selected and also subjected alongside with ethyl acetate fraction using xylene induced topical edema model. A total of sixty five mice of either sex (23.32 ± 0.26 g) were randomized into thirteen groups (n = 5). The groups include; control, Indomethacin, VLC-E13, VLC-E5, VLC-E14, VLC-E7 and ethyl acetate fraction. Two concentrations, 50 and 100 µg/ml were used for each test sample. They were screened using the method and procedure described above.

Also, Sephadex fractions that were selected based on the outcome of bioassay guided activity studies were screened using similar method and procedure described above. A total of sixty five

mice of either sex (28.22 ± 0.36 g) were randomized into 13 groups (n = 5). The groups include; control, Indomethacin, VLCE-7, SPH-E5, SPH- E6, SPH- E3, SPH- E4. Each sample had two dose levels (50 and 100 µg/ear).

3.2.5.4 EFFECTS OF EXTRACT AND FRACTIONS ON HEAT AND HYPOTONIC INDUCED HAEMOLYSIS

This assay was carried out on the extract and four fractions (n-hexane, ethyl acetate, butanol and water fractions) as well as selected vacuum liquid chromatography (VLC) fractions from ethyl acetate using the method described by Umapathy *et al* (2010) and Murugan and Parimelazhagan (2014).

Preparation of erythrocyte suspension:

Five mililitre of blood sample was obtained from a healthy human volunteer and transferred into a test tube containing anticoagulant, ethylene-diamine-tetra-acetic acid (EDTA) and then centrifuged at 3000 rpm for five minutes. The supernatant was removed and the red cell was washed three times with equal volume of normal saline until the supernatant was clear. Thereafter, the volume of the red cells was reconstituted as 40% (V/V) suspension with an isotonic buffer solution pH 7.4, containing NaH_2PO_4 (0.2 g/L), Na_2HPO_4 (1.15 g/L) and NaCl (9.0 g/L).

Heat induced haemolysis assay:

Two milliliter (2 ml) of isotonic buffer solution containing various concentrations (200, 400 and 800 µg/ml) of extract and fractions were placed in duplicate. The control tube contained only 2 ml of the vehicle while the standard groups contained 2 ml of 200, 400 and 800 µg/ml of Indomethacin and Prednisolone respectively. Thereafter, 20 µL of erythrocyte suspension was

added to each tube and gently mixed and the tubes were heated in a water bath at 54 ^{0}C for twenty minutes. After heating, the reaction mixture was allowed to cool. Thereafter, it was centrifuged at 1500 rpm for three minutes and absorbance of the supernatant was measured using a spectrophotometer at 540 nm, the reqired wavelength for hemoglobin estimation. Percentage inhibition of haemolysis or membrane stabilization of tested samples was calculated using the equation below.

% Inhibition of haemolysis = 100 x (A_1-A_2/A_1)

Where: A_1 = Absorption of control, A_2 = Absorption of test sample.

Hypotonic induced haemolysis:

Two milliliter (2 ml) of hypotonic solution containing various concentrations (200, 400 and 800 µg/ml) of extract and fractions were placed in duplicate. The control tube contained only 2 ml of the vehicle (distilled water) while the standard groups contained 2 ml of 200, 400 and 800 µg/ml of Indomethacin and Prednisolone prepared with distilled water.

Thereafter, 20 µL of erythrocyte suspension was added to each tube and gently mixed and the tubes were kept at room temperature for one hour. After 1 hour of incubation, the reaction mixture was centrifuged at 1500 rpm for three minutes and absorbance of the supernatant was measured using a spectrophotometer at 540 nm. The percentage inhibition of haemolysis or membrane stabilization was calculated using the equation described above for heat induced haemolysis (Umapathy *et al.*, 2010).

3.2.5.5: EFFECT OF EXTRACT AND FRACTIONS ON FORMALDEHYDE INDUCED ARTHRITIS

This test was carried out following the method described by Nworu *et al* (2012) and Ajaghaku *et al* (2013). Fifty rats of either sex (118.06 ± 0.81 g) were randomized into ten groups of five animals each as follows: Group 1 (control, distilled water 10 ml/kg), group 2 (Indomethacin, 5 mg/kg), groups 3-10 (received 200 and 400 mg/kg doses of the extract, ethyl acetate, butanol and water fractions respectively). One hour after oral treatment, paw diameter was measured with the aid of cotton thread and arthritis was induced by injection of 100 µl (0.1 ml) of 2.5% formaldehyde solution on the sub-plantar region of the left hind paw. On the 4^{th} day of the experiment, arthritis induction was repeated for the maintenance of arthritis. Arthritis level was assessed by measuring the paw diameter once daily for ten days. The level of inhibition of arthritis relative to untreated control was calculated from the area under curve using Microsoft excel, 2007.

3.2.5.6 ULCEROGENIC STUDY ON EXTRACT AND FRACTIONS

This test was carried out following the method described by Moke *et al* (2015) and Mezui *et al* (2017). Forty mice of either sex (20.02 ± 0.44 g) were fasted for eighteen hours prior to the experiment, weighed and randomized into ten groups of five animals each. They were treated as follows; control (distilled water, 10 ml/kg), Indomethacin (50 mg/kg), extract (200 and 400 mg/kg), butanol fraction (200 and 400 mg/kg) and ethyl acetate fraction (200 and 400 mg/kg).

Four hours after treatment, animals were sacrificed by cervical dislocation and the stomachs were removed and cut open along the greater curvature and washed with tap water and observed for presence or absence of lesions. Lesions on the mucosal surface were scored according to an

arbitrary scale, as follows; 0 = no lesion; 0.5 = hyperemia; 1= one or two lesions; 2 = severe lesions; 3 = very severe lesions; 4 = mucosa full of lesions (Main and Whittle, 1975).

3.2.5.7 VASCULAR PERMEABILITY TEST

This test was carried out following the method described by Nwabunike *et al* (2014). Forty five male mice (25.08 ± 0.40 g) were randomized into groups (n =5) as follows; group 1 (control, distilled water, 10 ml/kg), group 2 (Indomethacin, 50 mg/kg), group 3 (Diclofenac sodium, 10 mg/kg), groups 4 -9 (200 and 400 mg/kg of extract, ethyl acetate fraction and butanol fractions). One hour after treatment, 0.1 ml of Evans blue (1% w/v, in normal saline) was injected intravenously through the lateral tail vein (at 1 cm to the base of the tail). After thirty minutes, animals were injected intraperitoneally with 10 ml/kg of 1%v/v acetic acid prepared in normal saline. Then, after another thirty minutes, the mice were sacrificed by cervical dislocation and the abdominal cavity was washed with 5 ml of normal saline into a petri dish and the abdominal fluid was centrifuged at 3000 rpm for five minutes. Two milliliter (2 ml) of the supernatant was pipetted and the absorbance was measured using a Spectrophotometer set at 610 nm to determine the amount of dye leakage in the supernatant.

3.2.5.8 LEUKOCYTES MIGRATION TEST

This test was carried out following the method described by Ajaghaku *et al* (2013) and Nwabunike *et al* (2014). Fifty mice were randomized into groups (n =5) as follows; group 1 (induced control distilled water, 10 ml/kg), group 2 (Indomethacin, 50 mg/kg), group 3 (Diclofenac sodium, 10 mg/kg), groups 4 -9 (200 and 400 mg/kg of extract, ethyl acetate fraction and butanol fractions) and group 10 (non-induced control, distilled water, 10 ml/kg).

One hour after administration, leukocyte migration was induced by intraperitoneal administration of 10 ml/kg of 3% carrageenan. Four hours later, animals were anesthetized with diethyl ether and the peritoneal fluid was washed with 5 ml of phosphate buffered saline (containing 10 % EDTA) into a petri dish and was used for the determination of total and differential leukocyte count using Abacus Junior Haematology Analyzer.

3.2.5.9 EFFECTS OF SELECTED VLC FRACTION ON THE ACTIVITIES OF CYCLOOXYGENASE (COX-1 AND COX-2) ENZYMES

Selected VLC fraction, VLC-E7 was subjected to cyclooxygenase assays using Cayman chemical COX (Ovine) Colorimetric Inhibitor Screening Assay kit (Catalog number 760111) as described by Rafik *et al.,* (2014). Composition of assay kits include; Assay buffer, Heme, COX-1 (ovine), COX-2 (ovine), Arachidonic acid (substrate), Potassium hydroxide, Colorimetric substrate, 96-Well plate (Colorimetric Assay) and 96 –Well cover sheet.

The background wells (3 wells) consisted of 160 µl assay buffer, 10 µl of heme, and 10 µl of methanol. Initial activity wells (3 wells) consisted of 150 µl of assay buffer, 10 µl of heme, 10 µl of methanol and 10 µl of COX -1 or COX -2 enzyme. Positive wells (3 wells) consisted of 150 µl of the ssay buffer, 10 µl of heme, 10 µl of COX -1 or COX -2 enzyme and 10 µl of Diclofenac (0.296 µg/ml, 0.296 µg dissolved in 1 ml buffer).

Inhibitor wells consisted of of 150 µl of the assay buffer, 10 µl of heme, 10 µl of COX -1 and COX -2 enzyme and 10 µl of various concentrations (25, 50 and 100 µg/ml) of test the test samples (VLC-E7).

After the various samples were added to their respective wells, the plate was shaken for a few seconds and was incubated for ten minutes at 25℃. Then 20 µl of colorimetric substrate solution

was added to each well followed by the addition of 20 µl of arachidonic acid to all wells. The plate was shaken for a few seconds and was incubated for 2 min at 25 ℃.

Thereafter, the absorbance was read at 590 nm using a microplate reader (TECAN Safira 2, Vector Scientific, NI, UK). Assays were carried out in triplicate and average absorbances for various concentrations were taken. Percentage inhibition of the enzymes was determined by comparing the rate reaction of the sample with the negative control (blank). Diclofenac was used as positive control. Sample well containing the buffer and enzymes was used as sample blank.

Absorbances of background wells were subtracted from absorbance of various concentrations of samples to have the actual samples absorbances. Thereafter, inhibition was calculated using the following formula.

$$\text{Inhibition} = \frac{\text{Absorbance of initial activity} - \text{Actual sample absorbance}}{\text{Absorbance of initial activity}} \times \frac{100}{1}$$

3.2.6 ANTIOXIDANT ASSAY

3.2.6.1 FREE RADICAL (DPPH) SCAVENGING TEST

This assay was carried out as described by Patel and Patel (2010). DPPH (1, 1-diphenyl-2-picrylhydrazil) solution (0.6 mM) was freshly prepared using methanol. The reaction mixtures which contain 0.1 ml of various concentrations, 6.25, 12.5, 25, 50, 100, 200, 400 and 800 µg/ml of extract and fractions, 0.1 ml of 0.6 mMol DPPH and 0.8 ml of methanol were incubated in the dark for thirty minutes at room temperature. Thereafter, absorbances of the samples were measured at 517 nm against blank (methanol) using a Spectrophotometer. Ascorbic acid and Quercetin were used as standards. A tube containing 0.1 ml of DPPH solution and 0.9 ml methanol served as a control. Assays were carried out in duplicates. Free radical scavenging activities of samples were obtained using the formula below:

DPPH scavenging activity = 100 x (AC – AS)/ AC)

AC = Absorbance of control

AS = Absorbance of sample

A graph of percentage inhibition against concentration was plotted and the concentration that caused 50% inhibition (IC_{50}) was extrapolated using a regression analyses equation (Patel and Patel, 2010).

3.2.6.2 FERRIC REDUCING ANTIOXIDANT POWER (FRAP) ASSAY

FRAP assay was carried out following the method described by Habibur *et al* (2013) and Moein *et al* (2012). Two hundred and fifty microlitre (0.25 ml) of various concentrations, 6.25, 12.5, 25, 50, 100, 200, 400 and 800 µg/ml of extract and fractions were mixed with 0.625 ml of phosphate buffer and 0.625 ml of 1% potassium ferricyanide [K_3FeCN_6]. The mixtures were heated at 50^0C for twenty minutes. Then, 0.625 ml of 10% trichloroacetic acid (TCA) was added and the mixtures were centrifuged at 3000 rpm for ten minutes. From the upper layer, 0.625 ml was pipetted and mixed with 0.625 ml of distilled water and 0.125 ml of 0.1% (w/v) ferric chloride ($FeCl_3$) solution. Absorbances of the mixtures were measured at 700 nm against air using a Spectrophotometer. Ascorbic acid and Quercetin were used as standards. Tests were performed in duplicate and percentage inhibition was calculated using the formula below.

$$\% \text{ Inhibition} = \frac{\text{Average absorbance of sample} - \text{Average absorbance of blank}}{1} \text{ X } \frac{100}{1}$$

A graph of percentage inhibition against concentration was plotted and the effective concentration (EC_{50}) was extrapolated using a regression analyses equation.

3.2.6.3 NITRIC OXIDE SCAVENGING ASSAY

This assay was carried out following the method described by Habibur*et al* (2013) and Ezeja *et al* (2015). Two milliliter of 10 mM sodium nitroprusside prepared with phosphate buffer saline (pH 7.4) was mixed with 0.5 ml of samples at various concentrations (6.25, 12.5, 25, 50, 100, 200, 400 and 800 µg/ml). The mixture was incubated at room temperature for 150 min. Thereafter, 0.5 ml of the reaction mixture was withdrawn and mixed with 0.5 ml of Griess reagent (1% sulphanilamide + 0.1% naphthylethylenediamine dichloride + 3% phosphoric acid) was added to each test tube. The absorbance of the pink chromophore formed was measured at 540 nm. Ascorbic acid and Quercetin were used as standards. Assays were carried out in duplicates. Percentage inhibition was calculated using the formula below

$$\frac{\text{Abs Control} - \text{Abs Test}}{\text{Abs Control}} \times \frac{100}{1}$$

Inhibitory concentration, IC_{50} was estimated from a graph of % inhibition versus concentration using regression analyses equation.

3.2.7 PURIFICATION, ISOLATION AND STRUCTURAL ELUCIDATION

3.2.7.1 VACUUM LIQUID CHROMATOGRAPHY (VLC)

A portion of ethyl acetate fraction, 6.5 g was mixed with silica gel, air-dried and eluted with 500 ml of various ratios of n-hexane: ethyl acetate mixture (10:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9 and 0:10) as well as various ratio combinations of dichloromethane (DCM): Methanol mixtures (10:0, 9:1, 7:3, 5:5, 2:8, 0:10). These resulted to 17 fractions (VLC E1 – VLC E17). Samples were air dried and weighed (Cruz *et al.*, 2016).

3.2.7.2 SIZE EXCLUSION CHROMATOGRAPHY (SEPHADEX)

The ethyl acetate VLC fraction (VLC-E7, n-hexane: ethyl acetate, 0:10) was selected based on its promising biological activities for size exclusion chromatography. VLC-E7, 176.4 mg was dissolved in 2.5 ml of dichloromethane and methanol (1:1). The mixture was centrifuged and the supernatant was chromatographed in Sephadex LH-20. It was eluted with a combination of dichloromethane and methanol (1:1) at a flow rate of 0.20 - 0.25 (about 5 – 6 drops) ml/min. A total of 105 fractions of the eluents were collected. Each fraction contained about 2 - 2.5 ml of eluent. The fractions were spotted on thin layer chromatography (TLC) plate, 0.95 cm apart from neighboring fraction and 1 cm apart from the base. The spotted plate was developed and drawn up using dichloromethane and methanol, 9:1 as mobile phases. The fractions were bulked based on their color characteristic and their elution when visualized using UV lamp set at 254 nm. Bulked samples were air-dried and weighed (Okoye *et al.,* 2015; Cruz *et al.,* 2016).

3.2.7.3 SEMI-PREPARATIVE HIGH PRESSURE LIQUID CHROMATOGRAPHY (HPLC)

The separation column (125 × 4mm, i.d.) was prefilled with Eurosper C-18 (Kneuer, Berlin Germany) or Dynamax (250 × 21.4mm, L. ID). All samples for semi-preparative HPLC were analyzed thoroughly and pre-treated to make the separation to be optimal and maintain the lifespan of the HPLC system. Pretreatment was carried out by mixing the complex fraction with methanol. The mixture was centrifuged and the supernatant was used for HPLC injection. The mobile phase was a mixture of methanol and nano pure water. The flow rate was stabilized at 5 ml/minute. The eluted samples were recovered after detection by UV-VIS diode array detector. Isocratic condition was applied to achieve separations (Okoye *et al.,* 2015).

3.2.7.4 ANALYTICAL HIGH PRESSURE LIQUID CHROMATOGRAPHY (HPLC)

One milligram (1 mg) of the fraction was dissolved in 1 ml of HPLC grade methanol and was centrifuged for ten minutes. Then 100 µl of the supernatant was taken into the HPLC vial for analyses. The solvent gradient used started with 10:90 (MeOH: nanopure water, adjusted to pH 2 with phosphoric acid) increasing to 100 % methanol in 45 minutes and run till sixty minutes. Flow rate was 1000 µl/minute. Column temperature was 20^0C. HPLC analysis was carried out on sample using Dionex P580 HPLC system coupled to a photodiode array detector. Detection was carried out at 235, 245, 280 and 340 nm (Okoye *et al.*, 2015).

3.2.7.5 ELECTRON SPRAY IONIZATION MASS SPECTROMETRY (HPLC/ESI-MS)

Samples were subjected to Liquid Chromatography – Electrospray ionization Mass Spectroscopy (LC/ESI-MS) using a Thermo Finningnan LCQ-Deca mass spectrometry connected to a UV detector. Samples were injected into the HPLC/ESI-MS set-up. A solution of the sample was sprayed at atmospheric pressure through a 2-5 kV potential. HPLC was run on a Eurospher C-18 (6 × 2 nm, i.d) reverse phase column. The gradient eluent was composed of 0.1% formic acid in nanopure water and acetonitrile. The samples were dissolved in methanol and water. The flow rate was 0.4 ml/minute. Injected volume was 10 µl while sample concentration was 100 µg/ml. Column temperature was at room temperature. Absorbances were detected at 235, 254, 280 and 340 nm (Okoye *et al*, 2015).

3.2.7.6 NUCLEAR MAGNETIC RESONANCE (NMR) SPECTROSCOPY

The ^{1}H NMR spectra were recorded at 300^0K on Bruker DPX 300, ARX 400, 500 and 600 AVANCE DMX 600 NMR spectrometers. All 1D spectra was obtained using the standard

Bruker software. The samples were dissolved in a methanol. Tetramethylsilane (TMS) was used as an internal standard reference signal (Okoye *et al.,* 2015).

3.3 LONG-TERM TOXICITY STUDIES

3.3.1 TWO WEEKS REPEATED DOSE TOXICITY TEST

A total of twenty five rats of either sex (82.17 ± 1.52 g body weight) were randomized into five groups of five animals each as follows: Control, received distilled water (10 ml/kg) while groups 2, 3, 4 and 5 received extract at 250, 500, 1000 and 2000 mg/kg respectively.

After collecting blood samples for baseline assessment of haematological and biochemical parameters, animals were dosed once daily with the extract for a period of 14-days. At the end of the 14^{th} day (day 15), blood samples were collected from the retro-orbital plexus into EDTA tubes and were used for the determination of hematological parameters. Also, blood samples were collected into plain tubes and were centrifuged at 3500 rpm for ten minutes. Sera were aspirated into another set of labeled plain tubes and diluted 5-fold with normal saline and used for the assay of biochemical parameters including alkaline phosphatase (ALP), alanine aminotransferases (ALT), aspartate transaminases (AST), total protein, albumin, sodium, potassium, chloride, calcium, urea, creatinine, total cholesterol, triglyceride and high density lipoprotein cholesterol using manufacturer's (Randox and Teco) kits leaflets procedures with little modifications. Normal saline was added to reagent blank and the resulting absorbance of samples were multiplied by five. Body weights and organ weights were recorded.

Liver and kidney were fixed in 10 % formal saline for histopathological analyses. Animals' stomachs were removed and cut open along the greater curvature and washed with tap water and observed for presence or absence of lesions.

3.3.2 SUB-CHRONIC TOXICITY TEST

This test was carried out following the method described by Asma *et al* (2016). From results of 14 – day repeated toxicity test, doses that did not show toxicity were selected for sub-chronic toxicity test. A total of sixty rats of either sex (84.00 ± 2.89 g body weight) were randomized into five groups of twelve animals each. The control group received 10 ml/kg of distilled water while the test groups received 62.5, 125, 250 and 500 mg/kg of the extract. After collection of blood samples for determination of baseline hematological and biochemical parameters, animals were dosed once daily for a period of 90-days. On 31, 61 and 91st days, blood samples were collected from the retro-orbital plexus into EDTA and plain tubes for the determination of haematological and biochemical parameters respectively.

Body and organ weights were recorded. Liver and kidney were fixed in 10 % formal saline for histopathological analyses. Animals' stomachs were removed and cut open along the greater curvature and washed with tap water and observed for presence or absence of lesions.

At the end of 90-days, animals were placed on food and water *ad libitum* for 28-days without administration of the extract. At the end of 28th day (day 29), blood samples collected from retro-orbital plexus of animals were used for the determination of haematological and biochemical parameters. Body weights and organ weight were recorded. Liver and kidney were fixed in 10 % formal saline for histopathological analyses. Animals' stomachs were removed and cut open along the graeter curvature and washed with tap water and observed for presence or absence of lesions.

3.3.3 CHRONIC TOXICITY TEST

Following the method described by Asma *et al* (2016), maximum tolerable dose (MTD) obtained from sub-chronic toxicity study was determined and used for chronic toxicity test. On this basis, MTD (125 mg/kg), ½ of MTD (62.5 mg/kg) and 1/4th of MTD (31.25 mg/kg) were selected for the chronic toxicity study.

A total of forty eight rats of either sex (66.15 ± 1.41 g body weight) were randomized into four groups of twelve animals each. Control group received 10 ml/kg of distilled water while the test groups received 31.25, 62.5 and 125 mg/kg of the extract. After collection of blood samples for the determination of baseline hematological and biochemical parameters, animals were dosed once daily for a period of 180-days (6-months). At the end of 180 days (day 181), blood samples were collected from retro-orbital plexus of animals for the determination of haematological and biochemical parameters. Body and organ weights were also recorded. Liver and kidney were fixed in 10 % formal saline for histopathological analyses. Their stomachs were removed and cut open along the greater curvature and washed with tap water and observed for presence or absence of lesions.

At the end of 180-days, animals were placed on food and water *ad libitum* for 28-days without administration of the extract. At the end of the 28 day (day 29), samples were collected for haematological, biochemical and histopathology analyses. Body weights and organ weights were also recorded. Their stomachs were removed and cut open along the greater curvature and washed with tap water and observed for presence or absence of lesions (Chatchai *et al.,* 2013; Prasanth *et al.,* 2014; Asma *et al.,* 2016).

3.3.4 ASSAY OF BIOCHEMICAL PARAMETERS

3.3.4.1 DETERMINATION OF LIVER ENZYMES

Determination of serum alkaline phosphatase:

The method of Roy (1970) which uses phosphate thymolpthalein monophosphate was used for the determination of serum alkaline phosphatase.

Two hundred and fifty microlitres (250 µl) of alkaline phosphatase substrate reagent was added to test tubes labeled as blank, samples and standard. Then 25 µl of normal saline, samples and alkaline phosphatase standard reagent were added to the test tubes labeled blank, samples and standard respectively. They were mixed and incubated for ten minutes at 37^0C. This was followed by the addition of 1250 µl of alkaline phosphatase color developer to all tubes and absorbances of standard and sample tubes were measured against reagent blank at 590 nm using Spectrophotometer.

Alkaline phosphatase Concentration (IU/L) = $\frac{Abs.of\ sample}{Abs.of\ standard}$ x Concentration of standard

Determination of serum alanine aminotransferase (ALT):

Serum ALT was determined using the method of Reitman and Frankel (1957).

Test tubes were labeled blank and samples. To the sample and blank test tubes, 20 µL of normal saline and serum from samples were added respectively. Then 100 µl of ALT reagent 1 was added to samples and blank test tubes and all test tubes were mixed and incubated for 30 min at 37 ^{0}C. After thirty minutes incubation, 100 µ of ALT second reagent was added to all tubes and the mixtures were incubated at room temperatures for twenty minutes. Thereafter, 1000 µl of 0.4 mol/L sodium hydroxide solution was added to the mixture and absorbance of samples were

measured against reagent blank after five minutes at 546 nm using a Spectrophotometer. ALT activity was obtained from ALT calibration curve.

Determination of serum aspartate aminotransferase (AST):

Serum AST was determined using the method of Reitman and Frankel (1957).

Test tubes were labeled blank and samples. To the sample and blank test tubes, 20 µl of normal saline and serum were added respectively. Then 100 µl of AST reagent 1 was added to blank and samples test tubes and all test tubes were incubated for thirty minutes at 37 ^{0}C. After 30 minutes incubation, 100 µl of AST reagent 2 was added and the mixture was incubated at room temperatures for twenty minutes. Thereafter, 1000 µl of 0.4 mol/L of sodium hydroxide solution was added to the mixture and absorbance of samples were measured against a reagent blank after five minutes at 546 nm. AST activity was obtained from AST calibration curve.

Determination of serum total protein:

The method of the American Association for Clinical Chemistry (AACC) and National Committee for Clinical Laboratory Standard (NCCLS, 1979) was used for the determination of total protein level in serum.

Total protein reagent (1500 µl) was transferred into test tubes labeled as blank, samples and standard. Then, 10 µl of normal saline, samples and standard total protein reagent were added to their respective test tubes and mixed. The mixtures were incubated at room temperature for ten minutes and absorbance of samples and standard were measured against reagent blank at 540 nm using a Spectrophotometer.

Total protein (g/dl) in an unknown sample was calculated as follows:

$$\frac{Absorbance\ of\ sample}{Absorbance\ of\ standard} \times \text{Concentration of standard.}$$

Determination of serum albumin:

Bromcresol green method of Doumas and Biggs (1976) was used in the determination of serum albumin level in serum.

The working reagent (1500 µl) was transferred into test tubes labeled as blank, samples and standard. Thereafter, 10 µl of normal saline, samples and standard albumin reagent were added to their respective test tubes and mixed. The mixtures were incubated at room temperature for five minutes and absorbance of samples and standard were read against reagent blank at 630 nm using a Spectrophotometer.

Albumin level (mg/dl) in the sample was calculated using the formula below;

$\frac{Absorbance\ of\ sample}{Absorbance\ of\ standard}$ × Concentration of standard.

3.3.4.2 DETERMINATION OF RENAL BIOMARKERS

Estimation of serum chloride:

Serum chloride determination was based on the modified colorimetric method of Skeggs and Hochestrasser (1964).

Chloride reagent (1500 µl) was added into test tubes labeled blank, samples and standard respectively. This was followed by the addition of 10 µl of normal saline, samples and standard chloride reagent to their respective test tubes. The mixtures were incubated at room temperature for five minutes and absorbance of samples and standard were measured against reagent blank at 480 nm using a Spectrophotometer.

Concentration of chloride in the sample was calculated using the formula:

Chloride concentration (mEq/L) = $\frac{Abs.of\ sample}{Abs.of\ standard}$ x Concentration of standard.

Determination of serum sodium:

Modified method of Maruna (1958) was used for the determination of serum sodium.

Sodium filtrate reagent (500 µl) was pipetted into test tubes labeled as blank, standard and samples. Thereafter, 25 µl of normal saline, samples and standard chloride reagent were added to their respective tubes and mixed continuously for three minutes. Tubes were centrifuged at 1500 rpm for ten minutes. Thereafter, 50 µl of supernatant from blank, samples and standard were mixed with 1000 µl of chloride acid reagent in another set of test tubes. Thereafter, 50 µl of color developer reagent was added to the final mixture and absorbances of all tubes were measured against water blank at 550 nm.

Sodium concentration in sample (mEq/L) = $\frac{Abs.of\ Blank - Abs.of\ Sample}{Abs.of\ Blank - Abs.of\ Standard}$ x Conc. of Standard

Determination of serum potassium:

The method of Terri and Sesin (1958) was used for the determination of serum potassium.

Potassium reagent (1000 µl) was added into test tubes labeled as blank, samples and standard. Thereafter, 10 µl of normal saline, samples and standard potassium reagent were added to their respective test tubes and mixed. The mixtures were incubated at room temperature for three minutes. Thereafter, absorbance of samples and standard were measured against reagent blank at 500 nm using Spectrophotometer.

Potassium concentration in sample (mEq/L) = $\frac{Abs.of\ sample}{Abs.of\ standard}$ x Concentration of standard.

Determination of serum creatinine:

The endpoint method modified by Heinegard and Tiderstom (1973) was used for the determination of serum creatinine.

Creatinine reagent (1500 µl) was added to test tubes labeled as blank, samples and standard. Then 50 µl of normal saline, samples and standard creatinine reagents were added to their respective test tubes and the mixtures were incubated at 37^0C for fifteen minutes. Absorbance of samples and standard were measured against reagent blank at 510 nm.

Creatinine concentration (mEq/L) in sample = $\frac{\text{Absorbance of sample}}{\text{Absorbance of standard}}$ x Concentration of standard

Determination of serum blood urea nitrogen (BUN):

The modified Berthelot reaction method of Tobacco, (1979) was employed for the determination of serum blood urea Nitrogen.

Exactly 1500 µl of BUN enzyme reagent was added into test tubes labeled as blank, samples and standard. Then 10 µl of normal saline, samples and standard BUN reagent were added to blank, samples and standard tubes respectively and mixtures were incubated for five minutes at 37^0C. Thereafter, 1500 µl of BUN enzyme color developer was added to the mixture and further incubated for five minutes at 37^0C. Absorbance of samples and standard, blue color complex were measured against reagent blank at 630 nm.

BUN concentration (mg/dl) in sample = $\frac{\text{Absorbance of sample}}{\text{Absorbance of standard}}$ x Concentration of standard

Determination of serum calcium:

The complex metric procedure of Gitelman (1967) was used in the determination of calcium level in serum.

Calcium working reagent (1000 µl) was transferred into test tubes labeled as blank, sample and standard. Thereafter, 20 µl of normal saline, samples and standard calcium reagents were added to their respective test tubes and mixed. The mixtures were incubated for at least one minute at

room temperature and absorbance of samples and standard were read against reagent blank at 570 nm using a Spectrophotometer.

Calcium level (mg/dl) in sample was calculated using the formula below;

$\frac{Absorbance\ of\ sample}{Absorbance\ of\ standard}$ × Concentration of standard.

3.3.4.3 DETERMINATION OF LIPID PROFILE

Determination of serum triglyceride:

The colorimetric method of Tietz (1990) which involves enzymatic hydrolysis with lipase was used for the determination of serum triglyceride.

Triglyceride reagent (1000 µl) was added to test tubes labeled as blank, samples and standard. Thereafter, 10 µl of normal saline, samples and standard triglyceride reagent were added to test tubes labeled samples and standard respectively. The mixture were incubated for five minutes at 37 ^{0}C and absorbance of the samples and standard were measured against reagent blank at 500 nm within sixty minutes using a Spectrophotometer.

Triglyceride concentration (mg/dl) = $\frac{Absorbance\ of\ sample}{Absorbance\ of\ standard}$ x Concentration of standard

Determination of serum total cholesterol:

The enzymatic procedure of Roeschlaw *et al* (1974) which replaced chemical saponification with enzymatic saponification was used for the determination of serum cholesterol.

Total cholesterol reagent (1000 µl) was added to test tubes labeled as blank, samples and standard. Thereafter, 10 µl of normal saline, samples and standard cholesterol reagent were added to test tubes labeled samples and standard respectively. Mixture was incubated for five minutes at 37 ^{0}C and absorbance of the samples and standard were measured against reagent blank at 500 nm within sixty minutes using a Spectrophotometer.

Total cholesterol in sample (mg/dl) = $\frac{\text{Absorbance of sample}}{\text{Absorbance of standard}}$ x Concentration of standard.

Determination of serum high density lipoprotein cholesterol:

The method developed by the National Institute of Health Consensus Development Conference Statement **(**NIHCDCS, 1992) was employed in the determination of HDL-Cholesterol in serum. Normal saline (100 µl), samples and standard cholesterol reagent were added to the test tubes containing 250 µl of HDL cholesterol precipitate (R1) respectively. The mixtures were centrifuged at 4000 rpm for ten minutes. After centrifuging, 100 µL of the blank, samples and standard supernatants were added to another set of test tubes labeled as blank, samples and standard containing 1000 µl of cholesterol reagent. After incubating for five minutes at 37 ^{0}C, absorbance of standard and samples were measured against reagent blank at 500 nm within sixty minutes using a Spectrophotometer.

HDL cholesterol in sample (mg/dl) = $\frac{Absornace\ of\ sample}{\text{Absorbance of Standard}}$ x Concentration of standard.

Low density lipoprotein cholesterol (LDL-c) estimation:

LDL-cholesterol was calculated from triglyceride, total cholesterol and HDL-cholesterol values by the formula below.

LDL cholesterol (mg/dl) = Total cholesterol - $\frac{Triglyceride}{5}$ - HDL cholesterol.

(Friedewald *et al.*, 1972).

3.3.5 ASSAY OF HAEMATOLOGICAL PARAMETERS

Abacus Junior Haematology Analyzer was used for the determination of haematological parameters. It works based on Coulter method by detecting and measuring changes in electrical impedance when a particle in a conductive liquid passes through a small aperture. For each cell that is passing through the aperture, there is a constant current passing between the external and internal electrode thereby causing some change in the impedance of the conductive cell suspension. These changes are recorded as an increase in voltage between the electrodes. The number of pulses is proportional to the volume of the particle.

Parameters analyzed include hemoglobin, hematocrit (PCV), red blood cell (RBC), white blood cell (WBC), platelet counts, mean platelet volume (MPV), platelet percentage (PCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), lymphocytes, granulocytes and mid size cells count (MID).

3.3.6 HISTOPATHOLOGY ANALYSES

Histopathology analysis was carried out as described by Bancroft and Gamble (2010). Organs (liver, kidney, heart and lungs) were excised from animals and were fixed in 10% formal saline. They were passed through ascending grades of ethanol (70, 80, 90 and 100%), cleared with chloroform, impregnated and embedded in paraffin wax. Organs were routinely processed and sectioned at 4-5 mm thickness. Sections were mounted on albuminized glass slides and stained with Hematoxylin and Eosin (H and E) stain. The slides were mounted in cover slips with a mixture of distyrene, tricresyl phosphate and xylene (DPX) and the sections were examined by a histopathologist using a light microscope attached to a digital camera. Photomicrographs were captured using ×400 magnifications.

3.3.7 BODY AND ORGAN WEIGHTS DETERMINATION

Basal body weights of all animals were taken before administration of extract using electronic top weighing balance. Then, weekly body weights and final body weight at the end of the experiment were measured.

After blood collection from the retro-orbital plexus, animals were sacrificed by cervical dislocation, after which their liver, kidneys, heart, spleen and lungs were excised and weighed using an electronic weighing balance. Relative organ weights were calculated by dividing organ weight of each animal by their final body weight and multiplying the quotient by 100.

Percentage weight gain was calculated by subtracting initial body weight from final body weight and dividing the difference by final body weight. Thereafter, the quotient was multiplied by 100 (Ugbogu *et al.,* 2016).

Relative organ weight = $\frac{\text{Organ weight}}{\text{Final body weight}}$ x 100.

Percentage weight gain = $\frac{\text{Final body weight} - \text{Initial body weight}}{\text{Final body weight}}$ x 100.

3.3.8 EVALUATION OF STOMACH MUCOSA FOR ULCER

Stomachs of animals were removed, cut open along the greater curvature, washed with tap water and observed for presence or absence of lesions. Lesions on the mucosal surface were scored according to an arbitrary scale, as follows; 0 = no lesion; 0.5 = hyperemia; 1= one or two lesions; 2 = severe lesions; 3 = very severe lesions; 4 = mucosa full of lesions (Main and Whittle, 1975; Moke *et al,* 2015).

3.4 TERATOGENICITY TEST

A total of thirty two virgin adult female mice and fifteen adult male mice (23.70 ± 0.55 g) were used for this study. The visual method described by Richard *et al* (2016) was used for the

determination of the ovulation stage of estrus cycle based on the appearance of the external genitalia characterized by gaping vagina, light pink and less moist tissue with pronounced striation.

Twenty six female mice were selected and mated with male adult mice (2 males to 3 female mice in the same cage) overnight (7 pm to 7 am). The following morning, mating was confirmed by checking for the presence of plug in vagina of female mice. Females with plugs were confirmed as pregnant (day 0 of pregnancy).

Twenty mice with evidence of plug were divided into four groups of five animals each. Group 1, control, received 10 ml/kg of distilled water; groups 2, 3 and 4 received 250, 500 and 1000 mg/kg of extract respectively from the 6^{th} – 15^{th} days of gestation period.

Animals were allowed to give birth between the 20^{th} and 21^{st} days. Pulps were weighed and then examined for gross malformations or deviations from normal growth. Liver, kidney, heart, lung and femur of pulps from various groups were harvested and fixed in 10% buffered saline for histopathology analyses (Golalipour *et al.*, 2011; Saleem *et al.*, 2014; Madu *et al.*, 2015).

3.5 DATA ANALYSES

Results gathered from the study were presented as Mean ± Standard error of mean (SEM). Significant difference between control and treatment groups were compared using one way analysis of variance (ANOVA) followed by post hoc Tukey's test. $P<0.05$ was considered to be statistically significant, while $p>0.05$ was considered to be statistically non-significant. Statistical package for social science (SPSS-20, for windows) was the software used for data analyses. Graphical presentations of parameters, calculation of area under the curve and fifty percent inhibitory concentration (IC_{50}) of the extract and fractions were done using Microsoft Excel, 2010.

CHAPTER FOUR

4.1 RESULTS:

4.1.1 YIELD OF EXTRACT AND FRACTIONS

From table 1 below, 969.12 g (21%w/w) was recovered after extracting 4700 g of the powdered leaf by cold maceration. Yields of various fractions in increasing order include; butanol fraction (10.56 g, 3.77%w/w) < ethyl acetate fraction (16.10 g, 5.75%w/w) < water fraction (52.61 g, 18.79%w/w) < n- hexane fraction (68.10 g, 24.32%w/w).

Fraction VLC-E8 had the highest yield followed by VLC-E10, VLC-E14, VLC-E4, VLC-E7, VLC-E3 and others. Fraction VLC –E1 had the lowest yield (Table 2).

4.1.2 PHYTOCHEMICAL ANALYSIS

Phytochemical screeninig revealed the presence of tannins, flavonoids, saponins, steroids, alkaloids, terpenoids and reducing sugar in the extract and fractions of *D. filix-mas*. Anthranquinolone was absent in all the samples screened (Table 3).

4.1.3 ACUTE TOXICITY RESULTS

No deaths were recorded at various doses, after 24 hour of exposure of rats and mice to the extract of *D. filix-mas*. No toxicity or deaths were also recorded after 14-days observation period.

Table 1: Yields of extract and fractions.

	Weight (g)	% yield (w/w)
Extract	969.12	20.62[a]
n-hexane fraction	68.10	24.32[b]
Ethyl acetate fraction	16.10	5.75[b]
Butanol fraction	10.56	3.77[b]
Water fraction	52.61	18.79[b]

[a] Yield calculated from 4700 g of powdered leaves.

[b] Yield calculated from 280 g of extract.

Table 2: Yield of ethyl acetate VLC fractions.

Solvent ratio	Sample code	Weight (mg)	Yield (%)
N(500): E (0)	VLC-E1	12.70	0.20
N(450): E (50)	VLC-E2	132.50	2.04
N(400): E(100)	VLC-E9	257.95	3.97
N(350): E(150)	VLC-E13	154.55	2.38
N(300):E(200)	VLC-E4	333.95	5.14
N(250): E(250)	VLC-E11	123.05	1.89
N(200):E(300)	VLC-E3	314.60	4.84
N(150):E(350)	VLC-E15	67.60	1.04
N(100):E(400)	VLC-E5	166.70	2.56
N(50): E(450)	VLC-E17	60.40	0.93
N(0):E(500)	VLC-E7	325.25	5.00
D(500):M(0)	VLC-E6	39.85	0.61
D(450):M(50)	VLC-E14	347.35	5.34
D(350):M(150)	VLC-E8	1793.35	27.59
D(250):M(250)	VLC-E10	497.80	7.66
D(100):M(400)	VLC-E12	219.95	3.38
D(0):M(500)	VLC-E16	149.85	2.31

N = n-hexane, **E** = ethyl acetate, **M** = methanol, **D:** Dicloromethane. Yield was calculated from ethyl acetate fraction (6.5 g) used for VLC (Vacuum Liquid chromatography).

Table 3: **Qualitative phytochemistry results.**

	Extract	**n-hexane fraction**	**Ethyl-acetate fraction**	**Butanol fraction**	**Water fraction**
Tannins	++	++	++	+++	++
Flavonoids	+++	+	++	++	++
Saponins	++	+	+	++	+
Steroids	++	++	++	++	++
Alkaloids	++	+	+	+	+
Terpenoids	++	+	+	+	++
Anthraquinolones	-	-	-	-	-
Cardiac glycosides	+	+	+	-	+
Reducing sugar	+	+	+	+	+

"-":absent, "+" :trace "++" : moderate and **"+++"**: **abundant.**

4.1.4 ANTI-INFLAMMATORY RESULTS

4.1.4.1 Effects of extract on egg-albumin induced paw edema:

Administration of various doses of extract of *D. filix-mas* to rats caused reduction in paw edema of at thirty minutes up to the sixth hour. Significant reduction was recorded at the 3rd and 4th hours at 200, 400 and 800 mg/kg (Table 4). The median effective dose (ED_{50}) of the extract was established to be 399.11 mg/kg at the 4th hour (Fig. 1).

4.1.4.2 Effect of extract and fractions on egg-albumin induced paw edema:

Administration of 200 and 400 mg/kg of extract and fractions of *D. filix-mas* caused reductions in paw edema when compared to the control group (Table 5). Ethyl acetate and butanol fractions elicited dose dependent inhibitions better than other groups against egg albumin induced paw edema. Their activities were more significant at the 3rd and 4th hours.

4.1.4.3 Effect of extract and fractions on xylene-induced ear edema:

There was a significant ($p<0.05$) reduction in xylene induced topical edema following administration of extract, ethyl acetate, butanol and water fractions. Reduction elicited by n-hexane fraction was not statistically significant ($p>0.05$). Ethyl acetate and butanol fractions produced more inhibition against paw edema when compared to other groups (Table 6).

4.1.4.4. Effects of extract and fractions on heat and hypotonic induced haemolysis:

Admininstration of the extract and fractions caused concentration-depended inhibition of hypotonic induced haemolysis at all the doses by all the tested samples. Indomethacin produced the highest inhibition followed by butanol fraction, ethyl acetate fraction, extract, n-hexane fraction and water fraction.

Also, there was concentration-dependent inhibition by all the samples against heat induced haemolysis. Indomethacin caused the highest inhibition followed by ethyl acetate fraction, prednisolone, butanol fraction, extract, water fraction and n-hexane fraction (Table 7).

4.1.4.5 Effect of extract and fractions on formaldehyde-induced arthritis:

Table 8 shows the effect of extract and fractions on formaldehyde-induced arthritis in rat. There was significant reduction ($p<0.05$) in area under curve (AUC) of groups treated with indomethacin, ethyl acetate and butanol fractions, except water fraction when compared to AUC in control group. Indomethacin elicited the highest inhibition of 35.77% which was similar to that of the higher dose of butanol fraction (31.63%).

4.1.4.6 Ulcerogenic effects of extract and fractions:

Spot ulcers were observed in indomethacin treated group. However, no ulcer was recorded among the various groups treated with extract, butanol and ethyl acetate fractions.

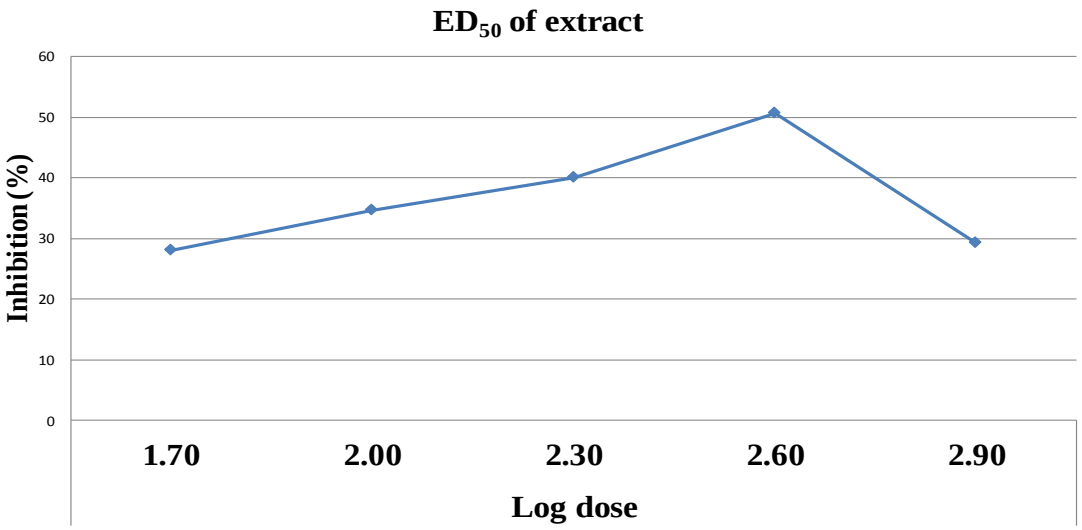
4.1.4.7 Effect of extract and fractions on vascular permeability:

From table 9, there was significant ($P<0.05$) reduction in dye content of peritoneal fluids of mice treated with extract and fractions of *D. filix-mas* when compared to control group. The extract (400 mg/kg) elicited the highest inhibition (39.55%) followed by Indomethacin (37.29%) and Diclofenac (36.16%). The butanol fraction caused the least inhibition.

Table 4: Effects of extract on egg-albumin induced paw edema.

Treatment	Change in paw size (cm) and inhibition in paw size relative to control (%)						
	0.5 hr	**1 hr**	**2 hr**	**3 hr**	**4 hr**	**5 hr**	**6 hr**
Control:	0.88 ± 0.07	0.70 ± 0.03	0.65 ± 0.07	0.76 ± 0.07	0.75 ± 0.08	0.62 ± 0.08	0.57 ± 0.07
50	0.66 ± 0.09	0.72 ± 0.05	0.66 ± 0.07	0.58 ± 0.07	0.54 ± 0.07	0.59 ± 0.10	0.45 ± 0.09
mg/kg	[25.00%]	[-2.86%]	[-1.54%]	[23.68%]	[28.00%]	[4.84%]	[21.05%]
100 mg/kg	0.60 ± 0.08	0.64 ± 0.05	0.68 ± 0.04	0.55 ± 0.04	0.49 ± 0.02	0.55 ± 0.05	0.40 ± 0.03
	[31.82%]	[8.57%]	[-4.62%]	[27.63%]	[34.67%]	[11.29%]	[29.82%]
200 mg/kg	0.55 ± 0.07	0.58 ± 0.10	0.52 ± 0.11	0.43 ± 0.05*	0.45 ± 0.08	0.36 ± 0.09	0.44 ± 0.09
	[37.50%]	[17.14%]	[20.00%]	[43.42%]	[40.00%]	[41.94%]	[22.81%]
400 mg/kg	0.58 ± 0.04	0.53 ± 0.05	0.48 ± 0.03	0.44 ± 0.04*	0.37 ± 0.05*	0.42 ± 0.05	0.41 ± 0.04
	[34.09%]	[24.29%]	[26.15%]	[42.11%]	[50.67%]	[32.26%]	[28.07%]
800 mg/kg	0.64 ± 0.07	0.65 ± 0.11	0.60 ± 0.10	0.52 ± 0.05*	0.53 ± 0.11	0.59 ± 0.08	0.50 ± 0.03
	[27.27%]	[7.14%]	[7.69%]	[31.58%]	[29.33%]	[4.84%]	[12.28%]

Values are presented as mean ± Standard error of mean (SEM), n =5. *$p<0.05$: Significantly different from control. Values in parenthesis indicate percentage inhibition relative to control at a specific time (0.5hr, 1, 2, 3, 4, 5, and 6 hr) respectively.



ED_{50} = Log 400 = 2.60

ED_{50} (mg/kg) = Antilog of 2.60

ED_{50} (mg/kg) = 399.11 mg/kg

Figure 2: Effective dose of the extract.

Table 5: Effect of extract and fractions on egg-albumin induced paw edema.

		Change in paw size (cm) and inhibition in paw size relative to control (%)				
	mg/kg	**1 hour**	**2 hour**	**3 hour**	**4 hour**	**5 hour**
Control:	-	0.78 ± 0.05	0.80 ± 0.04	0.80 ± 0.04	0.75 ± 0.04	0.67 ± 0.05
Standard	10	0.72 ± 0.06 (7.69%)	0.64 ± 0.07 (20.00%)	0.57 ± 0.06* (28.75%)	0.49 ± 0.04* (34.67%)	0.51 ± 0.07 (23.88%)
Extract	200	0.70 ± 0.05 (10.26%)	0.64 ± 0.05 (20.00%)	0.63 ± 0.06* (21.25%)	0.57 ± 0.05* (23.73%)	0.52 ± 0.05 (22.39%)
	400	0.64 ± 0.04 (17.95%)	0.62 ± 0.01* (22.50%)	0.59 ± 0.01* (26.25%)	0.56 ± 0.02* (25.33%)	0.48 ± 0.03* (28.36%)
NHF	200	0.79 ± 0.03 (-1.28%)	0.78 ± 0.04 (2.50%)	0.73 ± 0.03 (8.75%)	0.70 ± 0.03 (6.67%)	0.68 ± 0.03 (-1.49%)
	400	0.73 ± 0.02 (6.41%)	0.71 ± 0.03 (11.25%)	0.72 ± 0.04 (10.00%)	0.70 ± 0.03 (6.67%)	0.64 ± 0.02 (4.48%)
EAF	200	0.70 ± 0.03 (10.26%)	0.65 ± 0.02 (18.75%)	0.59 ± 0.01* (26.25%)	0.58 ± 0.03* (22.67%)	0.52 ± 0.02 (23.39%)
	400	0.57 ± 0.05* (26.92%)	0.57 ± 0.03* (28.75%)	0.56 ± 0.03* (30.00%)	0.50 ± 0.03* (33.33%)	0.50 ± 0.03 (25.37%)
BF	200	0.59 ± 0.02* (24.36%)	0.59 ± 0.03* (26.25%)	0.55 ± 0.02* (31.25%)	0.53 ± 0.01* (29.33%)	0.50 ± 0.02 (25.37%)
	400	0.58 ± 0.03* (25.64%)	0.54 ± 0.01* (32.50%)	0.53 ± 0.03* (33.75%)	0.48 ± 0.02 (36.00%)	0.44 ± 0.02* (34.33%)
WF	200	0.75 ± 0.03 (3.85%)	0.72 ± 0.03 (10.00%)	0.68 ± 0.03 (15.00%)	0.66 ± 0.02 (12.00%)	0.61 ± 0.02 (8.96%)
	400	0.70 ± 0.03 (10.26%)	0.71 ± 0.02 (11.25%)	0.65 ± 0.02 (18.75%)	0.63 ± 0.04 (16.00%)	0.57 ± 0.05 (14.93%)

Values are presented as mean ± Standard error of mean (SEM) of sample replicates (n =5).

*P<0.05: Sigificantly different from control group.

Key: NHF: n-hexane fraction**, EAF:** Ethyl acetate fraction**, BF:** Butanol fraction**, WF:** Water fraction**.**

Table 6: Effect of extract and fractions on xylene-induced ear edema.

	Weight of ear (mg)				
	Dose (µg/ear)	**Left ear**	**Right ear**	**Edema (mg)**	**Percentage Inhibition (%)**
Control:	-	4.16 ± 0.04	6.54 ± 0.05	2.38 ± 0.04	
Indomethacin	50	4.04 ± 0.08	5.00 ± 0.07	0.96 ± 0.06*	59.66
	100	4.10 ± 0.03	4.86 ± 0.05	0.76 ± 0.02*	68.07
Extract	50	3.72 ± 0.02	4.60 ± 0.03	0.88 ± 0.02*	63.03
	100	3.94 ± 0.07	4.58 ± 0.04	0.66 ± 0.05*	72.27
N-hexane fraction	50	3.30 ± 0.05	5.34 ± 0.05	2.04 ± 0.09	14.29
	100	3.48 ± 0.06	5.40 ± 0.03	1.92 ± 0.04	19.33
Ethyl acetate	50	4.06 ± 0.05	4.80 ± 0.05	0.74 ± 0.04*	68.91
fraction	100	4.06 ± 0.02	4.56 ± 0.05	0.50 ±0.04*	78.99
Butanol fraction	50	3.96 ± 0.09	4.64 ± 0.05	0.68 ± 0.06*	71.43
	100	4.00 ± 0.03	4.62 ± 0.04	0.62 ± 0.04*	73.95
Water fraction	50	3.32 ± 0.02	4.58 ± 0.06	1.26 ± 0.06	47.06
	100	3.56 ± 0.05	4.58 ± 0.04	1.02 ± 0.04	57.14

Values are presented as mean ± Standard error of mean (SEM), n=5. *p<0.05: Significantly different from control.

Table 7: Effects of extract and fractions on heat and hypotonic induced haemolysis.

	Conc. (µg/ml)	Heat Induced haemolysis		Hypotonic induced haemolysis	
		Absorbance	**Inhibition (%)**	**Absorbance**	**Inhibition (%)**
Control:	-	0.492 ± 0.002		0.627 ± 0.002	
Indomethacin	200	0.237 ± 0.002	51.93	0.523 ± 0.004	16.67
	400	0.229 ± 0.002	53.56	0.475 ± 0.003	24.32
	800	0.215 ± 0.004	56.40	0.455 ± 0.004	27.51
Prednisolone	200	0.293 ± 0.005	40.45	0.530 ± 0.002	15.47
	400	0.277 ± 0.003	43.80	0.519 ± 0.003	17.22
	800	0.236 ± 0.003	52.03	0.510 ± 0.002	18.74
Extract	200	0.327 ± 0.002	33.54	0.538 ± 0.002	14.19
	400	0.327 ± 0.002	33.64	0.537 ± 0.005	14.43
	800	0.314 ± 0.002	36.18	0.503 ± 0.001	19.78
n-hexane	200	0.349 ± 0.003	29.07	0.567 ± 0.003	9.65
fraction	400	0.335 ±0.004	32.01	0.534 ± 0.005	14.91
	800	0.320 ± 0.003	35.06	0.505 ± 0.002	19.46
Ethyl acetate	200	0.280 ± 0.003	43.19	0.540 ± 0.004	13.96
fraction	400	0.262 ± 0.003	46.75	0.504 ± 0.003	19.70
	800	0.246 ± 0.001	50.00	0.490 ± 0.004	21.93
Butanol	200	0.367 ± 0.005	25.51	0.529 ± 0.001	15.63
fraction	400	0.304 ± 0.003	38.31	0.490 ± 0.004	21.93
	800	0.286 ± 0.003	41.97	0.458 ± 0.001	27.03
Water	200	0.366 ± 0.003	25.61	0.615 ± 0.003	1.91
fraction	400	0.310 ± 0.005	37.09	0.589 ± 0.003	6.14
	800	0.278 ± 0.001	43.50	0.504 ± 0.007	19.70

Values are presented as mean ± Standard error of mean absorbance in duplicate.

Table 8: Effect of extract and fractions on formaldehyde-induced arthritis.

	Dose (mg/kg)	AUC	Percentage inhibition (%)
Control (vehicle)	-	7.24 ± 0.11	
Indomethacin	5	4.65 ± 0.12*	35.77
Extract	200	6.09 ± 0.15*	15.88
	400	6.03 ± 0.32*	16.77
Ethyl acetate	200	5.86 ± 0.19*	19.12
fraction	400	5.35 ± 0.17*	26.08
Butanol fraction	200	5.25 ± 0.25*	27.51
	400	4.95 ± 0.16*	31.63
Water fraction	200	7.11 ± 0.12	1.80
	400	6.82 ± 0.08	5.86

Values are presented as mean ± Standard error of mean (SEM) of sample replicates (n=5).

*$p<0.05$: Significantly different from control group.

Table 9: Effect of extract and fractions on vascular permeability.

Treatment	**Doses (mg/kg)**	**Mean absorbance**	**Inhibition (%)**
Control (distilled water, 10 ml/kg)	-	0.177 ± 0.01	
Indomethacin,	50	0.111 ± 0.01*	37.29
Diclofenac,	10	0.113 ± 0.00*	36.16
Extract	200	0.123 ± 0.00*	30.51
	400	0.107 ± 0.00*	39.55
Ethyl acetate fraction	200	0.131 ± 0.00*	25.99
	400	0.128 ± 0.00*	27.68
Butanol fraction	200	0.136 ± 0.00*	23.16
	400	0.125 ± 0.00*	29.38

Values are presented as mean ± Standard error of mean (SEM), n =5. *P<0.05: Statistically significantly different from control.

4.1.4.8 Effects of extract and fractions on leukocytes migration:

There was reduction in leukocyte count in extract (11.36 and 18.18%), ethyl acetate (6.82 and 31.82%) and butanol fraction (2.27 and 34.09%) when compared to untreated control group (0%). Un-induced control had a lower leukocyte count ($0.37 \pm 0.05\ 10^3/\mu l$) than induced control ($0.44 \pm 0.08\ 10^3/\mu l$). There was no inhibition in lymphocyte count in Diclofenac (0%), 200 mg/kg dose of ethyl acetate (0%). Inhibition in lymphocyte count was higher in 200 mg/kg extract, ethyl acetate and butanol fractions (11.98, 10.64 and 16.98%). MID levels in Diclofenac, extract, 400 mg/kg ethyl acetate fraction and butanol fraction were higher than MID level in untreated control. MID level in non-induced group was higher than other groups. There was inhibition in granulocyte count in extract, ethyl acetate fraction and 200 mg/kg butanol fraction, except Indomethacin and 400 mg/kg butanol fraction (Table 10).

4.1.4.9 Effect of ethyl acetate VLC fractions on heat and hypotonic induced haemolysis:

Indomethacin and Prednisolone showed more inhibition against heat induced haemolysis. These were followed by VLC-E7, VLC-E5 ethyl acetate fraction, VLC-E13, VLC-E16, VLC-E14, VLC-E15, VLC-E17, VLC-E1, VLC-E6, VLC-E8, VLC-E12 and VLC-E9. No activities were recorded in VLC-E10 and VLC-E4. Indomethacin showed most inhibition against hypotonic induced haemolysis. These were followed by VLC-E7, VLC-E5 Prednisolone, ethyl acetate fraction, VLC-E9, VLC-E16, VLC-E14, VLC-E6, VLC-E17, VLC-E1, VLC-E15 and VLC-E8. No activities were found in VLC-E10 and VLC-E4 (Table 11).

Table 10: Effects of extract and fractions on leukocytes migration.

Treatment	Dose (mg/kg)	WBC (10^3/µl)	Lymp (%)	MID (%)	Gran (%)
Control	-	0.44 ± 0.08 (0.0%)	52.52 ± 7.17	6.74 ± 1.20	41.19 ± 6.06
Indo.	50	0.39 ± 0.03 (11.36%)	52.46 ± 5.53 (0.11%)	5.56 ± 0.79	41.98 ± 5.90 (0.00%)
Diclofenac sodium	10	0.41 ± 0.06 (6.82%)	58.20 ± 3.69 (0.00%)	13.13 ± 3.17	28.69 ± 1.35 (30.35%)
Extract	200	0.39 ± 0.05 (11.36%)	52.23 ± 3.66 (0.55%)	12.05 ± 0.89	35.73 ± 3.77 (13.26%)
	400	0.36 ± 0.05 (18.18%)	46.23 ± 4.65 (11.98%)	15.85 ± 0.58	37.93 ± 4.31 (7.91%)
Ethyl acetate fraction	200	0.41 ± 0.06 (6.82%)	52.73 ± 3.35 (0.00%)	16.55 ± 1.70*	30.73 ± 4.49 (25.39%)
	400	0.30 ± 0.06 (31.82%)	46.93 ± 3.80 (10.64%)	14.60 ± 0.95	38.48 ± 3.43 (6.58%)
Butanol fraction	200	0.43 ± 0.05 (2.27%)	51.68 ± 3.51 (1.60%)	15.18 ± 1.27	33.15 ± 3.92 (19.52%)
	400	0.29 ± 0.03 (34.09%)	43.60 ± 1.80 (16.98%)	14.80 ± 1.14	41.60 ± 1.28 (0.00%)
Non-induced	-	0.37 ± 0.05 (15.91%)	48.00 ± 2.29 (8.61%)	20.55 ± 4.48*	31.45 ± 2.74 (23.65%)

Values are presented as mean ± Standard error of mean (SEM), n= 5. *$p<0.05$: Statistically significantly different from control. Values in parenthesis indicate inhibition in leukocyte count. **WBC:** White blood cell, **Lym:** Lymphocyte, **MID:** medium size cell count, **Gran:** Granulocyte. **Indo:** Indomethacine.

Table 11: Effect of ethyl acetate VLC fractions on heat and hypotonic induced haemolysis.

	Conc. (µg/ml)	Heat induced haemolysis		Hypotonic induced haemolysis	
		Absorbance	**Inhibition (%)**	**Absorbance**	**Inhibition (%)**
Control:	-	0.440 ± 0.003		0.539 ± 0.002	
Indo	200	0.228 ± 0.002	48.18	0.381 ± 0.002	29.31
	400	0.218 ± 0.004	50.57	0.295 ± 0.001	45.36
Pred	200	0.269 ± 0.005	38.98	0.455 ± 0.002	15.58
	400	0.224 ± 0.004	49.09	0.311 ± 0.001	42.39
EAF	200	0.316 ± 0.012	28.30	0.425 ± 0.006	21.15
	400	0.278 ± 0.007	36.93	0.350 ± 0.004	35.06
VLC-E1	200	0.422 ± 0.005	4.20	0.523 ± 0.002	3.06
	400	0.402 ± 0.006	8.64	0.509 ± 0.002	5.66
VLC-E9	200	0.440 ± 0.004	0.11	0.468 ± 0.003	13.27
	400	0.436 ± 0.010	1.02	0.330 ± 0.001	38.87
VLC-E13	200	0.342 ± 0.004	22.27	0.502 ± 0.002	6.96
	400	0.299 ± 0.002	32.05	0.453 ± 0.002	16.05
VLC-E4	200	0.450 ± 0.002	0.00	0.540 ± 0.002	0.00
	400	0.456 ± 0.001	0.00	0.540 ± 0.004	0.00
VLC-E15	200	0.430 ± 0.004	2.27	0.527 ± 0.005	2.32
	400	0.348 ± 0.001	20.91	0.521 ± 0.001	3.43
VLC-E7	200	0.280 ± 0.004	36.48	0.380 ± 0.009	29.50
	400	0.250 ± 0.004	43.30	0.314 ± 0.004	41.84
VLC-E17	200	0.417 ± 0.011	5.34	0.520 ± 0.001	3.53
	400	0.372 ± 0.004	15.45	0.493 ± 0.003	8.63
VLC-E5	200	0.339 ± 0.004	23.07	0.463 ± 0.003	14.19
	400	0.273 ± 0.004	38.07	0.431 ± 0.003	20.13
VLC-E6	200	0.422 ± 0.006	4.20	0.490 ± 0.002	9.18
	400	0.402 ± 0.004	8.64	0.473 ± 0.003	12.24
VLC-E14	200	0.378 ± 0.009	14.20	0.499 ± 0.006	7.42
	400	0.357 ± 0.016	18.98	0.435 ± 0.004	19.29
VLC-E8	200	0.433 ± 0.005	1.70	0.541 ± 0.004	0.00
	400	0.412 ± 0.006	6.48	0.518 ± 0.010	3.99
VLC-E10	200	0.460 ± 0.003	0.00	0.550 ± 0.018	0.00
	400	0.444 ± 0.006	0.00	0.562 ± 0.007	0.00
VLC-E12	200	0.435 ± 0.003	1.25	0.492 ± 0.003	8.81
	400	0.420 ± 0.004	4.55	0.487 ± 0.005	9.74
VLC-E16	200	0.394 ± 0.008	10.57	0.451 ± 0.001	16.42
	400	0.339 ± 0.004	23.07	0.413 ± 0.002	23.47

Values are presented as mean ± Standard error of mean absorbance in duplicate. VLC: Vacuum liquid chromatography. EAF: Ethyl acetate fraction. Pred: Prednisolone, Indo: Indomethacin.

4.1.4.10 Effect of selected VLC fractions on xylene-induced ear edema:

All selected samples from ethyl acetate and butanol VLC fractions showed activities against xylene induced topical ear edema in mouse. All the samples showed activity above 50% (Table 12).

4.1.4.11 Effects on COX-1 and COX -2 activities:

VLC-E7 fraction did not ellicite inhibition against the activity of COX -1 enzyme. Diclofenac produced an inhibition of 21.53% against COX-1 enzyme activity. VLC-E7 fraction inhibited COX - 2 enzyme by 1.53 and 25.44% at 50 and 100 µg/ml respectively when compared to Diclofenac which did not produce inhibition against COX -2 activity (Table 13).

4.1.4.12 Effect of selected Sephadex fractions on xylene-induced ear edema:

From table 14, selected samples from Sephadex fractions showed activities against xylene induced topical ear edema in mice. All tested samples produced inhibition above 50% except 50 µg of SPH-E4.

Table 12: Effect of selected VLC fractions on xylene-induced ear edema.

	Weight of ear (mg)				
	Dose/ear (µg)	**Left ear**	**Right ear**	**Edema (mg)**	**Inhibition (%)**
Control: Xylene 50 µl	-	4.72 ± 0.15	8.76 ± 0.64	4.04 ± 0.54	
Indomethacin	50	5.50 ± 0.05	7.08 ± 0.11	1.58 ± 0.16*	60.89
	100	5.06 ± 0.07	6.38 ± 0.16	1.32 ± 0.11*	67.33
VLC-E13	50	5.53 ± 0.02	6.95 ± 0.05	1.43 ± 0.07*	64.70
	100	4.86 ± 0.28	5.70 ± 0.32	0.84 ± 0.25*	79.21
VLC-E5	50	4.60 ± 0.10	5.40 ± 0.14	0.80 ± 0.05*	80.20
	100	5.20 ± 0.16	6.50 ± 0.54	1.30 ± 0.39*	67.82
VLC-E14	50	4.98 ± 0.07	6.88 ± 0.39	1.90 ± 0.45*	52.97
	100	4.82 ± 0.12	5.42 ± 0.07	0.60 ± 0.08*	85.15
VLC-E7	50	5.20 ± 0.29	6.36 ± 0.20	1.16 ± 0.37*	71.29
	100	4.82 ± 0.04	5.82 ± 0.19	1.00 ± 0.19*	75.25
Ethyl acetate fraction	50	4.88 ± 0.06	6.50 ± 0.06	1.62 ± 0.04*	59.90
	100	4.62 ± 0.20	5.98 ± 0.23	1.36 ± 0.14*	66.34

Values are presented as mean ± Standard error of mean (SEM), n=5. *$p<0.05$: Significantly different from control. VLC: Vacuum liquid chromatography.

Table 13: Effect of selected VLC fraction on COX- 1 and COX -2 enzymes.

		COX -1		COX-2	
Treatment	**Conc (µg/ml)**	**Mean absorbance**	**Inhibition (%)**	**Mean absorbance**	**Inhibition (%)**
VLC-E7	25	0.1885 ± 0.013	0.00	0.3177 ± 0.007	0.00
	50	0.1854 ± 0.013	0.00	0.2899 ± 0.019	1.53
	100	0.2215 ± 0.026	0.00	0.2195 ± 0.048	25.44
Diclofenac	0.296	0.1002 ± 0.009	21.53	0.3177 ± 0.007	0.00
Blank	-	0.1277 ± 0.008	0.00	0.2899 ± 0.019	1.53

Values are presented as mean ± Standard error of mean absorbance in triplicate. COX -1: Cyclooxygenase 1, COX-2: Cyclooxygenase -2.

Table 14: Effect of selected Sephadex fractions on xylene-induced ear edema.

	Weight of ear (mg)				
	Dose/ear	**Left Ear**	**Right Ear**	**Edema (mg)**	**Percentage Inhibition (%)**
Control (Xylene 50 µl)	-	4.62 ± 0.25	9.90 ± 0.45	5.28 ± 0.24	
Indomethacin	50	4.26 ± 0.05	6.50 ± 0.07	2.24 ± 0.09*	57.58
	100	4.46 ± 0.05	5.82 ± 0.21	1.36 ± 0.17*	74.24
VLC- E7	50	4.38 ± 0.10	6.96 ± 0.12	2.58 ± 0.06*	51.14
	100	4.30 ± 0.08	5.78 ± 0.22	1.48 ± 0.19*	71.97
SPH- E4	50	4.46 ± 0.05	7.26 ± 0.18	2.80 ± 0.14*	46.97
	100	4.30 ± 0.14	6.26 ± 0.19	1.96 ± 0.07*	62.88
SPH-E5	50	4.24 ± 0.08	6.70 ± 0.11	2.46 ± 0.16*	53.41
	100	4.46 ± 0.09	6.08 ± 0.11	1.62 ± 0.12*	69.32
SPH- E6	50	4.02 ± 0.13	6.32 ± 0.13	2.30 ± 0.03*	56.44
	100	4.22 ± 0.09	5.82 ± 0.13	1.60 ± 0.14*	69.70
SPH- E3	50	3.98 ± 0.12	6.38 ± 0.21	2.40 ± 0.14*	54.55
	100	4.08 ± 0.11	5.76 ± 0.25	1.68 ± 0.19*	68.18

Values are presented as mean ± Standard error of mean (SEM), n=5. *$p<0.05$: Significantly different from control. SPH: Sephadex.

4.1.5 TOTAL PHENOLIC CONTENTS, TOTAL FLAVONOID CONTENTS AND ANTI-OXIDANT RESULTS

4.1.5.1 Total phenolic and flavonoid contents and free radical scavenging activity of extract and fractions:

Total phenolic content was found to be highest in ethyl acetate fraction (34.93 ± 0.04 mgGAE/g) followed by butanol fraction (31.35 ± 0.07 mgGAE/g), extract (21.06 ± 0.14 mgGAE/g) and least in n-hexane fraction (15.78 ± 0.11 mgGAE/g). However, total phenolic content of tested samples were less than that of Quercetin (89.29 ± 0.07 mgGAE/g) and Ascorbic acid (88.79 ± 0.07 mgGAE/g).

The increasing order in total flavonoid content among samples tested include water fraction (12.50 ± 0.83 mgQEq/g) < butanol fraction (40.83 ± 1.67 mgQEq/g) < extract (44.17 ± 1.67 mgQEq/g) < n-hexane fraction (54.17 ± 0.83 mgQEq/g) < ethyl acetate fraction (107.50 ± 0.83 mgQEq/g) < ascorbic acid (140.00 ± 1.67 mgQEq/g).

For the DPPH scavenging activity of extract and fractions, the lowest IC_{50} value was ontained with the ethyl acetate fraction (45.72 ± 0.16 µg/ml) while n-hexane has the highest IC_{50} (134.12 ± 2.88 µg/ml). Ethyl acetate and butanol fractions have higher total phenolic content, total flavonoid content and better free radical scavenging activities than extract, water and n-hexane fractions (Table 15).

Table 15: Total flavonoid content, total phenolic content and free radical scavenging activities of extract and fractions.

	Total phenolic content (mgGAE/g)	Total flavonoid content (mgQEq/g)	DPPH, scavenging activity , IC_{50}(µg/ml)
Extract	21.06 ± 0.14	44.17 ± 1.67	63.91 ± 1.29
n-hexane fraction	15.78 ± 0.11	54.17 ± 0.83	134.12 ± 2.88
Ethyl-acetate fraction	34.93 ± 0.04	107.50 ± 0.83	45.72 ± 0.16
Butanol fraction	31.35 ± 0.07	40.83 ± 1.67	62.09 ± 0.21
Water fraction	19.40 ± 0.04	12.50 ± 0.83	94.97 ±0.14
Ascorbic acid	88.79 ± 0.07	140.00 ± 1.67	12.26 ± 0.20
Quercetin	89.29 ± 0.07	-	10.46 ± 0.15

Values are presented as mean ± Standard error of mean of experiment in duplicate.

4.1.5.2 DPPH scavenging activities of VLC fractions:

VLC fractions obtained from ethyl acetate fraction having inhibition above 50 % against DPPH include VLC-E1 (78.68%), VLC-E3 (52.33%), VLC-E7 (72.38%), VLC-E6 (73.84%), VLC-E14 (65.60%), VLC-E8 (73.06%), VLC-E10 (74.81%), VLC-E12 (72.09%), VLC-E16 (69.67%). The ethyl acetate fraction had inhibition of 73.26% against DPPH (Table 16).

4.1.5.3 Total phenolic content of VLC fractions:

Total phenolic content was higher in samples VLC-E2 (28.40 ± 0.11 mgGAE/g), VLC-E5 (34.43 ± 0.18 mgGAE/g), VLC-E7 (43.26 ± 0.14 mgGAE/g), VLC-E8 (36.28 ± 0.04 mgGAE/g), VLC-E10 (26.31 ± 0.78 mgGAE/g) compared to other samples. Ethyl acetate fraction had total phenolic content of 33.51 ± 1.38 mgGAE/g (Table 17).

4.1.5.4 Ferric reducing antioxidant power (FRAP) and Nitric oxide (NO) scavenging activities:

VLC-E7 caused ferric reduction with EC_{50} values of 256.33 ± 15.94 µg/ml while ethyl acetate and butanol fractions had EC_{50} values of 2906.84 ± 98.36 and 25424.86 ± 1336.32 µg/ml respectively.

Ascorbic acid exhibited the highest inhibition (14.74 ± 0.11 µg/ml) against nitric oxide generated by sodium nitropuside. This was followed by ethyl acetate fraction (15.38 ± 3.65 µg/ml), butanol fraction (15.45 ± 2.72µg/ml), VLC-E7 fraction (16.66 ± 0.48 µg/ml), quercetin (18.14 ± 2.57 µg/L) and extract (38.51 ± 2.23 µg/ml) (Table 18).

Table 16: Inhibition of ethyl acetate VLC fractions at 100 µg/ml against DPPH.

	Ethyl acetate VLC fractions	
Solvent ratio	**Sample code**	**Inhibition at 100 µg/ml**
	Ethyl acetate fraction	73.26
N(500): E (0)	VLC-E1	78.68
N(450): E (50)	VLC-E2	19.38
N(400): E(100)	VLC-E9	18.12
N(350): E(150)	VLC-E13	23.06
N(300):E(200)	VLC-E4	28.00
N(250): E(250)	VLC-E11	36.34
N(200):E(300)	VLC-E3	52.33
N(150):E(350)	VLC-E15	16.86
N(100):E(400)	VLC-E5	12.79
N(50): E(450)	VLC-E17	37.79
N(0):E(500)	VLC-E7	72.38
D(500):M(0)	VLC-E6	73.84
D(450):M(50)	VLC-E14	65.60
D(350):M(150)	VLC-E8	73.06
D(250):M(250)	VLC-E10	74.81
D(100):M(400)	VLC-E12	72.09
D(0):M(500)	VLC-E16	69.67

N = n-hexane, **E** = ethyl acetate, **M** = methanol. **VLC**: Vacuum liquid chromatography.

Table 17: Total phenolic content of ethyl acetate VLC fractions.

	Ethyl acetate VLC fractions	
Solvent ratio	**Sample code**	**Total phenolic content**
	Ethyl acetate fraction	33.51 ± 1.38
N(500): E (0)	VLC-E1	7.62 ± 0.11
N(450): E (50)	VLC-E2	28.40 ± 0.11
N(400): E(100)	VLC-E9	11.28 ± 0.07
N(350): E(150)	VLC-E13	9.29 ± 0.07
N(300):E(200)	VLC-E4	9.65 ± 2.48
N(250): E(250)	VLC-E11	8.44 ± 0.14
N(200):E(300)	VLC-E3	7.13 ± 0.04
N(150):E(350)	VLC-E15	14.61 ± 0.43
N(100):E(400)	VLC-E5	34.43 ± 0.18
N(50): E(450)	VLC-E17	13.40 ± 0.07
N(0):E(500)	VLC-E7	43.26 ± 0.14
D(500):M(0)	VLC-E6	15.04 ± 0.07
D(450):M(50)	VLC-E14	14.26 ± 0.07
D(350):M(150)	VLC-E8	36.28 ± 0.04
D(250):M(250)	VLC-E10	26.31 ± 0.78
D(100):M(400)	VLC-E12	9.29 ± 0.07
D(0):M(500)	VLC-E16	6.06 ± 0.18

Values are presented as mean ± Standard error of mean experiments in duplicate. **N** = n-hexane, **E** = ethyl acetate, **M** = methanol. **VLC**: Vacuum liquid chromatography.

Table 18: Ferric reducing antioxidant power (FRAP) and Nitric oxide (NO) scavenging activities of extract and selected fractions.

Sample	**FRAP, EC_{50} (µg/ml)**	**NO scavenging activity, IC_{50} (µg/ml)**
Extract	20084.00 ± 1968.93	38.51 ± 2.23
Ethyl aceate fraction	2906.84 ± 98.36	15.38 ± 3.65
Butanol fraction	25424.86 ± 1336.32	15.45 ± 2.72
VLC-E7	256.33 ± 15.94	16.66 ± 0.48
Ascorbic acid	161.39 ± 4.21	14.74 ± 0.11
Quercetin	65.78 ± 1.64	18.14 ± 2.57

Values are presented as mean ± Standard error of mean experiments in duplicate. **N** = n-hexane, **E** = ethyl acetate, **M** = methanol. **VLC**: Vacuum liquid chromatography.
FRAP: Ferric reducing antioxidant power, **NO:** Nitric oxide.

4.1.5.5 Total phenolic and flavonoid contents of Sephadex fractions:

Total phenolic content in various samples decreased in the following order: Quercetin (89.29 ± 0.07 mgGAE/g) > ascorbic acid (88.79 ± 0.07 mgGAE/g) > SPH-E5 (77.27 ± 0.04 mgGAE/g) > VLC-E7 (73.94 ± 0.11 mgGAE/g) > SPH-E6 (71.77 ± 0.07 mgGAE/g) > SPH-E7 (60.07 ± 0.07 mgGAE/g) > SPH-E4 (54.65 ± 0.11 mgGAE/g) > SPH-E3 (43.33 ± 0.07 mgGAE/g) > SPH-E2 (31.95 ± 0.18 mgGAE/g) (Table 19).

Total phenolic content in various samples decreased in the following order: Ascorbic acid (140.00 ± 1.67 mgQEq/g) > SPH-E4 (125.83 ± 1.67 mgQEq/g) > SPH-E6 (57.50 ± 1.67 mgQEq/g) > SPH-E5 (55.00 ± 0.00 mgQEq/g) > SPH-E7 (54.17 ± 0.83 mgQEq/g) >VLC-E7 (49.17 ± 1.67 mgQEq/g) > SPH-E3 (20.83 ± 0.83 mgQEq/g) > SPH-E2 (11.67 ± 2.50 mgQEq/g)

4.1.5.6 DPPH, scavenging activity of Sephadex fractions:

DPPH scavenging activities in Sephadex fractions decreased in the following order; Quercetin (10.46 ± 0.15 µg/ml) > Ascorbic acid (12.26 ± 0.20 µg/ml) > SPH-E3 (26.35 ± 1.38 µg/ml) > VLC-E7 (26.87 ± 0.24 µg/ml) > SPH-E6 (33.44 ± 0.38 µg/ml) > SPH-E5 (37.30 ± 0.30 µg/ml) > SPH-E7 (46.95 ± 0.61 µg/ml) > SPH-E4 (86.10 ± 7.69 µg/ml) (Table 19).

4.1.6 HPLC – DIODE ARRAY DETECTOR (HPLC-DAD) ANALYSIS RESULTS

Table 20 depicts the major compound detected in the extract and fraction of *D. filix-mas.* HPLC chromatogram and UV spectrals revealed quercitrin to be the major compound present (Fig. 3 - 13, appendix).

Table 19: Free radical scavenging activity, total phenolic and total flavonoid contents of Sephadex (SPH) fractions.

	Quantity recovered (mg)	Total phenolic content (mgGAE/g)	Total flavonoid content (mgQEq/g)	DPPH, scavenging activity , IC_{50}(µg/ml)
VLC-E7	-	73.94 ± 0.11	49.17 ± 1.67	26.87 ± 0.24
SPH-E1	0.4	-	-	-
SPH-E2	3.6	31.95 ± 0.18	11.67 ± 2.50	22.10 ± 0.62
SPH-E3	41.5	43.33 ± 0.07	20.83 ± 0.83	26.35 ± 1.38
SPH-E4	14.6.2	54.65 ± 0.11	125.83 ± 1.67	86.10 ± 7.69
SPH-E5	10.3.9	77.27 ± 0.04	55.00 ± 0.00	37.30 ± 0.30
SPH-E6	12.5	71.77 ± 0.07	57.50 ± 1.67	33.44 ± 0.38
SPH-E7	3.7	60.07 ± 0.07	54.17 ± 0.83	46.95 ± 0.61
Ascorbic acid	-	88.79 ± 0.07	140.00 ± 1.67	12.26 ± 0.20
Quertcetin	-	89.29 ± 0.07	-	10.46 ± 0.15

Values are presented as mean ± Standard error of mean experiments in duplicate.

Table 20: HPLC analysis of major compounds present in the extract and fractions.

Sample	**Major compound present**	**Retention time and library hit**	**Figure in appendix**
crude extract	**A =** Quercitrin	25.33 min, 998.90	Fig. 3
	B = Kmpf-3-O-rham	27.37, 996.14	
n-hexane fraction	**A** = Quercitrin	21.32 min, 998.28	Fig. 4
	B = R1/L2.0	27.52 min, 996.45	
ethyl acetate	**A** = Caffeoylglycolic acid	11.63 min, 996.33	Fig. 5
fraction	**B** = Quercitrin	21.32 min, 997.46	
butanol fraction	**A**: Quercitrin	21.30 min, 998.82	Fig. 6
	B =R1/L2.0	27.53, 998.12	
Water fraction	**A =** Quercitrin	20.17 min, 998.89	Fig. 7
VLC-E7	**A =** Quercitrin	20.69 min, 998.30	Fig. 8
	B = K,,mpf-3-O-rham	22.69 min, 998.40	
SPH-E2	**A =** Quercitrin	20.12 min, 999.13	Fig. 9
	B = Kmpf-O-rham	22.17 min, 998.54	
	C = Waol A	28.52 min, 996.20	
SPH-E3	**A =** Quercetin3Omethyl	19.33 min, 997.26	Fig. 10
	B = Kampf3Orhamnoglucside	22.23 min, 996.29	
SPH-E4	**A =** Septicine	7.65 min, 995.79	Fig. 11
	B = Kampf-3-O-rham	22.19 min, 998.25	
SPH-E5	**A =** Kmpf-3-O-rahm	22.19 min, 998.34	Fig. 12
SPH-E6	**A =** Quercitrin	21.07 min, 998.31	Fig. 13
	B = Kmpf-3-O-rahm	22.65 min, 994.79	

SPH: Sephadex, **VLC:** Vacuum liquid chromatography

4.1.7 NMR data of isolated compound, Quercetin-3O-αL-rhamnopyranoside:

The pure amorphous yellow powder was isolated from Sephadex LH-20 column chromatography using absolute MeOH eluent. MALDI-TOF-MS: m/z 449[M+H]+, 471[M+Na]+; UV λ_{max} nm (MeOH): 256.8 and 350.4; ^{1}H-NMR (600MHz, MeOH, δ): 0.94 (3H, d, *J*=6.0 Hz, H_3-6''), 3.40 (1H, d, H-5''), 3.54 (1H, m, H-4''), 3.75 dd (1H, *J*=9.2, 3.4 Hz, H-3''), 4.22 (1H, dd, *J*=3.4, 1.7 Hz, H-2''), 5.35 (1H, d, *J*=1.7 Hz, H-1''), 6.21 (1H,d, *J*=2.1 Hz, H-6), 6.38 (1H, d, *J*=2.1 Hz, H-8), 6.91 (1H,d, *J*=8.2 Hz, H-5'), 7.31 dd (1H, dd, *J*=8.2, 2.1 Hz, H-6'), 7.34 d (1H, d, *J*=2.0 Hz, H-2') (Table 21).

The presence of the aglycon was very evident, giving the proton signals at δ-7.34, 7.31 and 6.91 assigned to the protons H-2', H-6' and H-5' respectively. Also note the two metacoupled doublet at δ 6.21 and 6.35 were also identical to the protons H-6 and H-8 respectively. Looking at the above spectral data and when compared with the available literature, the aglycon was identified as quercetin (Ghaly *et al.,* 2010; Luiz *et al.,* 2013; Tae-Seong *et al.,* 2015). The proton NMR shows the characteristics of α-L-rhamnopyranoside; we can confirm that in the proton NMR with the presence of an anomeric proton signal at δ5.35 and a methyl proton signal at δ0.95; hence our compound is confirmed Quercetrin.

4.1.8 STRUCTURE OF ISOLATED COMPOUND

The chemical structure of the isolated compound was elucidated as Quercetin-3O-αL-rhamnopyranoside based on the analyses of its NMR and mass spectrometry (Fig. 14). UV: λ max (PDA) = 258.0 and 349.9 nm, 1H NMR (MeOH-d4).

Table 21: 1H NMR data of Quercetin-3O-αL-rhamnopyranoside compared with literatures.

No	Δh	Ghaly *et al*,[25] (δH in Acetone)	Tae-Seong *et al*.,[26] (δH MeOH)
1	-		
2	-		
3	-		
4	-		
5	-		
6	6.21 d (J=2.1 Hz, 1H)	6.22(1H,d, J=2.3Hz, H-6)	6.20(1H,d,J=2Hz,H-6)
7	-		
8	6.38 d (J=2.1 Hz, 1H)	6.43(1H, d, J=2.3Hz, H-8)	6.36(1H,d,J=2.0,H-8)
9	-		
10	-		
1'	-		
2'	7.34 d (J=2.0 Hz, 1H)	7.46 (J=2.0, d, 1H, H-2')	7.34 (J=2.2,1H,d,H-2')
3'	-		
4'	-		
5'	6.91 d (J=8.2 Hz, 1H)	6.95 (1H,d, H=8.4Hz, H-5')	6.9 (1H,d,J=8.5,Hz, H-5')
6'	7.31 dd (J=8.2, 2.1 Hz, 1H)	7.34(1H,dd,J=8.4,2.3Hz,H-6'	7.31(J=2.2Hz, 8.5Hz,1H,dd, H-6')
1''	5.35 d (J=1.7 Hz, 1H)	5.45(1H,d, J=1.5Hz, H-1'')	5.36 (1H,D,J=8.5Hz, H-1''
2''	4.22 dd (J=3.4, 1.7 Hz, 1H)	3.30-3.32 (4H,H2''-H5'')	4.23(1H,dd,,J=1.63Hz,H-2'')
3''	3.75 dd (J=9.2, 3.4 Hz, 1H)	3.30-3.32 (4H,H2''-H5'')	3.76(1H,dd,J=3.4,3.23Hz,H-3''
4''	3.54 (m 1H)	3.30-3.32 (4H,H2''-H5'')	3.66(1H,m,H-4'')
5''	3.4 (d 1H)	3.30-3.32 (4H,H2''-H5'')	3.42(1H,m,H-5'')
6''	0.94 d (J=6.0 Hz, 3H)	0.88(3H,d, J=5.6Hz, Me-6'')	0.95(3H,d,J=6.14Hz,H-6''

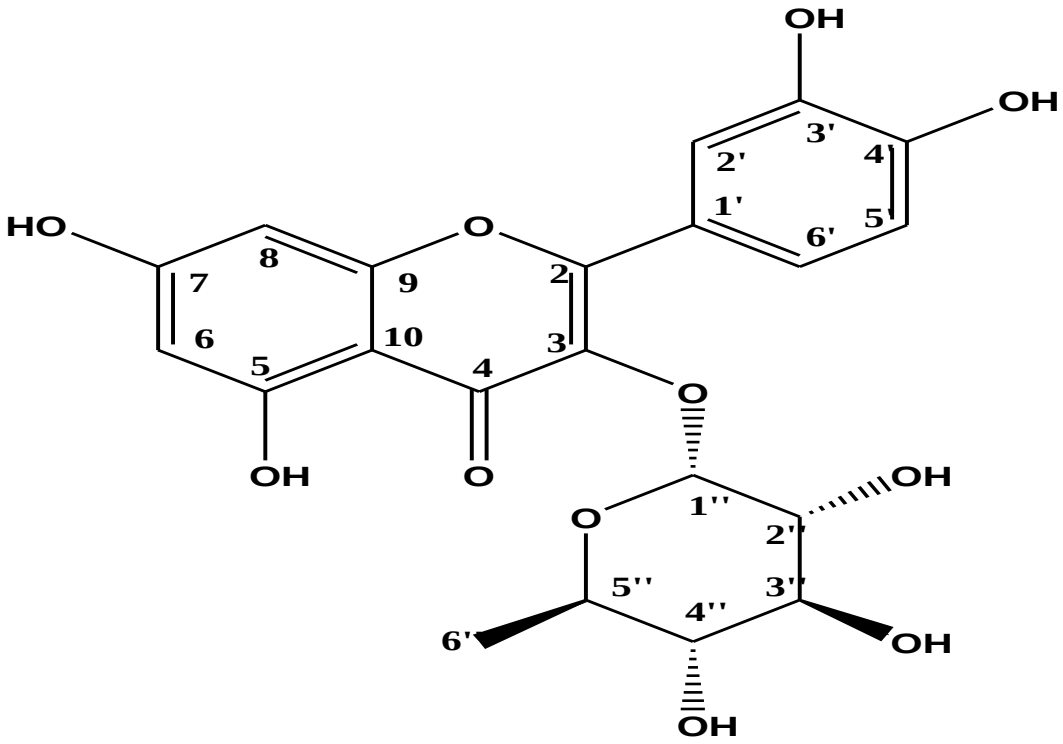


Figure 14: Structure of isolated compound (Quercetin-3O-αL-rhamnopyranoside).

4.1.9 TWO WEEKS REPEATED TOXICITY TEST RESULTS

4.1.9.1 Effect of extract on haematological parameters:

Two weeks repeated administration of various doses of *D. filix-mas* extract to rats did not cause significant change ($p>0.05$) in haematological parameters (PCV, RBC, hemoglobin, platelet, WBC, lymphocyte, granulocyte, and medium size cell count) when compared to haematological parameters of rats in control group (Table 22). The levels of PCT, MPV, MCV, MCH, and MCHC were not also significant when compared to those in control group (Table 23).

4.1.9.2 Effects of extract on lipid profile:

Two weeks repeated administration of various doses of *D. filix-mas* extract to rats did not cause significant change ($p>0.05$) in lipid profile parameters (total cholesterol, triglyceride, high density lipoprotein cholesterol and low density lipoprotein cholesterol when compared to lipid profile parameters of rats in control group (Table 24).

4.1.9.3 Effects of extract on liver enzymes:

Two weeks repeated administration of various doses of *D. filix-mas* extract to rats did not cause significant change ($p>0.05$) in liver enzyme parameters (ALT, AST and ALP, total protein and albumin) when compared to those of rats in control group (Table 25).

4.1.9.4 Effects of extract on renal function parameters:

Two weeks repeated administration of 1000 and 2000 mg/kg of *D. filix-mas* extract to rats caused significant ($*p<0.05$) increase in urea and creatinine levels when compared to those in control group. However, there was no significant difference ($p>0.05$) in sodium, potassium and chloride levels when compared to those in control group (Table 26)

Table 22: Effects of extract on haematological parameters.

Time	Treatment	PCV (%)	RBC (10^6/µl)	Hemoglobin (g/dl)	PLAT (10^3/ µl)
Baseline	**Control**	45.26 ± 1.00	7.14 ± 0.16	14.52 ± 0.39	860.60 ± 42.34
	250 mg/kg	42.63 ± 1.61	6.73 ± 0.25	14.92 ± 0.52	846.80 ± 38.55
	500 mg/kg	44.38 ± 0.73	6.59 ± 0.19	15.28 ± 0.48	811.80 ± 42.12
	1000 mg/kg	44.06 ± 1.46	6.82 ± 0.14	14.42 ± 0.41	694.60 ± 47.87
	2000 mg/kg	43.25 ± 1.15	6.81 ± 0.25	14.64 ± 0.52	845.20 ± 30.84
Day 15	**Control**	46.63 ± 1.44	7.02 ± 0.22	14.80 ± 0.50	899.40 ± 54.69
	250 mg/kg	41.08 ± 1.18	6.63 ± 0.13	13.68 ± 0.36	774.00 ± 68.96
	500 mg/kg	42.61 ± 0.35	6.87 ± 0.06	13.86 ± 0.18	697.00 ± 43.83
	1000 mg/kg	42.41 ± 1.30	6.81 ± 0.13	13.78 ± 0.25	658.60 ± 74.53
	2000 mg/kg	42.73 ±1.16	6.98 ± 0.13	14.16 ± 0.25	997.80 ± 37.96
	Treatment	**WBC (10^3/µl)**	**Lymp (%)**	**Gran (%)**	**MID (%)**
Baseline	**Control**	6.32 ± 0.37	61.50 ± 1.52	26.60 ± 1.71	11.90 ± 0.74
	250 mg/kg	6.36 ± 0.47	63.42 ± 2.13	25.70 ± 2.36	10.88 ± 0.42
	500 mg/kg	6.24 ± 0.43	64.05 ± 0.77	23.56 ± 0.66	12.39 ± 0.26
	1000 mg/kg	5.72 ± 0.16	63.37 ± 0.97	24.30 ± 0.74	12.33 ± 0.41
	2000 mg/kg	6.35 ± 0.33	64.02 ± 0.56	23.45 ± 0.66	12.53 ± 0.38
Day 15	**Control**	6.51 ± 0.37	61.18 ± 3.56	26.62 ± 4.93	12.20 ± 1.46
	250 mg/kg	9.97 ± 2.25	67.36 ± 2.72	21.14 ± 2.91	11.50 ± 1.11
	500 mg/kg	6.97 ± 0.98	66.50 ± 0.39	19.60 ± 0.93	13.90 ± 0.71
	1000 mg/kg	8.26 ± 1.37	69.48 ± 5.02	18.10 ± 3.78	12.42 ± 1.72
	2000 mg/kg	6.65 ± 0.89	56.82 ± 4.77	31.56 ± 5.29	11.62 ± 1.19

Values are presented as mean ± standard error of mean (n =5). $p>0.05$: Not significantly different from control group. PCV (packed cell volume), RBC (red blood cell), hemoglobin, platelet, WBC (white blood cell), Lymp (lymphocyte), Gran (granulocyte) and MID (medium size cell count).

Table 23: Effects of extract on haematological parameters.

Time	Treatment	PCT (%)	MPV (fL)	MCV (fL)	MCH (pg)	MCHC (g/dl)
Baseline	**Control**	0.75 ± 0.03	8.74 ± 0.25	64.40 ± 0.60	20.80 ± 0.24	31.74 ± 0.52
	250 mg/kg	0.65 ± 0.03	8.56 ± 0.02	64.60 ± 1.12	20.54 ± 0.29	32.32 ± 0.67
	500 mg/kg	0.68 ± 0.03	8.46 ± 0.14	63.80 ± 0.66	20.92 ± 0.35	32.22 ± 0.63
	1000 mg/kg	0.65 ± 0.03	8.60 ± 0.19	63.40 ± 0.51	20.30 ± 0.40	32.34 ± 0.45
	2000 mg/kg	0.72 ± 0.04	8.32 ± 0.13	64.80 ± 0.66	20.20 ± 0.27	33.14 ± 0.32
Day 15	**Control**	0.77 ± 0.03	8.68 ± 0.24	66.40 ± 0.51	21.06 ± 0.14	31.70 ± 0.43
	250 mg/kg	0.66 ± 0.07	8.50 ± 0.30	62.00 ± 0.89	20.62 ± 0.17	33.30 ± 0.43
	500 mg/kg	0.62 ± 0.05	8.90 ± 0.16	62.20 ± 0.73	20.18 ± 0.18	32.42 ± 0.39
	1000 mg/kg	0.58 ± 0.07	8.72 ± 0.14	62.00 ± 0.71	20.26 ±0.25	32.53 ± 0.53
	2000 mg/kg	0.82 ± 0.03	8.30 ± 0.15	61.20 ± 1.24	20.26 ± 0.22	33.10 ± 0.34

Values are presented as mean ± Standard error of mean (n =5). $p>0.05$: Not significantly different from control group. PCT (platelet percentage), MPV (mean platelet volume), MCV (mean corpuscular volume), MCH (mean corpuscular hemoglobin) and MCHC (mean corpuscular hemoglobin concentration).

Table 24: Effects of extract on lipid profile.

Time	Treatment	Total cholesterol (mg/dl)	Triglyceride (mg/dl)	HDL-Cholesterol (mg/dl)	LDL-Cholesterol (mg/dl)
Baseline	**Control**	147.93 ± 4.31	113.73 ± 2.80	50.86 ± 2.17	74.33 ± 3.67
	250 mg/kg	147.41 ± 2.08	112.31 ± 3.77	52.38 ± 2.61	72.56 ± 3.92
	500 mg/kg	139.73 ± 2.24	112.79 ± 2.83	48.52 ± 3.70	68.66 ± 3.82
	1000 mg/kg	144.41 ± 3.82	110.69 ± 2.88	49.22 ± 4.52	66.03 ± 5.54
	2000 mg/kg	144.69 ± 4.06	108.05 ± 2.84	51.54 ± 3.71	63.99 ± 6.42
Day 15	**Control**	150.42 ± 4.40	114.76 ± 2.95	51.49 ± 1.14	98.90 ± 3.76
	250 mg/kg	147.03 ± 1.44	113.60 ± 4.43	56.06 ± 1.40	90.95 ± 1.23
	500 mg/kg	142.95 ± 4.63	112.93 ± 2.54	58.34 ± 0.55	84.58 ± 4.66
	1000 mg/kg	143.65 ± 2.46	109.59 ± 2.67	54.40 ± 2.19	89.22 ± 1.91
	2000 mg/kg	137.48 ± 5.80	107.42 ± 3.59	54.40 ± 2.86	83.05 ± 4.95

Values are presented as mean ± Standard error of mean (n =5). $p>0.05$: Not different from control group.

Table 25: Effects of extract on liver enzymes.

Time	Treatment	ALT (U/L)	AST (U/L)	ALP (IU/L)	Albumin (g/dl)	Total protein (g/dl)
Baseline	**Control**	16.14 ± 0.75	38.11 ± 0.92	46.28 ± 1.86	3.148 ± 0.06	4.50 ± 0.23
	250 mg/kg	17.06 ± 0.55	34.57 ± 0.85	48.57 ± 1.76	3.130 ± 0.09	4.58 ± 0.19
	500 mg/kg	15.90 ± 0.49	37.30 ± 1.23	46.72 ± 2.06	3.15 ± 0.13	4.58 ± 0.59
	1000 mg/kg	16.15 ± 0.50	35.85 ± 1.02	43.82 ± 0.49	3.09 ± 0.07	3.99 ± 0.21
	2000 mg/kg	14.86 ± 0.54	36.31 ± 1.04	48.46 ± 1.77	3.17 ± 0.05	3.95 ± 0.29
Day 15	**Control**	16.39 ± 0.44	38.77 ± 0.75	48.55 ± 2.34	3.51 ± 0.18	5.18 ± 0.56
	250 mg/kg	16.98 ± 0.66	36.02 ± 1.23	50.93 ± 2.35	3.00 ± 0.17	4.98 ± 0.48
	500 mg/kg	17.65 ± 0.64	37.20 ± 0.66	55.20 ± 1.77	3.07 ± 0.16	4.19 ± 0.16
	1000 mg/kg	15.47 ± 0.62	36.85 ± 0.86	53.85 ± 1.73	3.03 ± 0.18	4.55 ± 0.21
	2000 mg/kg	15.77 ± 0.50	37.54 ± 0.63	55.48 ± 1.10	2.60 ± 0.23	5.59 ± 0.74

Values are presented as mean ± Standard error of mean (n =5). $p>0.05$: Not significantly different from control group. ALT: alanine aminotransferase, AST: aspertate aminotransferase, ALP: alkaline phosphatase.

Table 26: Effects of extract on renal function parameters.

Time	Treatment	Sodium (mEq/L)	Potassium (mEq/L)	Chloride (mEq/L)	Urea (mg/dl)	Creatinine (mg/dl)	Calcium (mg/dl)
Baseline	**Control**	143.73 ± 2.79	4.11 ± 0.09	106.77 ± 2.38	21.74 ± 1.24	3.82 ± 0.22	10.98 ± 0.75
	250 mg/kg	145.61 ± 5.91	3.99 ± 0.13	102.72 ± 1.63	22.62 ± 0.69	3.76 ± 0.16	8.65 ± 0.43
	500 mg/kg	141.24 ± 1.71	4.06 ± 0.23	103.34 ± 3.34	21.51 ± 0.65	3.43 ± 0.15	10.11 ± 0.50
	1000 mg/kg	144.07 ± 6.66	4.30 ± 0.18	104.64 ± 2.91	22.88 ± 0.38	3.48 ± 0.15	10.56 ± 0.46
	2000 mg/kg	146.01 ± 3.81	3.98 ± 0.22	99.63 ± 4.06	22.13 ± 0.60	3.67 ± 0.25	9.79 ± 0.65
Day 15	**Control**	149.55 ± 16.28	4.45 ± 0.20	103.06 ± 1.55	21.85 ± 0.38	3.37 ± 0.09	10.81 ± 0.59
	250 mg/kg	152.20 ± 14.07	5.03 ± 0.31	100.44 ± 2.67	21.01 ± 1.12	3.59 ± 0.95	8.57 ± 0.48
	500 mg/kg	140.98 ± 15.21	4.48 ± 0.35	97.57 ± 1.39	22.72 ± 1.32	3.44 ± 0.08	8.57 ± 1.86
	1000 mg/kg	153.71 ± 16.68	4.65 ± 0.10	98.41 ± 1.80	30.85 ± 1.40*	4.52 ± 0.09*	9.89 ±0.60
	2000 mg/kg	174.17 ± 16.02	4.64 ± 0.36	95.01 ± 2.68	33.57 ± 1.05*	5.75 ± 0.41*	8.27 ± 0.80

Values are presented as mean ± Standard error of mean (n =5). *p<0.05: Significantly different from control group.

4.1.9.5 Effects of extract on body weights:

There was no significant difference ($p>0.05$) in body weight gain of animals treated with various doses, 250, 500, 1000 and 2000 mg/kg of *D. filix-mas* extract when compared to those in control group (Table 27).

4.1.9.6 Effects of extract on relative organ weights:

No significant differences ($p>0.05$) were observed in relative organs weights of rats treated with various doses (250, 500, 1000 and 2000 mg/kg) of extract of *D. filix-mas* when compared to control group (Table 28).

4.1.9.7 Effects of extract on stomach mucosa:

No ulcer was recorded among the various groups of rats treated with extract.

4.1.9.8 Effects of extract on liver histology:

There was no distortion in liver architecture after 14 days of exposure of rats to 250, 500, 1000 and 2000 mg/kg dose of the extract (Fig. 15).

4.1.9.9 Effects of extract on kidney histology:

From figure 16, there was proliferative glomerulopathy with vascular congestion of the kidneys in rats that were exposed to 1000 and 2000 mg/kg doses of the extract (plates D and E).

Table 27: Effects of extract on body weight.

Treatement	Week 0 (g)	Week 1 (g)	Week 2 (g)	%Wt gain
Control	80.52 ± 5.32	87.82 ± 3.86	101.90 ± 0.23	20.98 ± 5.23
250 mg/kg	84.08 ± 3.39	96.02 ± 4.98	104.16 ± 2.14	19.24 ± 3.01
500 mg/kg	84.56 ± 2.50	94.16 ± 2.96	107.06 ± 5.08	20.25 ± 4.66
1000 mg/kg	78.56 ± 2.50	88.38 ± 3.01	107.12 ± 3.75	26.36 ± 2.93
2000 mg/kg	83.12 ± 3.32	94.98 ± 4.27	100.26 ± 4.61	16.19 ± 5.95

Values are presented as mean ± Standard error of mean (SEM) of sample replicates, n = 5. p>0.05: Not significantly different from control.

Table 28: Effects of extract on relative organ weights.

Treatment	Liver (%)	Kidney (%)	Heart (%)	Spleen (%)	Lung (%)
Control	4.34 ± 0.10	0.66 ± 0.02	0.35 ± 0.02	0.38 ± 0.03	0.69 ± 0.03
250 mg/kg	4.14 ± 0.30	0.65 ± 0.02	0.35 ± 0.02	0.29 ± 0.03	0.59 ± 0.03
500 mg/kg	4.27 ± 0.30	0.64 ± 0.04	0.32 ± 0.02	0.36 ± 0.03	0.59 ± 0.04
1000 mg/kg	3.73 ± 0.23	0.59 ± 0.05	0.30 ± 0.02	0.28 ± 0.03	0.57 ± 0.06
2000 mg/kg	4.13 ± 0.15	0.65 ± 0.03	0.32 ± 0.02	0.44 ± 0.04	0.61 ± 0.07

Values are presented as mean ± Standard error of mean (SEM) of sample replicates, n =5. $p>0.05$: Not significantly different from control.

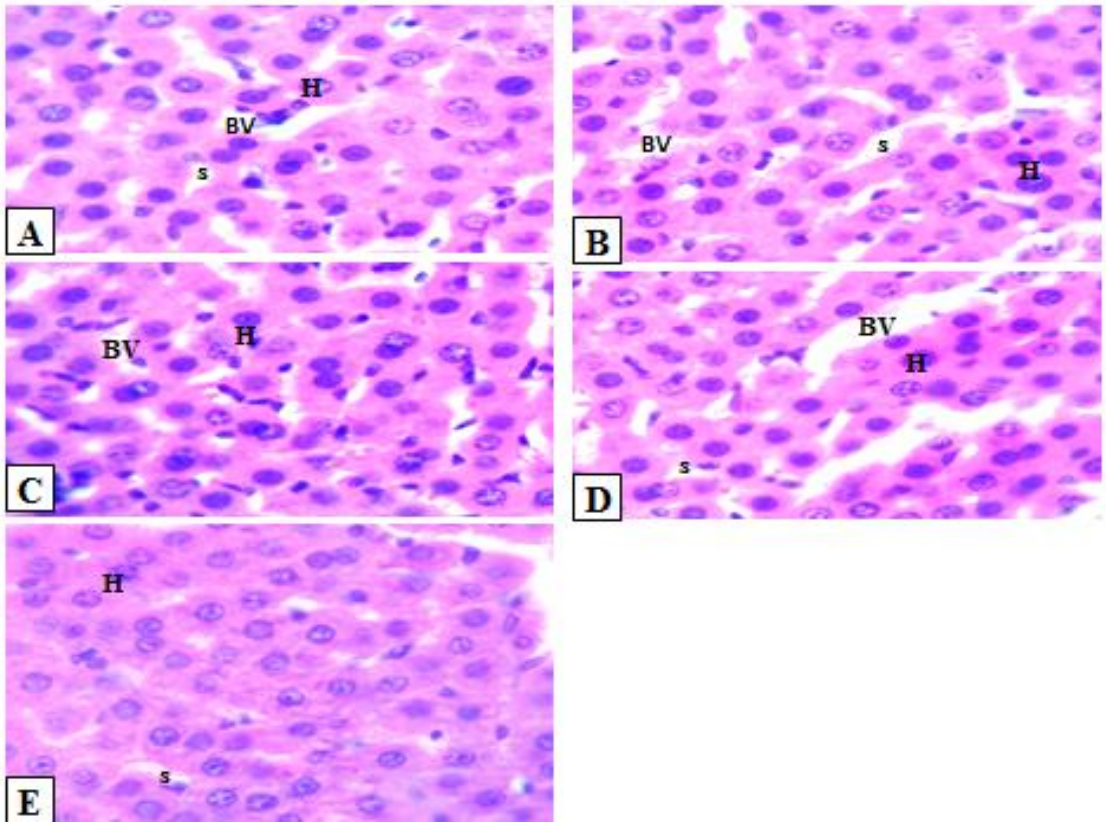


Figure 15: Photomicrograph of liver sections (two weeks toxicity study). H and E, ×400. **Plate A** (Control), **Plate B** (250 mg/kg), **Plate C** (500 mg/kg), **Plate D** (1000 mg/kg), **Plate E** (2000 mg/kg). **H:** Hepatocytes disposed in sheet. **S:** sinusoids, **BV:** Blood vessel. These are features of a normal liver histoarchitecture.

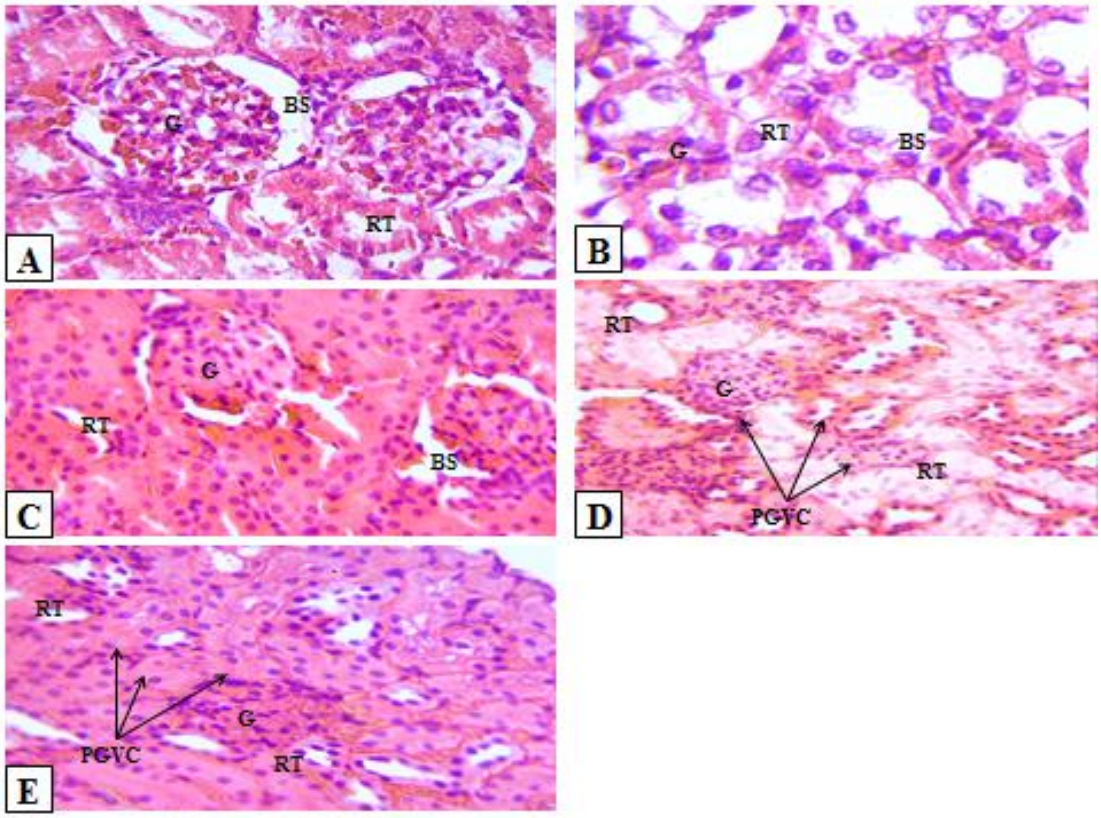


Figure 16. Photomicrograph of kidney sections (two weeks toxicity study). H and E x 400.

Plate A (Control), **Plate B** (250 mg/kg), **Plate C** (500 mg/kg), **Plate D** (1000 mg/kg), **Plate E** (2000 mg/kg). **G:** Glomeruli, **BS: B**owman's space, **RT:** Renal tubule. **PGVC:** Proliferative glomerulopathy with vascular congestion.

4.1.10 SUB-CHRONIC TOXICITY RESULTS

4.1.10.1 Effects of extract on haematological parameters:

From table 29, there was significant ($p<0.05$) increase in PCV, RBC and hemoglobin levels on 31st day following administration of 250 and 500 mg/kg doses of *D. filix-mas* extract to rats. The levels of platelete (PLAT) and platelete percentage (PCT) were not significant.

There was significant (*$p<0.05$) decrease in WBC and lymphocyte counts and significant (*$p<0.05$) increase in granulocyte and MID levels on 91 day following the administration of 250 and 500 mg/kg doses of *D. filix-mas* extract to rats. However, significant changes observed in these parameters on 91 day were reversible in recovery studies (Table 30)

4.1.10.2 Effects of extract on lipid profile:

There was significant (*$p<0.05$) decrease in total cholesterol and triglyceride levels of animals treated with 250 mg/kg doses of *D. filix-mas* extract on 61st day when compared with control group. Also, on the days 31 and 91, there were no significant ($p>0.05$) differences in total cholesterol, triglyceride, HDL-cholesterol and LDL-cholesterol when compared with the control (Table 31).

4.1.10.3 Effect of extract on liver enzymes:

Administration of extract at 250 and 500 mg/kg doses to rats for 90 days significantly ($p<0.05$) increased liver enzymes (ALT, AST, and ALP). However, these changes were reversible in recovery studies. Albumin level was not significant at various periods (Table 32).

4.1.10.4 Effect of extract on sodium, potassium, chloride and calcium:

Administration of *D. filix-mas* extract did not cause significant alterations ($p>0.05$) in sodium, potassium, chloride and calcium of rats treated with extract on days 31, 61 and 91 when compared with control group (Table 33).

Table 29: Effects of extract on haematological parameters.

Time	Treatment	PCV (%)	RBC (10^6/µl)	Hemoglobin (g/dl)	PLAT (10^3/ul)	PCT (%)
Baseline	**Control**	45.66 ± 1.00	7.49 ± 1.54	14.66 ± 0.29	864.40 ± 41.48	0.97 ± 0.04
	61.25 mg/kg	43.03 ± 1.61	7.24 ± 0.22	15.14 ± 0.51	847.80 ± 37.47	0.88 ± 0.04
	125 mg/kg	44.78 ± 0.74	7.18 ± 0.16	15.46 ± 0.49	832.20 ± 39.55	0.83 ± 0.02
	250 mg/kg	44.46 ± 1.50	7.51 ± 0.16	14.58 ± 0.41	795.60 ± 46.06	0.81 ± 0.06
	500 mg/kg	42.56 ± 0.93	7.35 ± 0.20	15.46 ± 0.27	847.40 ± 32.45	0.95 ± 0.05
Day 31	**Control**	41.03 ± 1.33	6.24 ± 0.30	14.74 ± 0.57	677.80 ± 54.22	0.52 ± 0.04
	61.25 mg/kg	39.97 ± 1.68	6.58 ± 0.25	14.05 ± 0.70	677.80 ± 48.59	0.53 ± 0.05
	125 mg/kg	39.77 ± 0.96	6.34 ± 0.28	13.86 ± 0.28	618.60 ± 37.94	0.47 ± 0.02
	250 mg/kg	54.56 ± 3.61*	8.39 ± 0.12*	19.55 ± 1.31*	664.00 ± 43.81	0.54 ± 0.03
	500 mg/kg	54.34 ± 4.81*	8.55 ± 0.19*	19.34 ± 1.79*	786.60 ± 86.02	0.62 ± 0.07
Day 61	**Control**	41.18 ± 0.67	6.63 ± 0.14	14.20 ± 0.21	675.20 ± 77.68	0.71 ± 0.07
	61.25 mg/kg	40.44 ± 0.65	6.63 ± 0.15	13.88 ± 0.16	667.40 ± 66.13	0.68 ± 0.05
	125 mg/kg	40.28 ± 1.50	6.52 ± 0.27	14.02 ± 0.54	633.40 ± 29.53	0.65 ± 0.02
	250 mg/kg	41.14 ± 1.13	6.70 ± 0.15	14.54 ± 0.41	561.00 ± 25.80	0.63 ± 0.03
	500 mg/kg	40.77 ± 0.56	6.64 ± 0.14	13.86 ± 0.16	599.20 ± 27.20	0.65 ± 0.02
Day 91	**Control**	42.74 ± 1.01	7.00 ± 0.22	13.68 ± 0.35	760.80 ± 42.92	0.60 ± 0.04
	61.25 mg/kg	42.47 ± 0.45	6.77 ± 0.37	13.99 ± 0.12	681.20 ± 23.22	0.60 ± 0.05
	125 mg/kg	43.71 ± 0.38	6.89 ± 0.07	14.06 ± 0.17	821.00 ± 54.83	0.65 ± 0.05
	250 mg/kg	42.19 ± 1.06	6.96 ± 0.16	13.12 ± 0.35	780.40 ± 30.97	0.66 ± 0.02
	500 mg/kg	44.26 ± 1.83	7.15 ± 0.32	13.62 ± 0.60	837.40 ± 37.43	0.68 ± 0.04
Recovery	**Control**	43.48 ± 1.18	6.94 ± 0.29	13.12 ± 0.39	797.80 ± 68.75	0.63 ± 0.06
	61.25 mg/kg	43.28 ± 0.94	6.95 ± 0.21	13.38 ± 0.36	793.20 ± 47.88	0.63 ± 0.04
	125 mg/kg	44.65 ± 1.36	7.11 ± 0.29	13.66 ± 0.46	707.80 ± 82.30	0.58 ± 0.06
	250 mg/kg	42.59 ± 0.82	6.72 ± 0.23	12.92 ± 0.34	734.60 ± 70.48	0.57 ± 0.06
	500 mg/kg	44.89 ± 0.89	7.04 ± 0.11	13.73 ± 0.19	835.40 ± 33.18	0.66 ± 0.04

Values are presented as mean ± Standard error of mean (n =5). *p<0.05: Significantly different from control group. PCV (packed cell volume), RBC (red blood cell), hemoglobin, platelet (PLAT) and platelete percentage (PCT) levels.

Table 30: Effects of extract haematological parameters.

Time	Treatment	WBC (10^3/µl)	Lymp (%)	Gran (%)	MID (%)
Baseline	**Control**	6.53 ± 0.32	62.92 ± 0.99	24.81 ± 0.81	12.27 ± 0.51
	61.25 mg/kg	6.60 ± 0.45	62.85 ± 1.10	25.33 ± 1.31	11.82 ± 0.60
	125 mg/kg	6.37 ± 0.30	62.65 ± 0.51	24.70 ± 0.45	12.65 ± 0.20
	250 mg/kg	5.94 ± 0.13	62.85 ± 0.73	25.15 ± 0.47	12.00 ± 0.36
	500 mg/kg	6.52 ± 0.24	62.80 ± 0.40	24.50 ± 0.34	12.70 ± 0.24
Day 31	**Control**	7.25 ± 0.88	76.04 ± 5.74	20.52 ± 2.16	9.18 ± 0.92
	61.25 mg/kg	6.86 ± 0.68	71.38 ± 5.72	19.86 ± 1.32	8.98 ± 0.66
	125 mg/kg	5.82 ± 0.36	71.24 ± 1.54	17.86 ± 2.35	9.90 ± 0.44
	250 mg/kg	6.58 ± 0.49	73.52 ± 3.19	18.56 ± 1.78	11.96 ± 1.96
	500 mg/kg	6.52± 0.88	71.70 ± 4.94	17.66 ± 1.93	13.30 ± 4.26
Day 61	**Control**	6.84 ± 1.16	69.54 ± 3.56	18.60 ± 2.44	11.86 ± 1.58
	61.25 mg/kg	6.17 ± 1.11	70.02 ± 0.21	19.47 ± 0.75	10.51 ± 0.59
	125 mg/kg	5.91 ± 0.51	69.82 ± 1.38	19.56 ± 1.17	10.62 ± 0.76
	250 mg/kg	5.57 ± 0.79	69.00 ± 3.34	20.10 ± 2.96	10.90 ± 0.58
	500 mg/kg	4.49 ± 0.37	66.88 ± 1.07	21.76 ± 1.01	11.36 ± 0.69
Day 91	**Control**	6.09 ± 0.50	72.44 ± 0.88	15.92 ± 0.37	11.50 ± 0.87
	61.25 mg/kg	5.94 ± 0.98	73.00 ± 1.11	15.82 ± 0.96	11.18 ± 0.55
	125 mg/kg	6.53 ± 0.62	71.22 ± 1.22	16.38 ± 1.24	12.40 ± 1.04
	250 mg/kg	4.46 ± 0.43*	61.88 ± 0.73*	22.22 ± 0.69*	15.90 ± 0.34*
	500 mg/kg	3.48 ± 0.15*	56.18 ± 1.37*	25.92 ± 1.30*	17.90 ± 0.76*
Recovery	**Control**	5.75 ± 0.75	69.80 ± 1.28	17.22 ± 0.77	13.00 ± 0.63
	61.25 mg/kg	5.85 ± 0.65	70.40 ± 0.77	16.62 ± 0.68	12.98 ± 0.17
	125 mg/kg	6.60 ± 0.54	68.60 ± 0.76	17.04 ± 0.29	14.36 ± 0.53
	250 mg/kg	5.76 ± 0.50	67.86 ± 0.95	17.95 ± 0.51	14.18 ± 0.66
	500 mg/kg	5.25 ± 0.78	68.42 ±0.30	17.12 ± 0.82	14.46 ± 0.68

Values are presented as mean ± Standard error of mean (n =5). *p<0.05: Significantly different from control group. White blood cell (WBC), lymphocyte (Lymp), granulocyte (Gran), and medium size cell counts (MID).

Table 31: Effects of extract on lipid profile.

Time	Treatment	Total cholesterol (mg/dl)	Triglyceride (mg/dl)	HDL-cholesterol (mg/dl)	LDL-cholesterol (mg/dl)
Baseline	**Control**	148.17 ± 3.39	114.34 ± 2.51	51.85 ± 2.03	73.46 ± 3.05
	61.25 mg/kg	149.27 ± 1.89	113.13 ± 3.53	52.92 ± 3.06	73.73 ± 4.38
	125 mg/kg	141.52 ± 2.02	113.49 ± 2.55	49.39 ± 3.42	69.43 ± 3.42
	250 mg/kg	146.00 ± 2.36	111.17 ± 2.81	50.68 ± 4.42	73.08 ± 3.88
	500 mg/kg	146.93 ± 2.16	108.78 ± 2.79	52.55 ± 3.58	72.62 ± 4.71
Day 31	**Control**	142.02 ± 5.37	111.24 ± 3.05	57.18 ± 1.23	62.59 ± 5.78
	61.25 mg/kg	130.82 ± 3.89	114.16 ± 2.79	57.65 ± 1.77	50.33 ± 5.23
	125 mg/kg	141.03 ± 8.28	110.45 ± 2.60	59.91 ± 1.23	59.02 ± 8.06
	250 mg/kg	132.13 ± 7.98	112.57 ± 4.83	60.63 ± 0.87	48.99 ± 7.04
	500 mg/kg	135.43 ± 3.95	112.04 ± 1.36	60.99 ± 1.15	52.03 ± 4.82
Day 61	**Control**	140.71 ± 6.33	117.47 ± 4.52	66.67 ± 1.84	50.55 ± 7.63
	61.25 mg/kg	137.65 ± 5.80	118.10 ± 3.40	71.59 ± 2.43	42.44 ± 5.72
	125 mg/kg	125.41 ± 3.71	95.84 ± 7.75	74.62 ± 7.08	31.63 ± 9.10
	250 mg/kg	115.47 ± 6.88*	81.22 ± 4.70*	68.56 ± 3.14	30.67 ± 5.98
	500 mg/kg	126.18 ± 6.51	98.87 ± 5.57	75.00 ± 2.21	31.40 ± 5.32
Day 91	**Control**	141.86 ± 4.19	119.93 ± 5.66	62.53 ± 5.49	55.34 ± 3.94
	61.25 mg/kg	146.20 ± 4.94	122.77 ± 2.43	61.61 ± 3.07	60.04 ± 7.80
	125 mg/kg	144.03 ± 8.52	116.99 ±2.92	56.05 ± 2.55	64.58 ± 8.88
	250 mg/kg	135.34 ± 3.25	123.68 ± 5.62	65.77 ± 4.34	44.83 ± 4.98
	500 mg/kg	151.26 ± 9.46	111.32 ± 5.77	70.64 ± 3.53	58.36 ± 13.72
Recovery	**Control**	142.45 ± 1.83	129.01 ± 5.54	65.44 ± 1.61	51.21 ± 2.77
	61.25 mg/kg	144.81 ± 3.05	132.83 ± 2.89	68.16 ± 1.56	50.09 ± 3.15
	125 mg/kg	143.80 ± 1.62	133.24 ± 3.96	69.92 ± 0.62	47.22 ± 2.23
	250 mg/kg	141.01 ± 2.06	123.06 ± 2.67	70.78 ± 0.80	45.62 ± 1.76
	500 mg/kg	139.41 ± 2.11	123.09 ± 3.92	69.66 ± 1.41	45.12 ± 2.24

Values are presented as mean ± Standard error of mean (n =5). *p<0.05: Significantly different from control group.

Table 32: Effect of extract on liver enzymes.

Time	Treatment	ALT (U/L)	AST (U/L)	ALP (IU/L)	Albumin (g/dl)	Total protein (g/dl)
Baseline	**Control**	16.54 ± 0.67	38.83 ± 1.11	46.45 ± 1.81	3.26 ± 0.06	4.23 ± 0.09
	61.25 mg/kg	17.32 ± 0.57	35.05 ± 0.90	48.64 ± 1.88	3.27 ± 0.09	4.61 ± 0.19
	125 mg/kg	16.26 ± 0.50	37.45 ± 1.27	47.41 ± 1.98	3.26 ± 0.14	4.64 ± 0.59
	250 mg/kg	16.34 ± 0.55	37.06 ± 1.50	43.63 ± 0.35	3.15 ± 0.07	4.24 ± 0.04
	500 mg/kg	15.39 ± 0.66	36.92 ± 1.06	48.73 ± 1.70	3.26 ± 0.04	4.27 ± 0.24
Day 31	**Control**	15.58 ± 0.86	38.47 ± 0.94	62.66 ± 7.71	3.43 ± 0.23	4.94 ± 0.30
	61.25 mg/kg	15.23 ± 0.85	36.33 ± 1.98	62.75 ± 7.40	3.47 ± 0.27	4.93 ± 0.30
	125 mg/kg	12.37 ± 1.21	32.52 ± 2.57	51.16 ± 4.30	3.80 ± 0.22	4.80 ± 0.15
	250 mg/kg	12.82 ± 1.15	34.76 ± 1.10	52.63 ± 6.65	3.92 ± 0.20	5.01 ± 0.16
	500 mg/kg	13.49 ± 1.59	35.56 ± 2.10	56.49 ± 10.32	3.54 ± 0.26	4.87 ± 0.08
Day 61	**Control**	19.78 ± 0.65	37.72 ± 0.55	55.22 ± 0.51	3.69 ± 0.11	4.54 ± 0.26
	61.25 mg/kg	18.93 ± 0.42	37.94 ± 0.59	53.66 ± 6.11	3.72 ± 0.08	4.56 ± 0.20
	125 mg/kg	17.42 ± 1.85	37.67 ± 0.56	56.99 ± 3.35	3.60 ± 0.14	4.24 ± 0.19
	250 mg/kg	16.43 ± 1.60	37.93 ± 1.07	56.67 ± 3.44	3.88 ± 0.13	4.73 ± 0.41
	500 mg/kg	17.12 ± 1.36	37.62 ± 0.53	56.08 ± 0.91	3.67 ± 0.23	4.42 ± 0.29
Day 91	**Control**	17.28 ± 0.93	35.00 ± 2.47	45.03 ± 3.82	3.94 ± 0.26	4.78 ± 0.34
	61.25 mg/kg	16.42 ± 0.58	39.13 ± 1.13	40.51 ± 1.25	4.45 ± 0.21	4.96 ±0.08
	125 mg/kg	18.24 ± 1.72	40.60 ± 2.62	55.10 ± 1.00*	4.08 ± 0.20	5.01 ± 0.25
	250 mg/kg	23.36 ± 1.30*	59.50 ± 1.92*	63.70 ± 2.10*	3.82 ± 0.13	5.13 ± 0.26
	500 mg/kg	30.72 ± 1.28*	73.50 ± 2.21*	78.02 ± 3.05*	3.81 ± 0.28	5.26 ± 0.10
Recovery	**Control**	15.92 ± 1.00	44.49 ± 1.09	42.30 ± 2.10	2.66 ± 0.17	4.06 ± 0.04
	61.25 mg/kg	16.16 ± 1.06	40.66 ± 1.55	41.34 ± 3.13	3.57 ± 0.26	4.11 ± 0.06
	125 mg/kg	14.18 ± 0.94	39.72 ± 1.68	43.25 ± 3.68	3.40 ± 0.23	4.08 ± 0.05
	250 mg/kg	14.30 ± 0.90	40.23 ± 0.83	37.64 ± 5.07	2.93 ± 0.11	4.01 ± 0.08
	500 mg/kg	13.26 ± 1.34	38.80 ± 1.86	42.23 ± 3.91	3.51 ± 0.26	4.10 ± 0.09

Values are presented as mean ± Standard error of mean (n =5). *p<0.05: Significantly different from control group. ALT (alanine aminotransferase), AST (aspertate aminotransferase), ALP (alkaline phosphatase).

Table 33: Effect of extract on sodium, potassium, chloride and calcium.

Time	Treatment	Sodium (mEq/L)	Potassium (mEq/L)	Chloride (mEq/L)	Calcium (mg/dl)
Baseline	**Control**	144.77 ± 2.68	4.47 ± 0.13	108.47 ± 2.13	10.80 ± 0.62
	61.25 mg/kg	146.37 ± 5.63	4.28 ± 0.21	104.24 ± 1.53	8.93 ± 0.45
	125 mg/kg	144.00 ± 2.75	4.49 ± 0.21	105.11 ± 3.04	9.95 ± 0.46
	250 mg/kg	145.27 ± 6.78	4.55 ± 0.09	106.24 ± 2.72	10.51 ± 0.31
	500 mg/kg	146.99 ± 3.46	4.39 ± 0.19	101.58 ± 3.65	9.73 ± 0.48
Day 31	**Control**	143.73 ± 0.69	4.08 ± 0.29	96.52 ± 2.75	9.95 ± 0.27
	61.25 mg/kg	145.28 ± 1.66	3.95 ± 0.23	97.15 ± 3.70	9.50 ± 0.23
	125 mg/kg	142.17 ±1.81	4.34 ± 0.22	92.22 ± 1.55	9.24 ± 0.12
	250 mg/kg	142.42 ± 2.36	4.18 ± 0.23	86.88 ± 1.66	9.48 ± 0.37
	500 mg/kg	139.96 ± 2.45	4.43 ± 0.20	90.95 ± 0.94	9.94 ± 0.33
Day 61	**Control**	131.17 ± 1.66	5.21 ± 0.14	95.99 ± 3.39	11.05 ± 0.77
	61.25 mg/kg	130.96 ± 1.79	4.92 ± 0.31	93.60 ± 3.68	10.74 ± 0.66
	125 mg/kg	133.42 ± 4.83	4.33 ± 0.76	88.72 ± 4.10	10.63 ± 0.44
	250 mg/kg	141.63 ± 3.12	3.81 ± 0.04	96.23 ± 2.82	10.93 ± 0.36
	500 mg/kg	141.26 ± 3.75	3.93 ± 0.53	100.20 ± 3.40	10.88 ± 0.30
Day 91	**Control**	132.01 ± 1.69	3.64 ± 0.22	93.26 ± 3.23	9.34 ± 0.71
	61.25 mg/kg	134.13 ± 1.28	3.68 ± 0.14	94.72 ± 1.31	9.35 ± 0.48
	125 mg/kg	129.49 ± 0.35	4.35 ± 0.09	84.15 ± 3.52	9.51 ± 0.66
	250 mg/kg	139.12 ± 6.68	3.14 ± 0.38	85.94 ± 4.76	9.32 ± 0.19
	500 mg/kg	136.52 ± 1.12	4.03 ± 0.50	92.39 ± 2.68	10.40 ± 0.19
Recovery	**Control**	134.23 ± 3.83	3.96 ± 0.26	98.03 ± 9.67	8.05 ± 0.34
	61.25 mg/kg	138.26 ± 5.11	3.42 ± 0.07	106.81 ± 4.88	8.49 ± 0.74
	125 mg/kg	136.02 ± 3.80	4.28 ± 0.48	102.38 ± 3.85	7.74 ± 0.32
	250 mg/kg	143.98 ± 1.75	5.15 ± 0.22	85.12 ± 6.37	8.50 ± 0.51
	500 mg/kg	141.74 ± 3.31	3.93 ± 0.53	91.81 ± 3.88	9.14 ± 0.37

Values are presented as mean ± Standard error of mean (n =5). $p>0.05$: Not significantly different from control group.

4.1.10.5 Effects of extract on urea and creatinine:

On 91st day, there was significant (*p<0.05) increased in urea and creatinine levels of rats treated with 250 and 500 mg/kg doses of *D. filix-mas* extract when compared to control group. However, these changes were reversible in recovery studies (Table 34).

4.1.10.6 Effect of extract on body weight:

From table 35, administration of extract of *D. filix-mas* did not cause significant (p>0.05) change in body weight gain on 31, 61 and 91st days when compared to control group.

4.1.10.7 Effect of extract on relative organs weight:

Administration of extract of *D. filix-mas* to Wistar rats did not cause significant (p>0.05) change in relative organs weights when compared with control groups on 91st day (Table 36).

4.1.10.8 Effects of extract on stomach mucosa:

There was no ulcer recorded among the various groups treated with extract after 91st day.

Table 34: Effects of extract on urea and creatinine.

Time	Treatment	Urea (mg/dl)	Creatinine (mg/dl)
Baseline	**Control**	19.88 ± 0.86	4.36 ± 0.21
	61.25 mg/kg	20.82 ± 0.45	4.28 ± 0.16
	125 mg/kg	19.86 ± 0.62	3.95 ± 0.10
	250 mg/kg	21.15 ± 0.49	4.00 ± 0.17
	500 mg/kg	20.40 ± 0.59	4.19 ± 0.21
Day 31	**Control**	19.65 ± 0.45	5.64 ± 0.19
	61.25 mg/kg	20.17 ± 0.61	5.29 ± 0.33
	125 mg/kg	22.08 ± 0.82	5.46 ± 0.22
	250 mg/kg	19.57 ± 0.71	5.67 ± 0.10
	500 mg/kg	20.29 ± 1.11	5.75 ± 0.24
Day 61	**Control**	19.06 ± 1.61	4.70 ± 0.32
	61.25 mg/kg	19.82 ± 1.75	4.32 ± 0.28
	125 mg/kg	20.35 ± 0.78	4.09 ± 0.14
	250 mg/kg	19.65 ± 1.01	4.00 ± 0.24
	500 mg/kg	21.12 ± 1.65	4.60 ± 0.22
Day 91	**Control**	19.83 ± 0.18	3.70 ± 0.08
	61.25 mg/kg	19.96 ± 0.40	3.60 ± 0.06
	125 mg/kg	20.80 ± 0.40	4.00 ± 0.06
	250 mg/kg	23.79 ± 0.29*	4.77 ± 0.04*
	500 mg/kg	24.97 ± 0.18*	5.54 ± 0.13*
Recovery	**Control**	18.36 ± 0.55	4.38 ± 0.54
	61.25 mg/kg	18.78 ± 0.38	5.15 ± 0.60
	125 mg/kg	18.32 ± 0.15	4.90 ± 0.42
	250 mg/kg	19.20 ± 0.20	5.50 ± 0.45
	500 mg/kg	18.61 ± 0.22	4.70 ± 0.31

Values are presented as mean ± Standard error of mean (n =5). *$p<0.05$: Significantly different from control group.

Table 35: Effect of extract on body weight.

	Body weight gain (%)			
Treatment	**Day 31**	**Day 61**	**Day 91**	**Recovery**
Control	35.02 ± 2.00	37.97 ± 5.11	57.22 ± 2.99	48.34 ± 9.89
61.25 mg/kg	33.87 ± 1.72	38.28 ± 4.95	46.25 ± 6.41	63.57 ± 3.93
125 mg/kg	36.51 ± 2.20	51.95 ± 3.57	60.73 ± 3.48	52.44 ± 10.51
250 mg/kg	38.37 ± 3.59	42.32 ± 6.48	55.69 ± 5.35	51.81 ± 9.29
500 mg/kg	35.79 ± 2.42	47.71 ± 6.07	57.87 ± 5.84	60.53 ± 5.32

Values are presented as mean ± Standard error of mean (n =5). $p>0.05$: Not significantly different from control group.

Table 36: Effect of extract on relative organs weight.

Time	Treatment	Liver (%)	Kidney (%)	Heart (%)	Spleen (%)	Lung (%)
Day 91	**Control**	2.96 ± 0.06	0.71 ± 0.02	0.44 ± 0.03	0.39 ± 0.02	0.67 ± 0.02
	61.25 mg/kg	3.14 ± 0.11	0.75 ± 0.01	0.47 ± 0.01	0.39 ± 0.02	0.70 ± 0.03
	125 mg/kg	3.10 ± 0.10	0.73 ± 0.01	0.46 ± 0.01	0.40 ± 0.02	0.71 ± 0.03
	250 mg/kg	3.10 ± 0.03	0.70 ± 0.02	0.41 ± 0.03	0.34 ± 0.01	0.73 ± 0.04
	500 mg/kg	3.09 ± 0.03	0.71 ± 0.03	0.45 ± 0.05	0.42 ± 0.01	0.71 ± 0.02
Recovery	**Control**	3.14 ± 0.12	0.65 ± 0.04	0.39 ± 0.03	0.31 ± 0.01	0.87 ± 0.11
	61.25 mg/kg	2.93 ± 0.25	0.59 ± 0.02	0.33 ± 0.03	0.30 ± 0.01	0.75 ± 0.12
	125 mg/kg	2.77 ± 0.10	0.68 ± 0.03	0.32 ± 0.01	0.35 ± 0.01	0.68 ± 0.03
	250 mg/kg	2.77 ± 0.10	0.62 ± 0.02	0.34 ± 0.03	0.29 ± 0.02	0.86 ± 0.20
	500 mg/kg	2.78 ± 0.10	0.62 ± 0.02	0.29 ± 0.01	0.36 ± 0.02	0.78 ± 0.10

Values are presented as mean ± Standard error of mean (n =5). $p>0.05$: Not significantly different from control group.

4.1.10.9 Effects of extract on liver and kidney histology:

From figure 17, there was no distortion in liver architecture on 31st day in the control group and groups treated with 62.5, 125, 250 and 500 mg/kg extract.

Ther was no distortion in kidney architecture on 31st day in control group and groups treated with 62.5, 125, 250 and 500 mg/kg extract (Fig. 18).

There was no distortion in liver architecture on 61st day in control group and groups treated with 62.5, 125, 250 and 500 mg/kg extract (Fig. 19).

There was no distortion in kidney architecture on 61st day in control group and groups treated with 62.5, 125, 250 and 500 mg/kg extract (Fig. 20).

Congestion of liver portal triad in groups that received 250 and 500 mg/kg doses of the extract was observed on day 91. There was no distortion in liver architecture of animals in control, 62.5 and 125 mg/kg treated groups (Fig. 21).

There was glomerular congestion in groups that received 250 and 500 mg/kg doses of the extract on 91st day. There was no distortion in kidney architecture of animals in control, 62.5 and 125 mg/kg treated groups (Fig. 22).

There was reversibility in liver toxicities observed on day 91 as normal liver architectures of groups treated with extract was observed (Fig. 23).

All groups treated with the extract showed normal kidney architecture. Kidney toxicities observed on 91st day was reversed in recovery studies (Fig. 24).

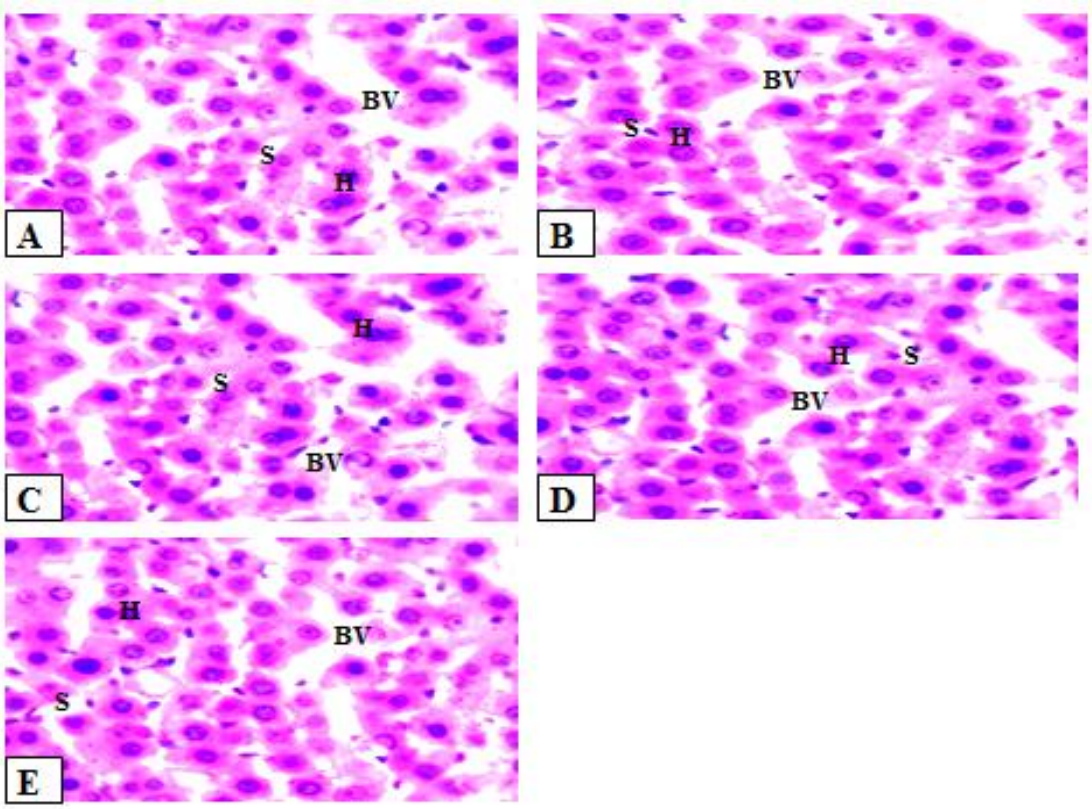


Figure 17. Photomicrograph of liver sections (day 31). H and E x 400.

Plate A (Control), **Plate B** (62.5 mg/kg), **Plate C** (125 mg/kg), **Plate D** (250 mg/kg), **Plate E** (500 mg/kg). **H:** Hepatocytes disposed in sheet. **S:** sinusoids, **BV:** Blood vessel. These are features of a normal liver histoarchitecture.

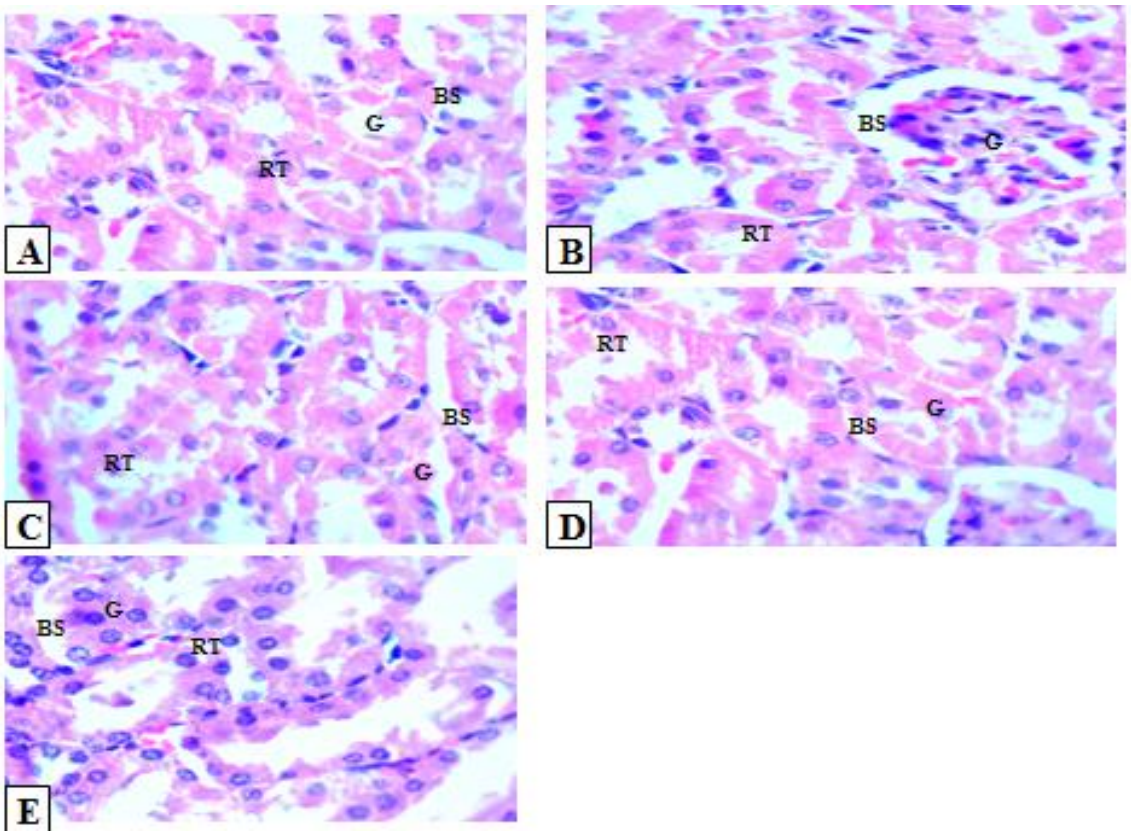


Figure 18. Photomicrograph of kidney sections (day 31). H and E x 400.

Plate A (Control), **Plate B** (62.5 mg/kg), **Plate C** (125 mg/kg), **Plate D** (250 mg/kg), **Plate E** (500 mg/kg). **G:** Glomeruli, **BS: B**owman's space, **RT:** Renal tubule, **BC:** Bowman's capsule. These are features of normal kidney histoarchitecture.

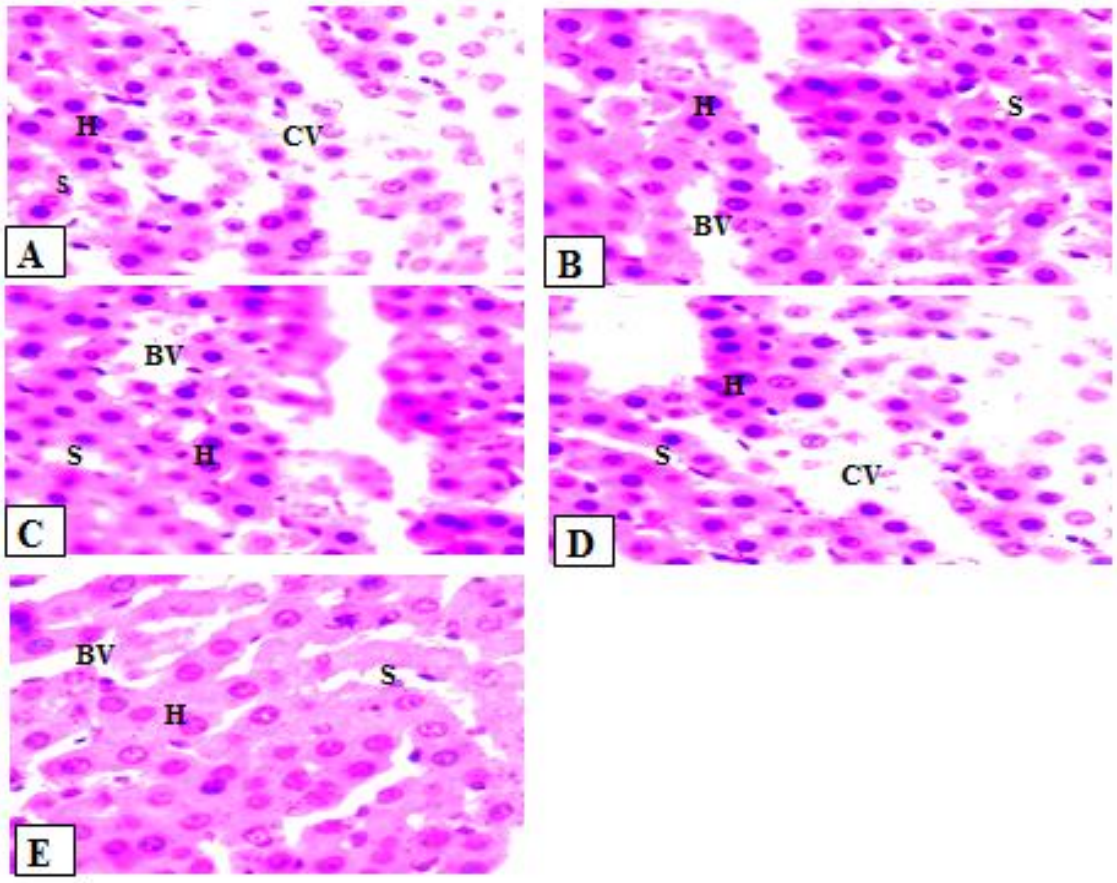


Figure 19. Photomicrograph of liver sections (day 61). H and E x 400.

Plate A (Control), **Plate B** (62.5 mg/kg), **Plate C** (125 mg/kg), **Plate D** (250 mg/kg), **Plate E** (500 mg/kg). **H:** Hepatocytes disposed in sheet. S**:** sinusoids, **BV:** Blood vessel. **CV:** Central vain. This are features of a normal liver histoarchitecture.

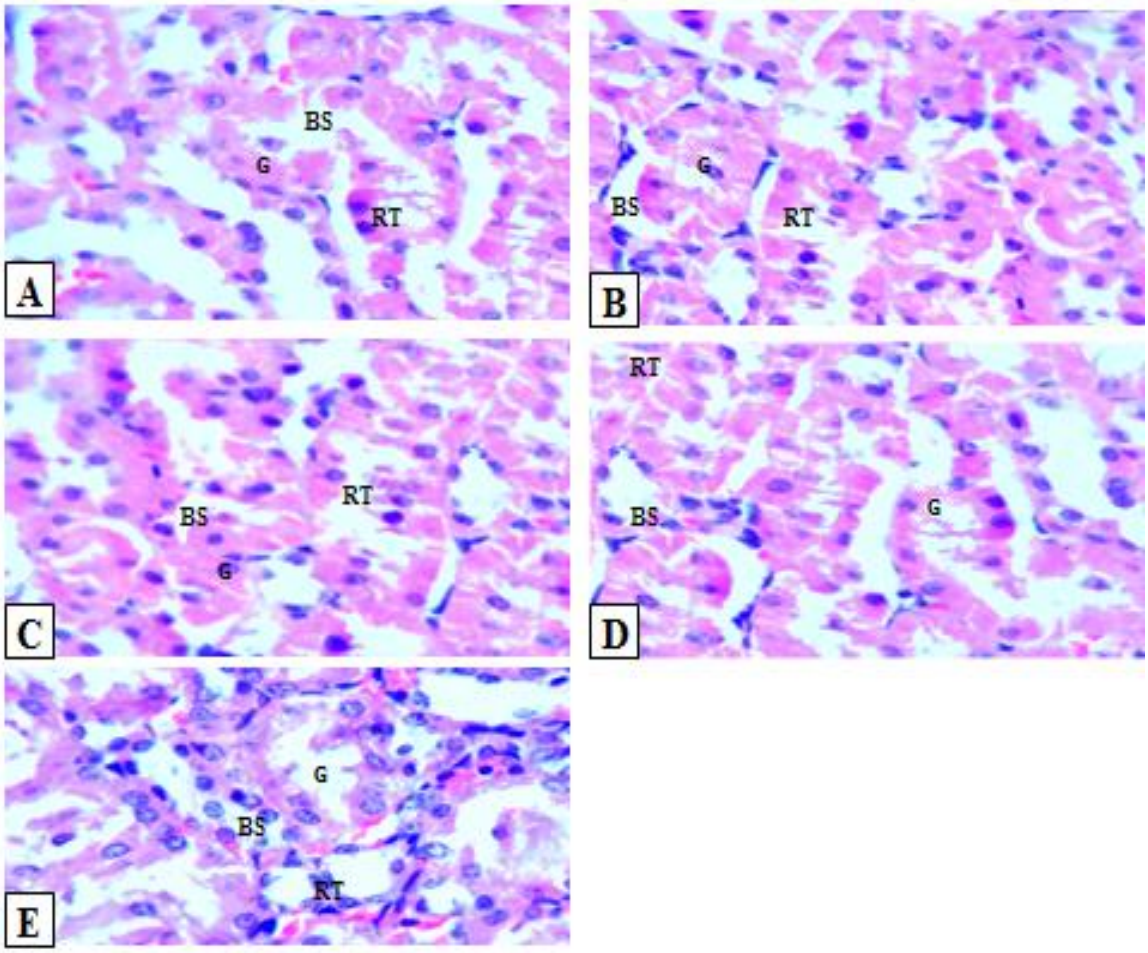


Figure 20. Photomicrograph of kidney sections (day 61). H and E x 400.

Plate A (Control), **Plate B** (62.5 mg/kg), **Plate C** (125 mg/kg), **Plate D** (250 mg/kg), **Plate E** (500 mg/kg). **G:** Glomeruli, **BS: B**owman's space, **RT:** Renal tubule, **BC:** Bowman's capsule. These are features of normal kidney histoarchitecture.

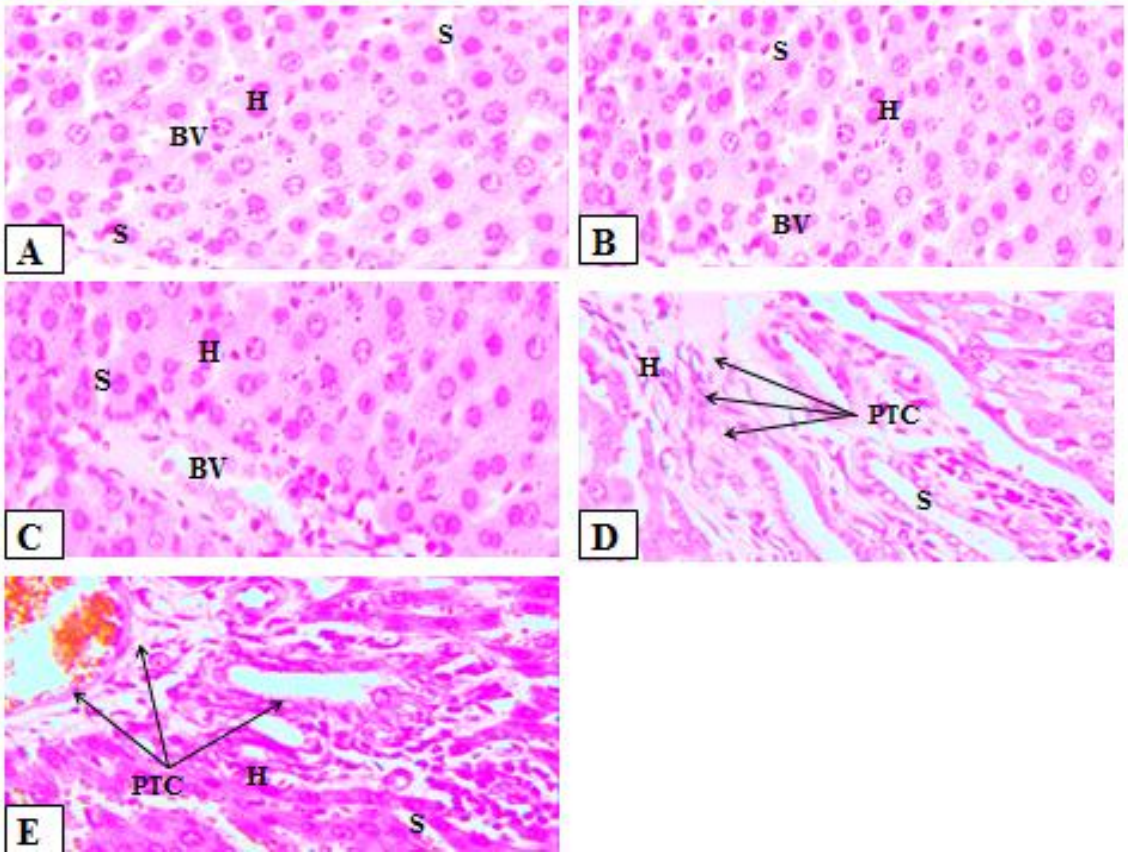


Figure 21. Photomicrograph of liver sections (day 91). H and E x 400. Plate A (Control), **Plate B** (62.5 mg/kg), **Plate C** (125 mg/kg), **Plate D** (250 mg/kg), **Plate E** (500 mg/kg). **H:** Hepatocytes, **BV:** Blood vessel, **S:** Sinosoid, **PTC**: Portal triad congestion).

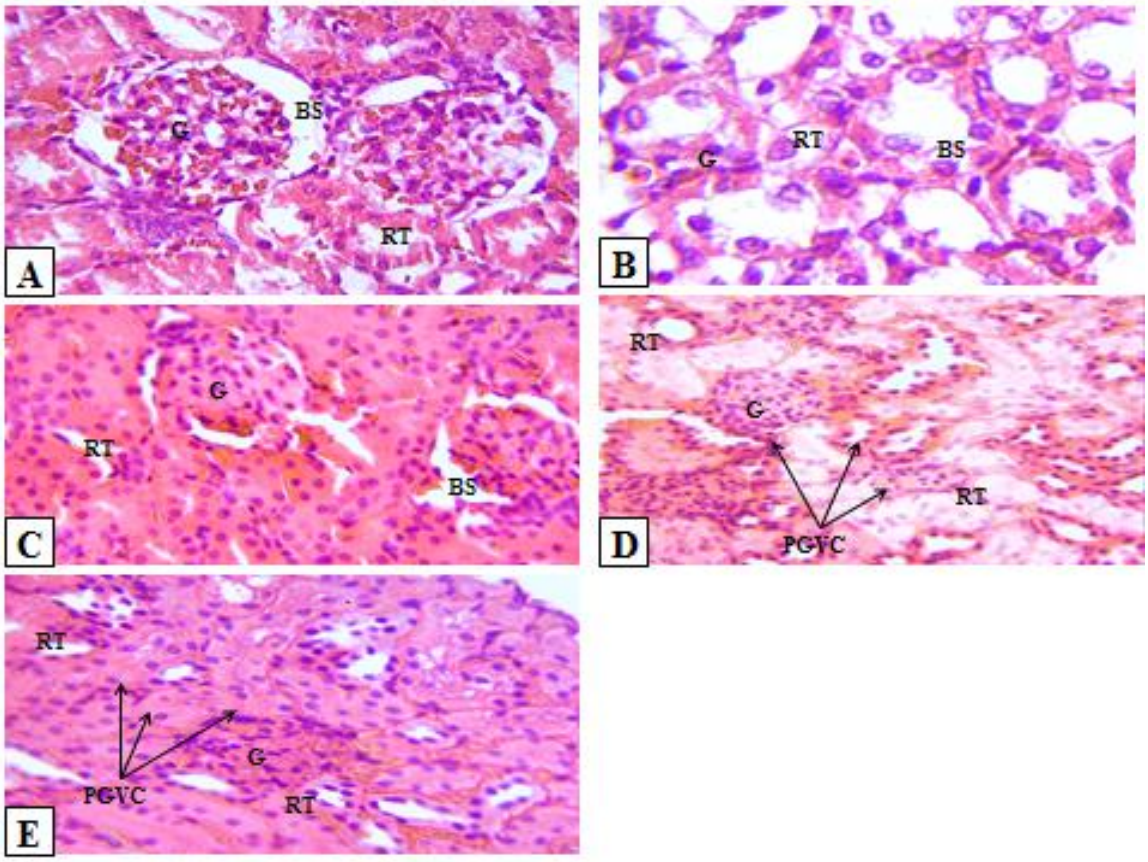


Figure 22. Photomicrograph of kidney sections (day 91). H and E x 400.

Plate A (Control), **Plate B** (62.5 mg/kg), **Plate C** (125 mg/kg), **Plate D** (250 mg/kg), **Plate E** (500 mg/kg). **G:** Glomeruli, **BS: B**owman's space, **RT:** Renal tubule. **GC:**Glomeruli congestion.

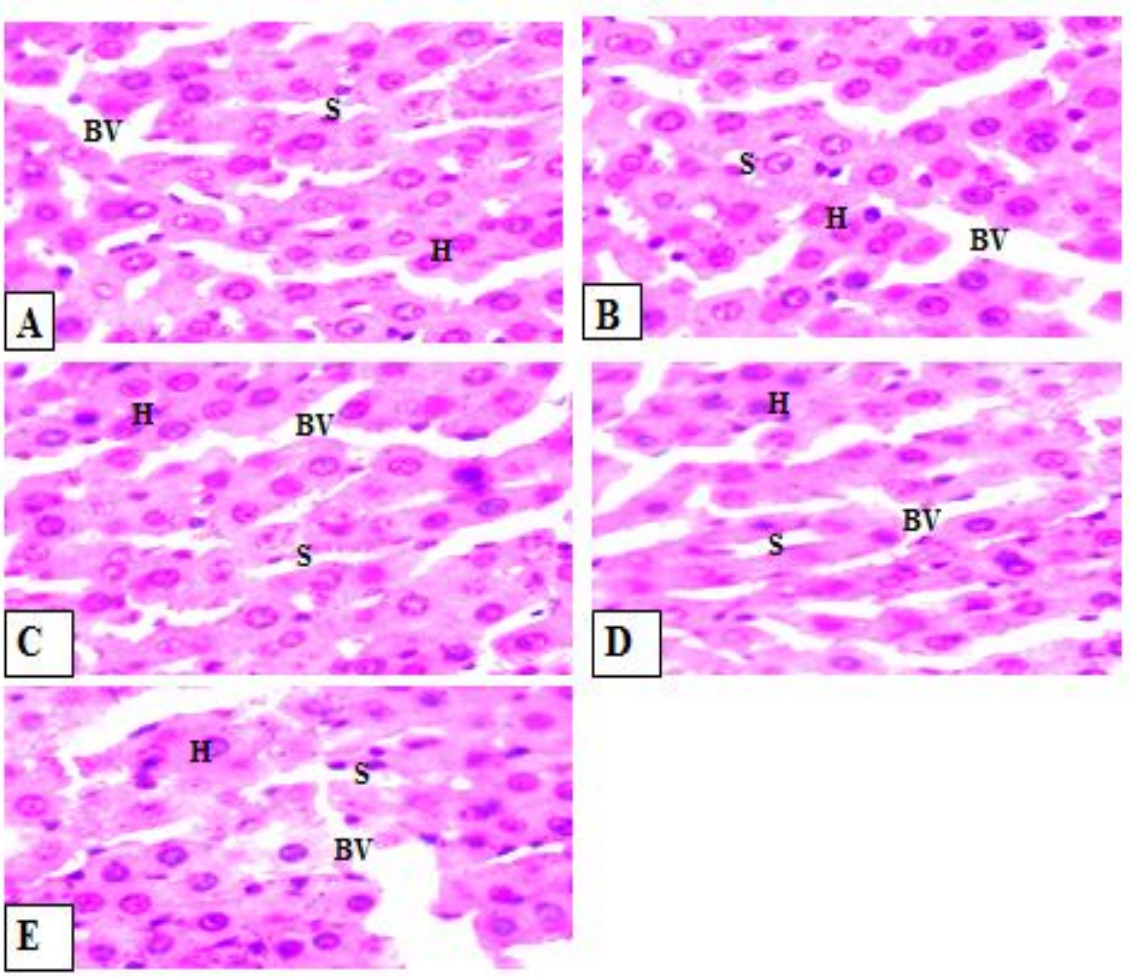


Figure 23. Photomicrograph of liver sections (recovery). H and E x 400.

Plate A (Control), **Plate B** (62.5 mg/kg), **Plate C** (125 mg/kg), **Plate D** (250 mg/kg), **Plate E** (500 mg/kg). **H:** Hepatocytes disposed in sheet. **S:** sinusoids, **BV:** Blood vessel. These are features of a normal liver histoarchitecture.

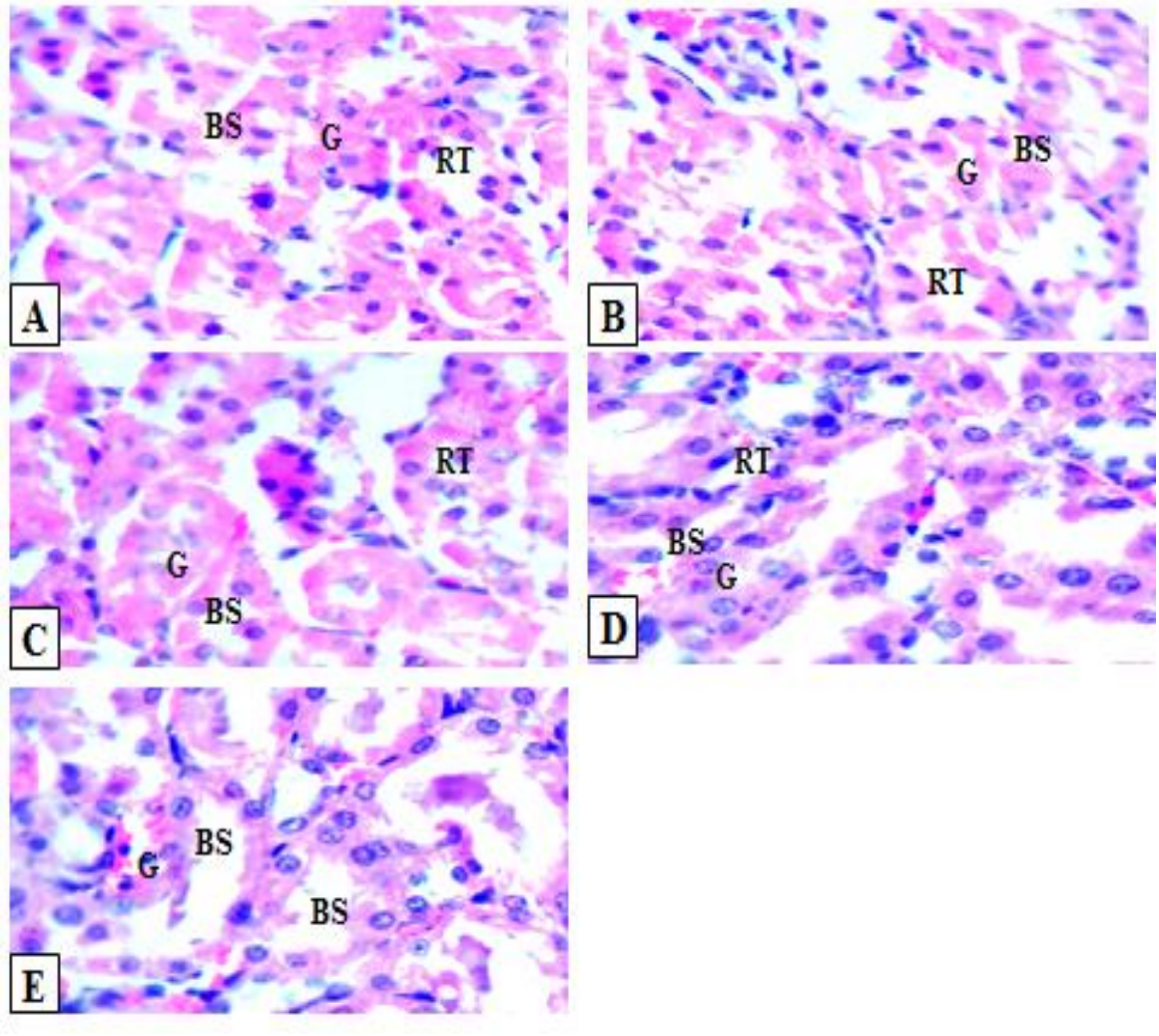


Figure 24. Photomicrograph of kidney sections (recovery). H and E x 400.

Plate A (Control), **Plate B** (62.5 mg/kg), **Plate C** (125 mg/kg), **Plate D** (250 mg/kg), **Plate E** (500 mg/kg). **G:** Glomeruli, **BS: B**owman's space, **RT:** Renal tubule. These are features of normal kidney histoarchitecture.

4.1.11 CHRONIC TOXICITY RESULTS

4.1.11.1 Effects of extract on haematological parameters:

Chronic administration of various doses of *D. filix-mass* extract did not cause significant changes ($p>0.05$) in any of the haematological parameters measured on day 181 (Tables 37).

4.1.11.2 Effects of extract on lipid profile:

Chronic administration of various doses of *D. filix-mass* extract did not cause significant change ($p>0.05$) in total cholesterol, triglyceride, LDL-cholesterol and HDL-cholesterol levels of rats on day 181 (Table 38).

4.1.11.3 Effects of extract on liver enzymes:

Administration of various doses of *D. filix-mass* extract caused significant reduction ($p<0.05$) in ALT and AST levels. However, no significant changes ($p>0.05$) were observed in ALP, total protein and albumin levels in extract treated groups relative to control. Changes in ALT and AST levels on day 181 were reversible in recovery studies (Table 39).

4.1.11.4 Effects of extract on sodium, potassium, chloride and calcium:

Chronic administration of various doses of *D. filix-mass* extract did not cause significant change ($p>0.05$) in sodium, potassium, chloride and total protein levels of rats on day 181 (Table 40).

Table 37: Effects of extract on haematological parameters.

Time	Treatment	PCV (%)	RBC (10^6/µl)	Hemoglobin (g/dL)	PLAT (10^3/µL)	WBC (10^3/µl)	PCT (%)
Baseline	**Control**	44.35 ± 0.83	7.10 ± 0.15	14.58 ± 0.34	856.00 ± 40.42	6.24 ± 0.34	0.75 ± 0.02
	31.25 mg/kg	42.56 ± 0.98	6.68 ± 0.23	14.90 ± 0.49	842.20 ± 38.91	6.31 ± 0.41	0.66 ± 0.02
	62.5 mg/kg	43.77 ± 0.59	6.55 ± 0.17	15.32 ± 0.47	806.80 ± 41.09	6.08 ± 0.42	0.66 ± 0.03
	125 mg/kg	43.66 ± 0.70	6.77 ± 0.11	14.58 ± 0.34	781.60 ± 35.05	5.56 ± 0.16	0.66 ± 0.03
Day 181	**Control**	40.29 ± 1.49	6.95 ± 0.46	13.87 ± 0.73	848.23 ± 70.82	7.01 ± 0.28	0.72 ± 0.06
	31.25 mg/kg	42.39 ± 0.57	7.01 ± 0.17	14.22 ± 0.52	831.81 ± 30.93	6.34 ± 0.32	0.64 ± 0.03
	62.5 mg/kg	41.68 ± 0.57	6.73 ± 0.08	13.69 ± 0.17	855.85 ± 36.47	5.65 ± 0.21	0.66 ± 0.03
	125 mg/kg	42.51 ± 0.57	6.95 ± 0.14	14.32 ± 0.19	907.73 ± 36.23	6.05 ± 0.46	0.73 ± 0.02
Recovery	**Control**	42.64 ± 0.74	7.18 ± 0.18	13.40 ± 0.16	852.04 ± 52.91	6.50 ± 0.50	0.67 ± 0.04
	31.25 mg/kg	41.06 ± 0.66	6.66 ± 0.15	12.68 ± 0.16	704.00 ± 36.38	6.10 ± 0.42	0.55 ± 0.02
	62.5 mg/kg	40.30 ± 0.47	6.45 ± 0.10	12.32 ± 0.13	804.68 ± 67.74	5.23 ± 0.64	0.62 ± 0.05
	125 mg/kg	40.73 ± 2.21	6.69 ± 0.25	12.60 ± 0.62	880.36 ± 64.07	6.02 ± 0.60	0.68 ± 0.04

Values are presented as mean ± Standard error of mean (n =5). $p>0.05$: Not significantly different from control group. PCV (packed cell volume), RBC (red blood cell) PLAT (platelet), WBC (white blood cell), PCT (Platelete percentage).

Table 38: Effects of extract on lipid profile.

Time	Treatment	Total cholesterol (mg/dl)	Triglyceride (mg/dl)	HDL-cholesterol (mg/dl)	LDL-cholesterol (mg/dl)
Baseline	**Control**	147.37 ± 3.86	113.65 ± 2.42	50.10 ± 2.04	74.54 ± 3.41
	31.25 mg/kg	147.85 ± 1.90	112.46 ± 3.45	51.71 ± 2.47	73.64 ± 3.81
	62.5 mg/kg	139.76 ± 1.20	112.39 ± 2.66	48.35 ± 3.38	68.94 ± 3.98
	125 mg/kg	143.89 ± 3.61	111.02 ± 1.98	48.43 ± 4.44	73.26 ± 5.49
Day 181	**Control**	144.96 ± 3.14	113.30 ± 4.18	53.86 ± 0.92	68.44 ± 2.99
	31.25 mg/kg	144.66 ± 2.36	118.08 ± 2.22	55.90 ± 0.62	65.15 ± 2.45
	62.5 mg/kg	146.73 ± 3.29	114.73 ± 3.62	57.39 ± 2.19	66.39 ± 4.10
	125 mg/kg	144.66 ± 1.51	114.25 ± 3.50	55.76 ± 1.71	66.05 ± 2.42
Recovery	**Control**	151.70 ± 3.50	106.26 ± 6.82	58.43 ± 2.33	72.02 ± 1.81
	31.25 mg/kg	148.80 ± 4.98	101.09 ± 3.06	56.58 ± 2.06	72.00 ± 3.55
	62.5 mg/kg	152.86 ± 2.13	109.24 ± 5.22	56.99 ± 0.70	74.02 ± 2.68
	125 mg/kg	151.70 ± 1.96	105.63 ± 5.69	56.17 ± 1.83	74.40 ± 1.09

Values are presented as mean ± Standard error of mean (n =5). P>0.05: Not significantly different from control group. HDL (High density liporprotein), LDL (Low density lipoprotein).

Table 39: Effects of extract on liver enzymes.

Time	Treatment	ALT (U/L)	AST (U/L)	ALP (IU/L)	Albumin (g/dl)	Total protein (g/dl)
Baseline	**Control**	15.59 ± 0.57	37.51 ± 0.68	46.29 ± 1.37	3.06 ± 0.05	4.61 ± 0.22
	31.25 mg/kg	16.53 ± 0.49	34.18 ± 0.87	48.09 ± 1.49	3.04 ± 0.06	4.76 ± 0.32
	62.5 mg/kg	15.39 ± 0.40	36.85 ± 0.92	46.81 ± 1.68	3.03 ± 0.12	4.54 ± 0.58
	125 mg/kg	15.63 ± 0.28	35.27 ± 0.88	46.36 ± 1.87	3.03 ± 0.05	4.06 ± 0.04
Day 181	**Control**	17.10 ± 0.83	40.30 ± 2.71	46.95 ± 4.31	3.11 ± 0.04	4.73 ± 0.29
	31.25 mg/kg	14.72 ± 0.36*	33.70 ± 0.46*	51.43 ± 5.83	3.08 ± 0.04	5.33 ± 0.14
	62.5 mg/kg	14.90 ± 0.33*	33.00 ± 0.91*	49.69 ± 3.98	2.98 ± 0.18	5.18 ± 0.43
	125 mg/kg	14.03 ± 0.16*	31.10 ± 0.37*	46.76 ± 3.45	2.46 ± 0.62	4.99 ± 0.26
Recovery	**Control**	15.82 ± 1.57	31.36 ± 3.54	47.82 ± 5.50	4.06 ± 0.10	5.24 ± 0.32
	31.25 mg/kg	16.71 ± 0.58	26.75 ± 2.45	46.17 ± 7.43	3.84 ± 0.11	5.40 ± 0.17
	62.5 mg/kg	15.86 ± 1.40	31.56 ± 1.67	46.45 ± 3.76	4.15 ± 0.12	5.24 ± 0.33
	125 mg/kg	16.53 ± 1.68	28.32 ± 3.39	40.81 ± 8.38	4.11 ± 0.14	5.43 ± 0.15

Values are presented as mean ± Standard error of mean (n =5). *p<0.05: Significantly different from control group. ALT (alanine aminotransferase), AST (aspertate aminotransferase), ALP (alkaline phosphatase).

Table 40: Effects of extract on electrolytes.

Time	Treatment	Sodium (mEq/L)	Potassium (mEq/L)	Chloride (mEq/L)	Calcium (mg/dl)
Baseline	**Control**	142.94 ± 2.46	4.04 ± 0.08	105.87 ± 2.07	10.57 ± 0.70
	31.25 mg/kg	145.00 ± 5.35	3.96 ± 0.09	102.00 ± 1.44	9.76 ± 0.32
	62.5 mg/kg	140.25 ± 1.65	4.04 ± 0.17	102.97 ± 3.15	9.77 ± 0.41
	125 mg/kg	143.43 ± 6.21	4.30 ± 0.14	104.04 ± 2.51	10.21 ± 0.39
Day 181	**Control**	132.94 ± 1.63	3.72 ± 0.19	102.91 ± 2.00	10.99 ± 0.75
	31.25 mg/kg	130.81 ± 1.04	4.60 ± 0.09	101.43 ± 2.18	10.88 ± 0.84
	62.5 mg/kg	131.68 ± 1.31	3.56 ± 0.39	103.71 ± 2.32	10.33 ± 0.48
	125 mg/kg	131.37 ± 0.70	3.77 ± 0.47	102.44 ± 2.13	10.85 ± 0.55
Recovery	**Control**	126.87 ± 3.03	3.53 ± 0.11	94.61 ± 2.85	9.68 ± 0.11
	31.25 mg/kg	132.40 ± 1.96	3.53 ± 0.11	101.39 ± 3.77	9.74 ± 0.29
	62.5 mg/kg	128.94 ± 3.30	3.53 ± 0.09	98.37 ± 2.88	9.47 ± 0.18
	125 mg/kg	131.02 ± 2.02	3.46 ± 0.11	97.06 ± 3.57	9.07 ± 0.64

Values are presented as mean ± Standard error of mean (n =5). $p>0.05$: Not significantly different from control group.

4.1.11.5 Effects of extract on urea and creatinine:

Chronic administration of various doses of *D. filix-mass* extract caused significant increase ($p<0.05$) in urea and creatinine levels of rats on day 181. However, these changes were reversible in recovery studies (Table 41).

4.1.11.6 Effects of extract on body weight:

Administration of various doses of *D. filix-mass* extract did not cause significant change ($p>0.05$) in body weights of rats from the 1st month through the 6th month (Table 42).

4.1.11.7 Effects of extract on relative organs weights:

From table 43, administration of extract did not cause significant ($p>0.05$) change in relative organs weights on 181 day when compared with control group.

4.1.11.8 Effects of extract on stomach mucosa:

No ulcer was recorded among the various groups treated with extract for a period of 6 months.

Table 41: Effects of extract on urea and creatinine.

Time	Treatment	Urea (mg/dl)	Creatinine (mg/dl)
Baseline	**Control**	20.19 ± 0.90	3.79 ±0.21
	31.25 mg/kg	21.31 ± 0.77	3.73 ± 0.15
	62.5 mg/kg	20.01 ± 0.29	3.43 ± 0.14
	125 mg/kg	20.68 ± 0.51	3.47 ± 0.15
Day 181	**Control**	18.90 ± 0.42	4.34 ± 0.16
	31.25 mg/kg	24.00 ± 1.46*	4.86 ± 0.20
	62.5 mg/kg	32.55 ± 1.39*	6.77 ± 0.49*
	125 mg/kg	35.14 ± 0.95*	8.07 ± 0.39*
Recovery	**Control**	19.78 ± 0.18	4.27 ± 0.11
	31.25 mg/kg	19.85 ± 0.25	4.34 ± 0.11
	62.5 mg/kg	20.00 ± 0.20	4.16 ± 0.17
	125 mg/kg	20.07 ± 0.31	4.43 ± 0.17

Values are presented as mean ± Standard error of mean (n =5). *$p<0.05$: Significantly different from control group.

Table 42: Effects of extract on body weight.

	Body weight gain (%)			
Time	**Control**	**31.25 mg/kg**	**62.5 mg/kg**	**125 mg/kg**
1st month	51.54 ± 3.44	44.90 ± 2.37	47.29 ± 3.20	45.21 ± 2.78
2nd month	63.51 ± 2.05	57.27 ± 3.13	60.15 ± 2.14	64.63 ± 1.59
3rd month	68.55 ± 1.28	61.85 ± 2.79	62.92 ± 2.14	69.01 ± 1.17
4th month	70.24 ± 1.17	65.54 ± 2.44	66.51 ± 1.90	71.13 ± 1.12
5th month	73.22 ± 0.74	69.92 ± 1.74	70.04 ± 1.99	73.41 ± 1.08
6th month	73.74 ± 0.80	69.24 ± 1.41	70.77 ± 2.98	73.90 ± 1.28
Recovery	72.06 ± 1.78	66.14 ± 2.63	74.93 ± 2.14	73.99 ± 3.55

Values are presented as mean ± Standard error of mean (n =5). $p>0.05$: Not significantly different from control group.

Table 43: Effects of extract on relative organs weights.

Time	Treatment	Liver (%)	Kidney (%)	Heart (%)	Spleen (%)	Lung (%)
Day 91	**Control**	2.86 ± 0.06	0.44 ± 0.02	0.27 ± 0.01	0.23 ± 0.01	0.54 ± 0.03
	31.25 mg/kg	2.96 ± 0.08	0.54 ± 0.05	0.27 ± 0.01	0.22 ± 0.01	0.63 ± 0.04
	62.5 mg/kg	2.74 ± 0.08	0.54 ± 0.02	0.32 ± 0.04	0.30 ± 0.04	0.59 ± 0.02
	125 mg/kg	2.63 ± 0.12	0.54 ± 0.02	0.34 ± 0.01	0.31 ± 0.05	0.74 ± 0.09
Recovery	**Control**	3.19 ± 0.22	0.55 ± 0.03	0.31 ± 0.01	0.30 ± 0.05	0.69 ± 0.04
	31.25 mg/kg	3.25 ± 0.19	0.57 ± 0.04	0.32 ± 0.02	0.30 ± 0.02	0.75 ± 0.08
	62.5 mg/kg	3.18 ± 0.17	0.53 ± 0.02	0.29 ± 0.01	0.25 ± 0.01	0.67 ± 0.03
	125 mg/kg	3.20 ± 0.15	0.54 ± 0.03	0.30 ± 0.01	0.23 ± 0.01	0.74 ± 0.05

Values are presented as mean ± Standard error of mean (n =5). $p>0.05$: Not statistically significantly different from control group.

4.1.11.9 Effects of extract on liver and kidney histology:

There was no distortion in liver architecture following the administration of 31.25, 62.5 and 125 mg/kg doses of extract to rats for a period of 6 months (Fig. 25).

There was distortion in kidney architecture characterized by glomerulonephritis of the glomerulus with acute tubular necrosis of the renal tubules in dose dependent manner following 6 months exposure of animals to 31.25, 62.5 and 125 mg/kg doses of extract (Fig 26).

In recovery study for 28 days, liver and kidney sections of rats in control and various doses of the extract showed normal architecture (Fig. 27 and 28).

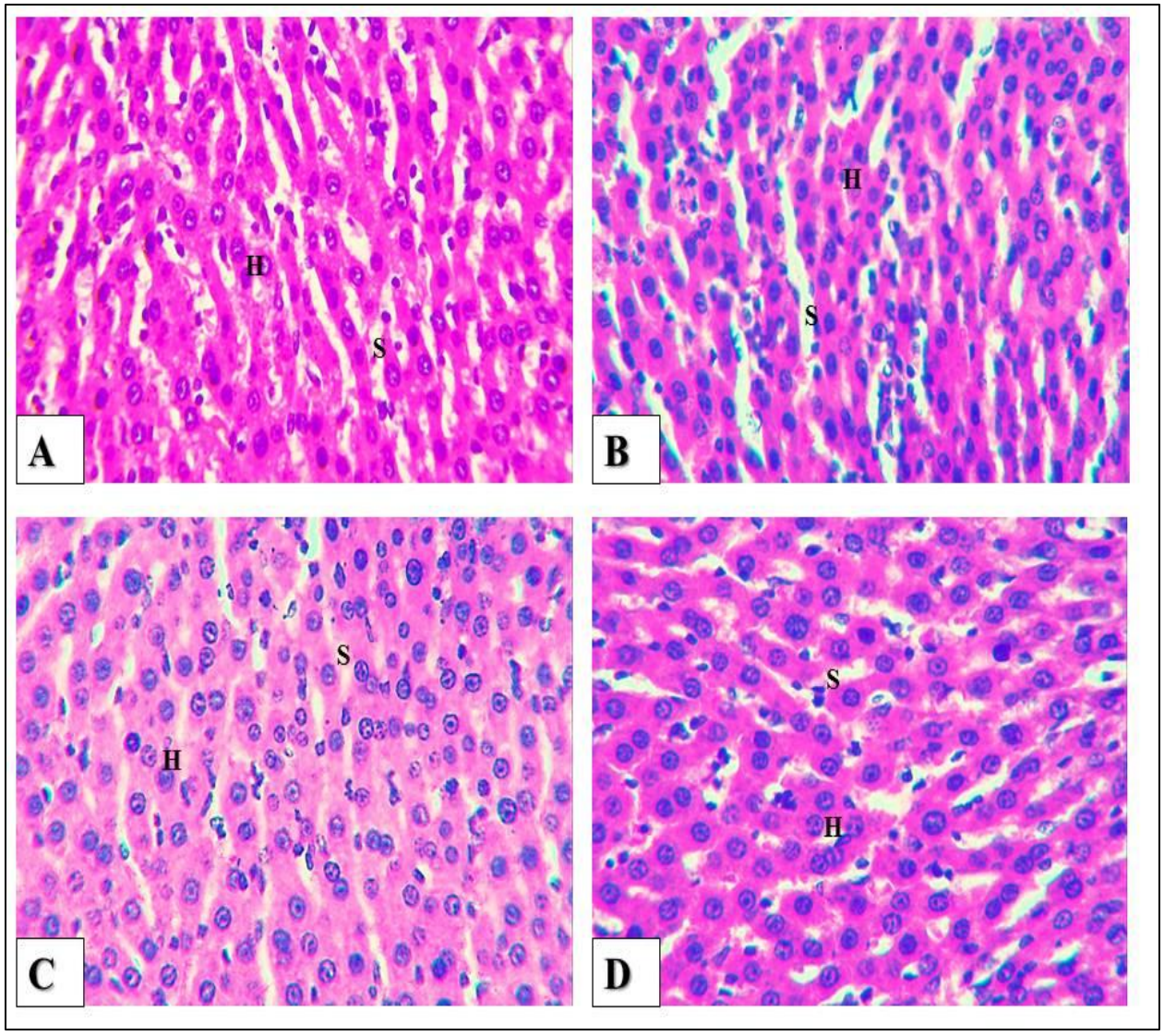


Figure 25: Photomicrographs of liver histology (day 181).

Liver sections of rats given distilled water (control, A) and extract at doses of 31.25 mg/kg (B), 62.5 mg/kg (C), 125 mg/kg (D) showing normal liver architecture. **H:** Hepatocytes disposed in sheet. **S:** sinusoids.

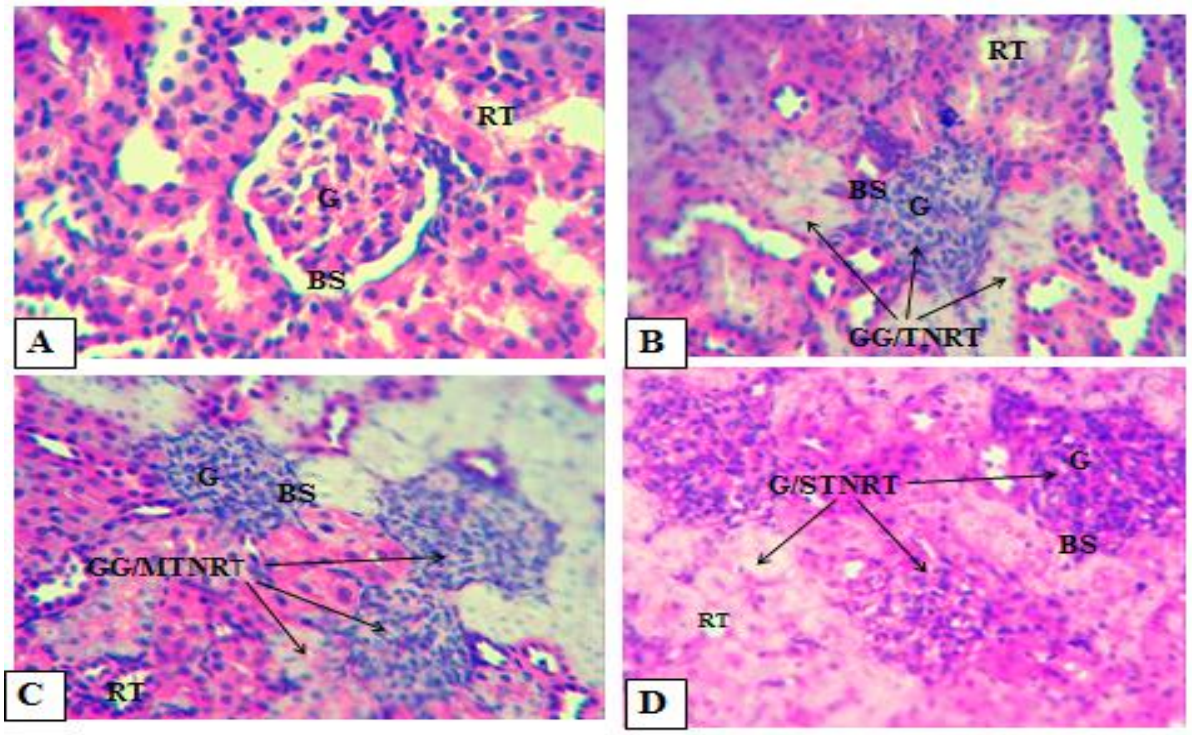


Figure 26: Photomicrographs of kidney histology (day 181). H and E x 400. **Plate A** (Control), **Plate B** (31.25 mg/kg), **Plate C** (62.5 mg/kg), **Plate D** (125 mg/kg). **G:** Glomeruli, **BS: B**owman's space, **RT:** Renal tubule. **GG/TNRT:** Glomerulonephritis of the glomerulus with tubular necrosis of the renal tubules. **GG/MTNRT:** Moderate glomerulonephritis of the glomerulus with morderate tubular necrosis of the renal tubules. **GG/STNRT:** Severe glomerulonephritis of the glomerulus with severe tubular necrosis of the renal tubules.

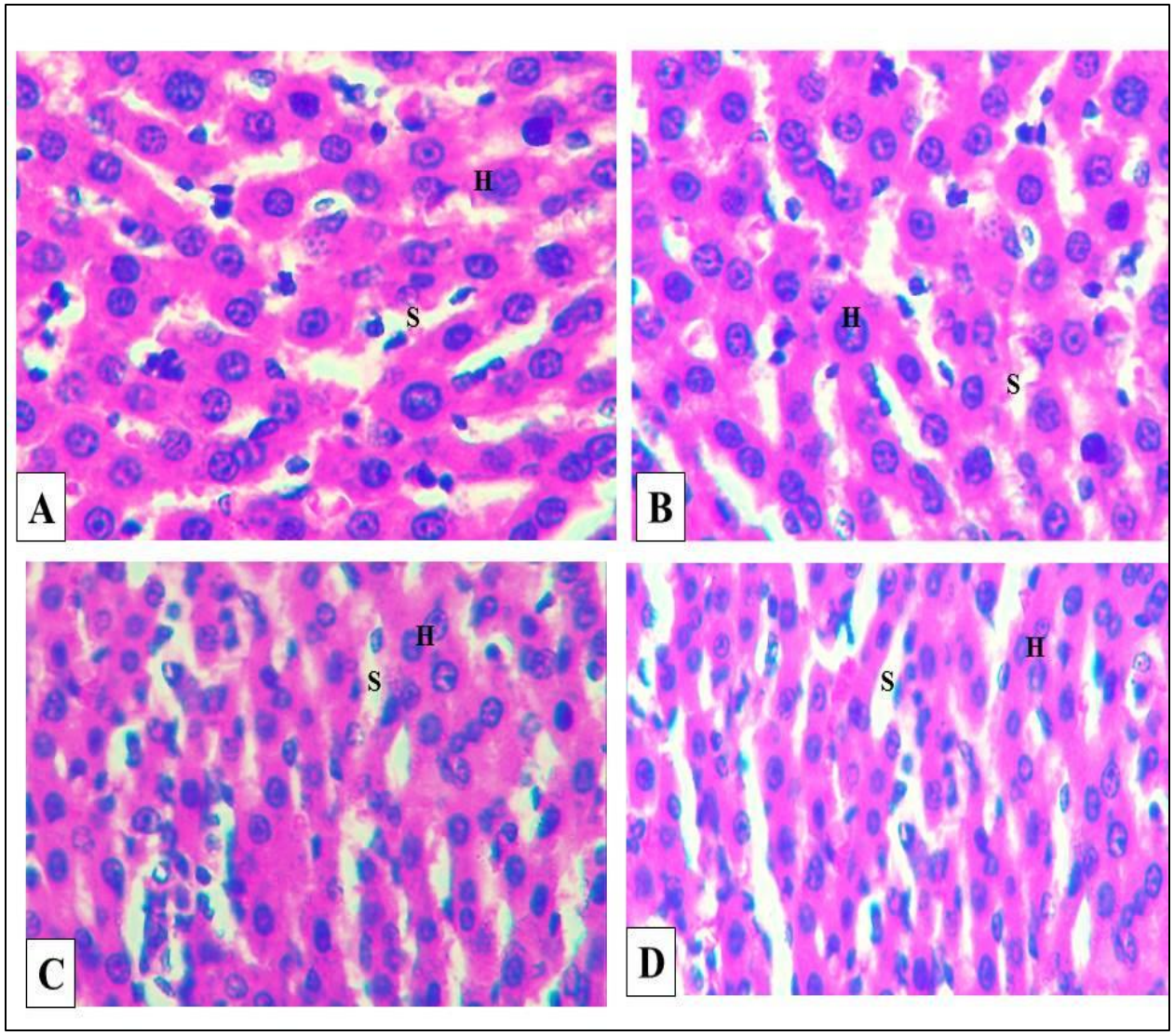


Figure 27: Photomicrographs of liver histology (recovery study). Liver sections of rats given distilled water (control, A) and extract at doses of 31.25 mg/kg (B), 62.5 mg/kg (C), 125 mg/kg (D) showing normal liver architecture. **H:** Hepatocytes disposed in sheet. **S:** sinusoids.

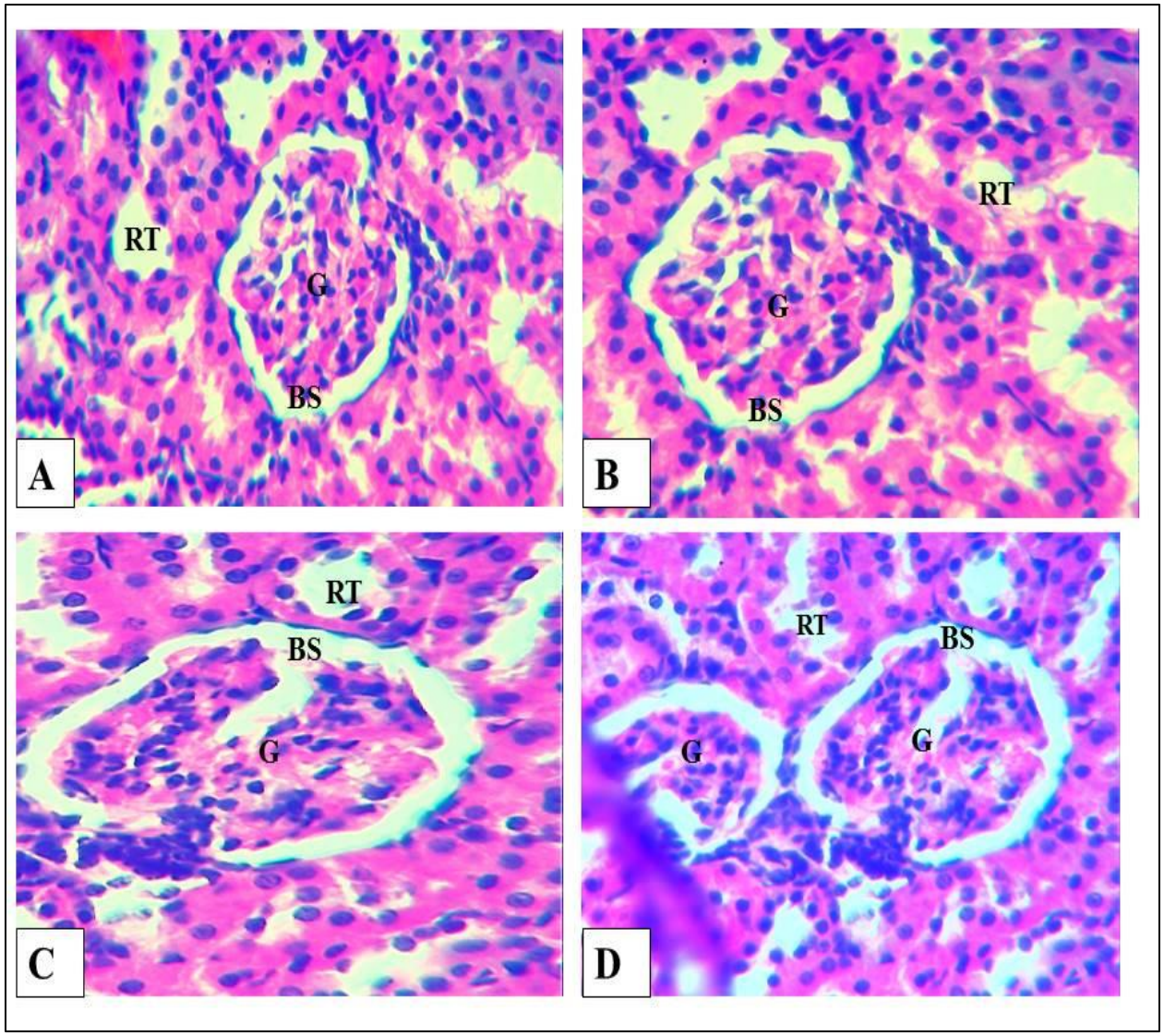


Figure 28: Photomicrographs of kidney histology (recovery study). Kidney sections of rats given distilled water (control, A) and extract at doses of 31.25 mg/kg (B), 62.5 mg/kg (C), 125 mg/kg (D) showing normal kidney architecture.

4.1.12 TERATOGENIC STUDY RESULTS

4.1.12.1 Effects of extract on body weights of pregnant mice:

There was a progressive increase in the weight of the pregnant mice from day 0 of pregnancy through day 6, day 16 and before delivery. After delivery, there was reduction in body weights in all groups (Table 44).

4.1.12.2 Effects of extract on pups' body weight and size:

From table 45, administration of the extract at 250, 500, 1000 mg/kg did not produce significant alteration in average number of pups, average fetal body weight, average tail length, and average crown rump length compared to control groups.

4.1.12.3 Effects of extract on pups' morphology and general appearance:

All littered pups had normal appearance. There was no middle ear disease in any of the pups. None of them was polydactyl neither were their eyes opened on delivery (Table 46).

4.1.12.4 Effects of extracts on pups' organs histology:

Figures 29, 30, 31 and 32 did not produce any observable histological changes in the liver, kidney, heart, and lungs respectively in pups whose mothers were treated with 250, 500 and 1000 mg/kg doses of extract.

Tthere was poor mineralization in the histo-architecture of pups femur whose mothers were treated with 1000 mg/kg of extract. No toxicologocal chages were observed in pups whose mothers were treated with 250 and 500 mg/kg of the extract.

Table 44: Effects of extract on body weights of pregnant mice.

	Body weight (g)				
Treatment	**Day 0**	**Day 6**	**Day 16**	**Before delivery**	**After delivery**
Control	21.88 ± 0.75	25.10 ± 0.86	31.24 ± 2.20	37.64 ± 1.24	29.18 ± 0.77
250 mg/kg	23.28 ± 1.38	26.94 ± 1.08	35.86 ± 1.10	38.06 ± 0.80	28.02 ± 0.71
500 mg/kg	24.70 ± 0.90	27.04 ± 1.27	34.80 ± 0.31	36.00 ± 0.71	29.76 ± 0.88
1000 mg/kg	24.06 ± 1.58	24.80 ± 1.45	36.42 ± 1.02	39.74 ± 0.52	29.22 ± 0.74

Values are presented as mean ± standard error of mean (SEM), n =5, $p>0.05$: Not significantly different from control group. Values in parenthesis indicate percentage weight gain relative to Day 0.

Table 45: Effects of extract on pups body weight and sizes.

Treatment	Number of pups	Body weight (g)	Tail length (cm)	Crown rump length (CRL), (cm)
Control	5.60 ± 0.24	1.65 ± 0.03	1.25 ± 0.02	3.05 ± 0.03
250 mg/kg	5.60 ± 0.40	1.48 ± 0.06	1.11 ± 0.11	2.91 ± 0.11
500 mg/kg	5.80 ± 0.58	1.47 ± 0.09	1.22 ± 0.01	2.91 ± 0.17
1000 mg/kg	5.40 ± 0.68	1.42 ± 0.07	1.11 ± 0.06	2.97 ± 0.05

Values are presented as mean ± Standard error of mean (SEM). $p>0.05$: Not significantly different from control group.

Table 46: Effects of extract on pups' morphology and general appearance.

Treatment	Middle ear disease (MED)	Open eye (OE)	Polydactyl	General appearance
Control	-	-	-	Normal
250 mg/kg	-	-	-	Normal
500 mg/kg	-	-	-	Normal
1000 mg/kg	-	-	-	Normal

"-": Indicates absence of deformity

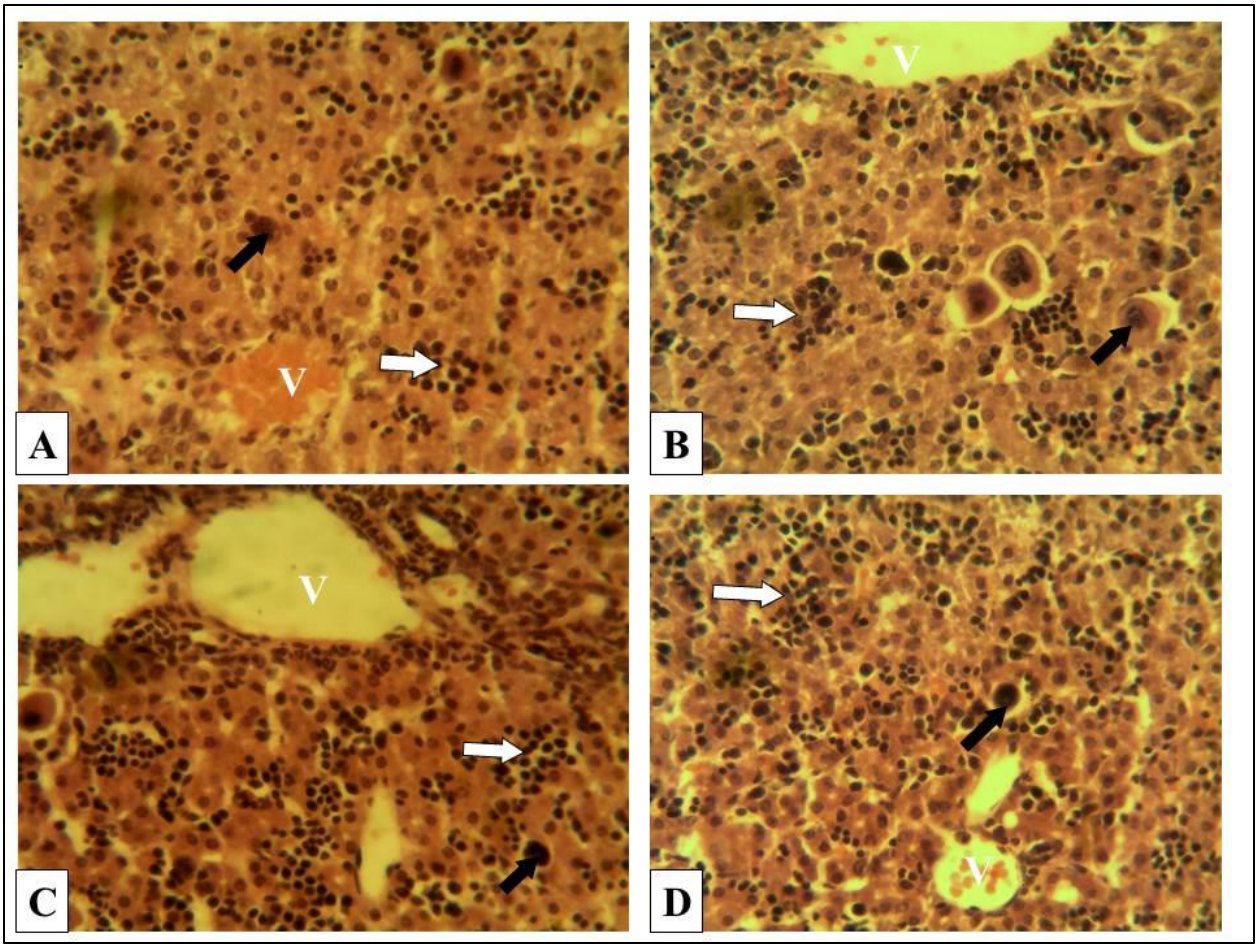


Figure 29: Photomicrographs of pups' liver sections. H and E x 400.

Distilled water, control (**A**), 250 mg/kg (**B**), 500 mg/kg (**C**) and 1000 mg/kg (**D**) groups showing the central vein (V). There is evidence of fetal hematopoiesis (blood cell formation) by the presence of megakaryocytes (black arrows) and aggregation of erythrocyte precursor cells (white arrows). There is no observable adverse reaction or lesion in all the groups.

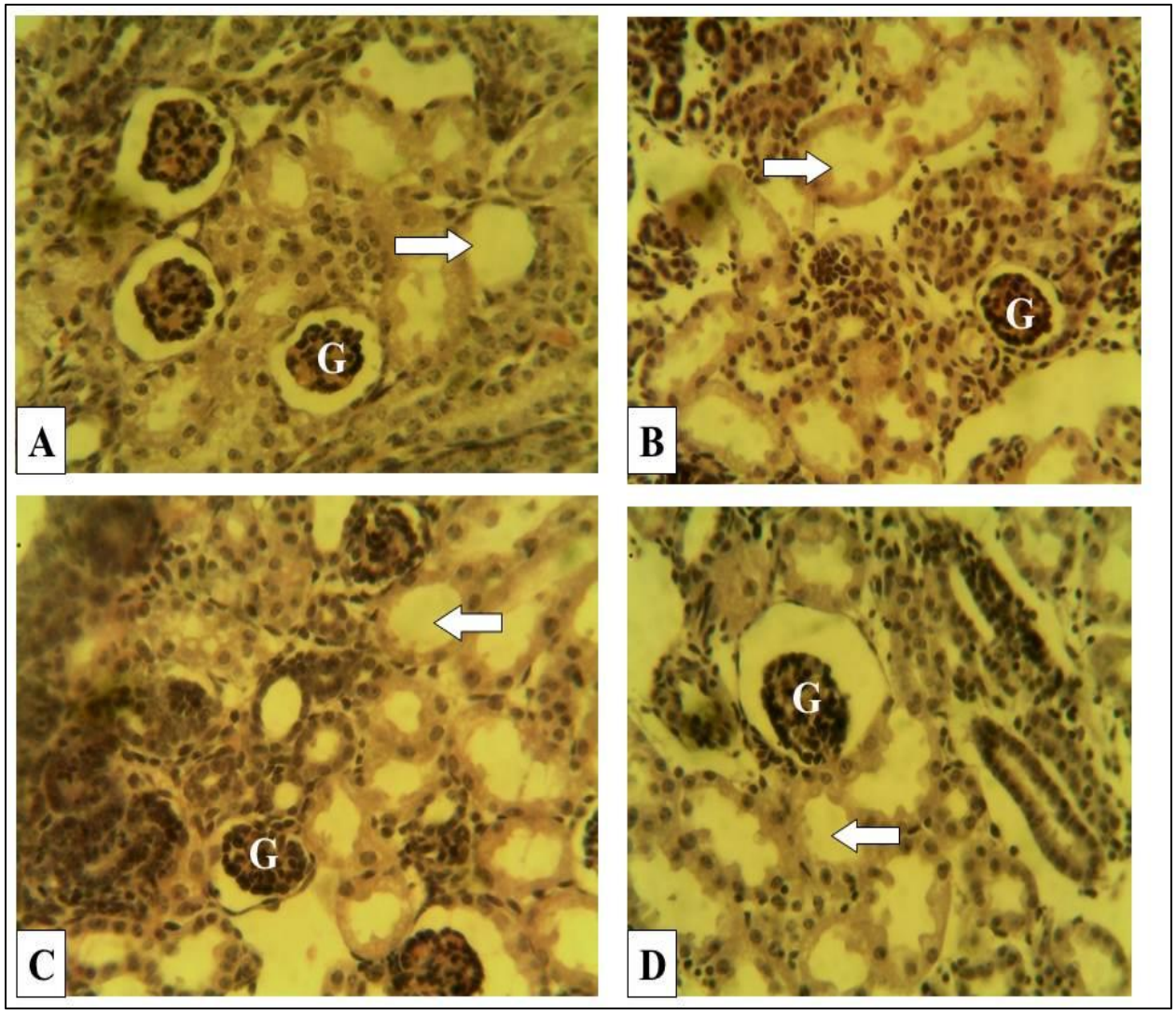


Figure 30:. **Photomicrographs of pups' kidney sections. H and E x 400.**

Distilled water, control (**A**), 250 mg/kg (**B**), 500 mg/kg (**C**) and 1000 mg/kg (**D**) showing the maturing glomerulus (G) and renal tubules (arrows). There is no observable adverse reaction or lesion in all the groups.

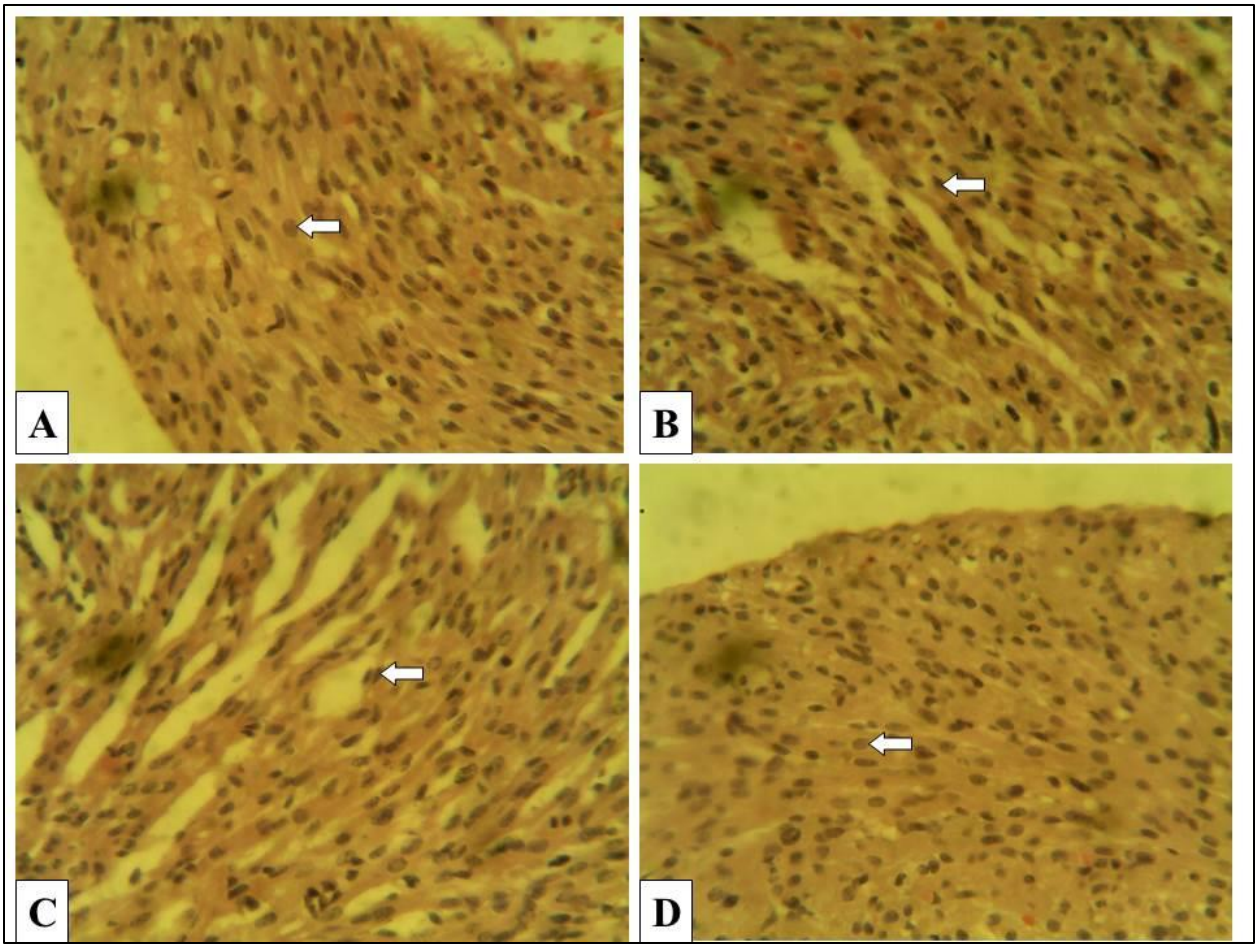


Figure 31: Photomicrograph of pups' heart sections. **H and E x 400.** Distilled water, control (**A**), 250 mg/kg (**B**), 500 mg/kg (**C**) and 1000 mg/kg (**D**) showing poorly separated muscle fibres (arrows). There is no observable adverse reaction or lesion in all the groups.

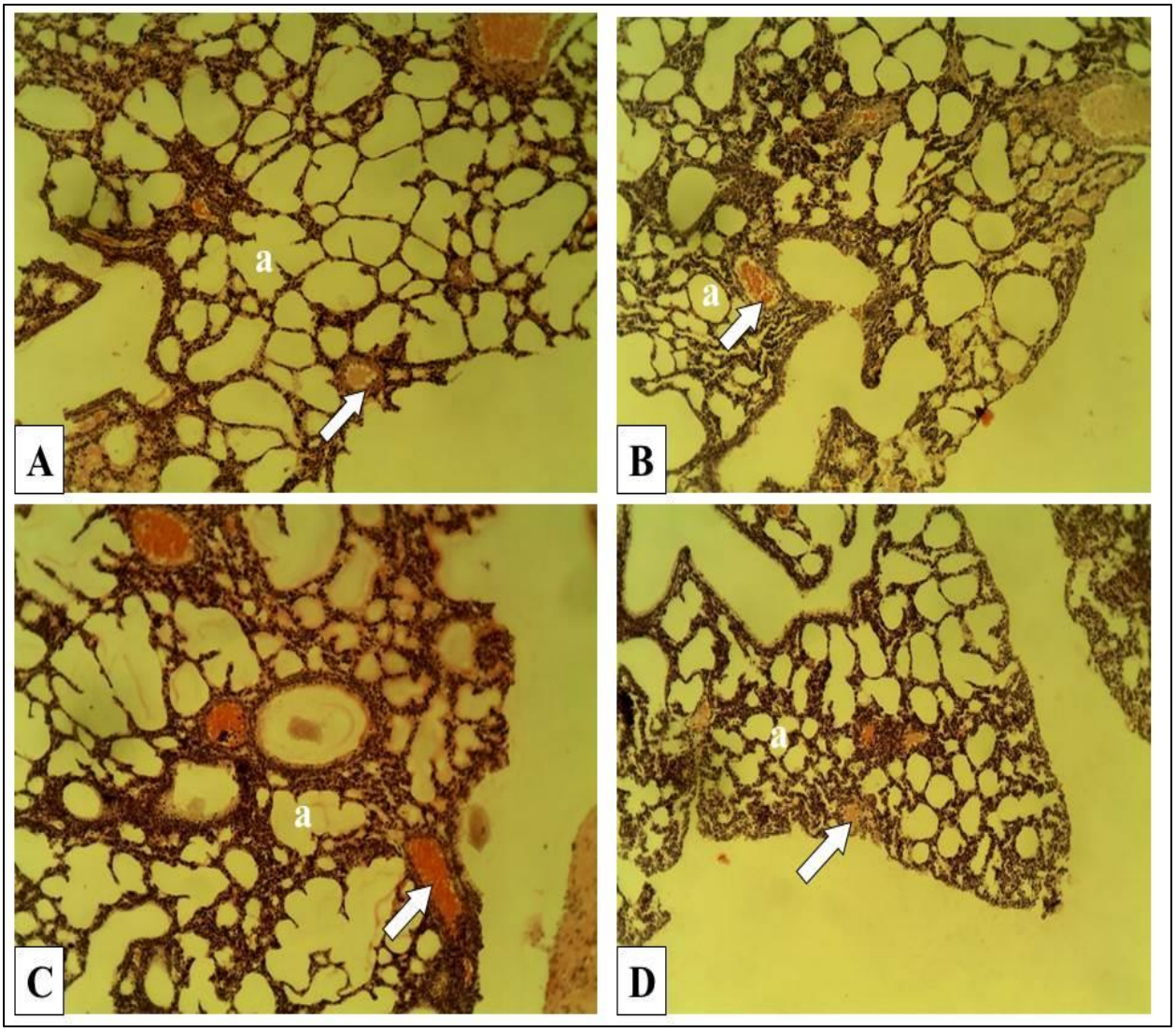


Figure 32: Phtomicrograph of pups' lungs sections. H and E x 400. Distilled water, control (**A**), 250 mg/kg (**B**), 500 mg/kg (**C**) and 1000 mg/kg (**D**) showing the maturing alveoli (a) and pulmonary blood vessels (arrows).There is no observable adverse reaction or lesion in all the groups.

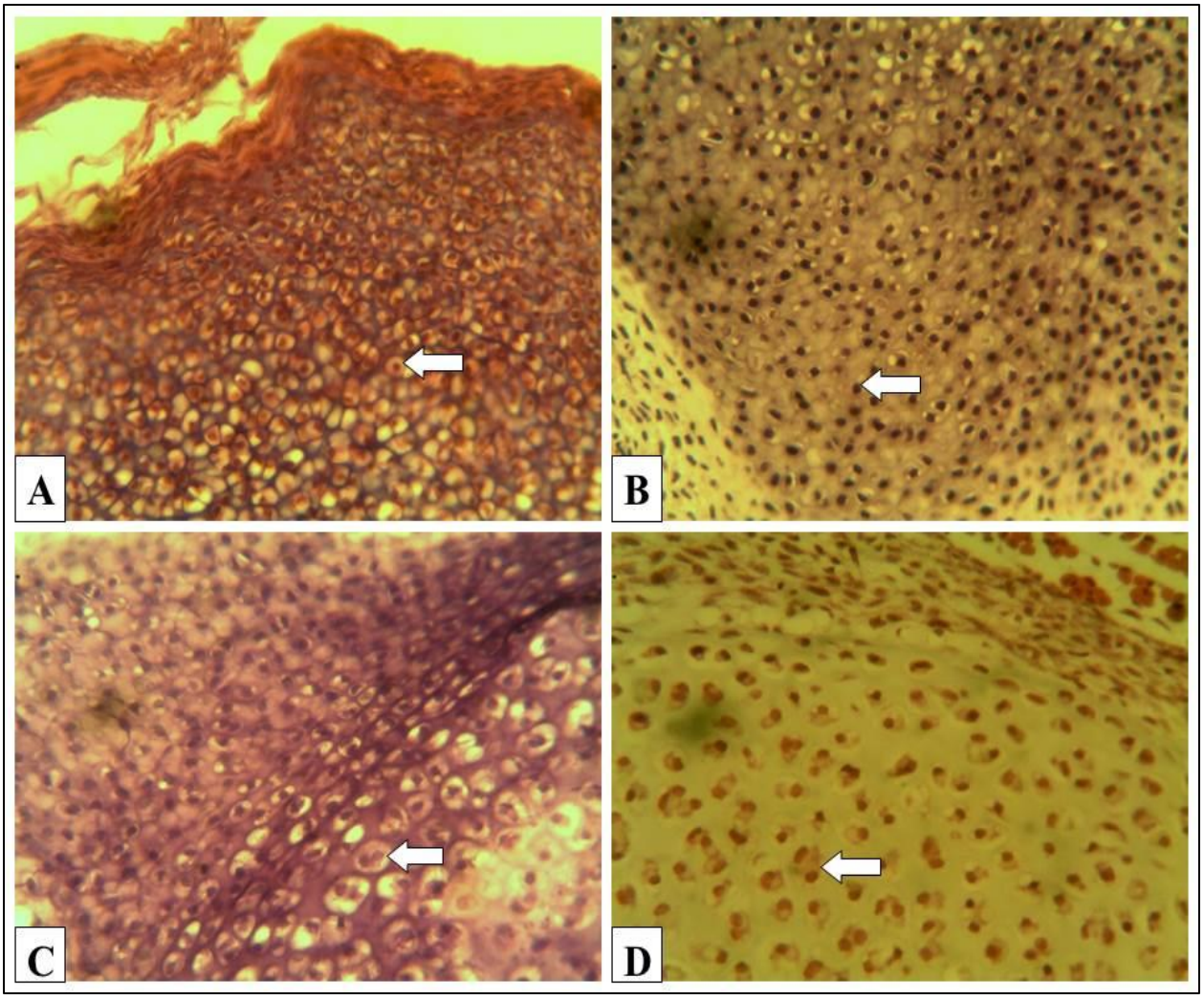


Figure 33: Photomicrographs of pups' femur sections. H and E x 400.

Distilled water, control (**A**), 250 mg/kg (**B**), 500 mg/kg (**C**) and 1000 mg/kg (**D**). High dose (**D**) groups showed actively proliferating and mineralizing cartilages (arrows). Note that there is poor mineralization in high dose group (less bluish staining, which means less calcium depositions).

4.2 DISCUSSION

This study revealed that the extract, ethyl acetate and butanol fractions of *D. filix-mas* elicited significant anti-inflammatory and antioxidant properties in various models. Also, toxicological studies revaled that the extract is relatively safe when used at lower doses.

From the acute toxicity result, absence of toxicity and death at 5000 mg/kg suggests that the leaf extract of *D. filix-mas* is safe when used for a short duration. Ihekwereme *et al.,* (2018) reported that LD_{50} values of test substances above 5000 mg/kg are considered safe. Ahmed (2015) also revealed that LD_{50} values above 5000 mg/kg are classified as practically non-toxic.

From the anti-inflammatory results, significant reduction in paw edema by the extract, ethyl acetate and butanol fractions of *D. filix-mas*, especially at the 3^{rd} and 4^{th} hours (Tables 4 and 5), suggests their ability to inhibit acute phases of inflammatory response which is characterized by release of inflammatory mediators such as histamine and serotonin (released during the first phase) as well as prostaglandin and bradykinin (released during the second phase). Suppression of rats paw edema may be attributed to the phytoconstituents such as flavonoids, alkaloids and saponins which are present in the extract and fractions (Table 3). These secondary metabolites of these plants have been reported to elicit anti-inflammatory activities (Okigbo *et al.,* 2009; Yadav and Agarwala, 2011). Qnais *et al.,* (2014) reported that the extract of *Artemisia Herba-Alba* exhibited anti-inflammatory properties in rats paw edema model due to presence of flavonoids. A study by Ilavarasana *et al* (2005) revealed that the extract of *Cassia fistula* elicited anti-inflammatory properties in carrageenan-induced paw edema model due to presence of flavonoids. Alkaloids from several medicinal plants have also been reported to reduce rat and mouse paw edema induced by egg-white and caragenaan (José *et al.,* 2006). Saponins from *Camellia sinensis*

was also reported to inhibit rat paw edema (Sur *et al*., 2001). Saponins isolated from Ivy plant belonging to the family of araliaceae (Gepdireman *et al.,* 2005) as well as steroidal saponins from *Polygala japonica* (Wang *et al.,* 2008) were also reported to inhibit rat paw edema.

Appreciable anti-inflammatory activities elicited by the extract, ethyl acetate and butanol fractions of *D. filix-mas* in egg-albumin induced paw edema prompted further evaluation of their possibilities to modulate chronic inflammatory condition using formalin. From the result of formaldehyde induced arthritis (Table 8), administration of extract, ethyl acetate and butanol fractions caused significant reduction in edematous response, evidenced by reduction in area under the curve, suggesting that *D. filix-mas* could be of use in the management of chronic arthritis. Presence of alkaloids, tannins and flavonoids in the extract, ethyl acetate and butanol fractions of *D. filix-mas* may have contributed significantly to its anti-arthritic activity. Presence of alkaloids in medicinal plants have been reported to elicit anti-rheumatoid arthritic activity during chronic inflammatory conditions caused by formaldehyde (José *et al.,* 2006). A study by Hosseinzadeh (2002) revealed that the extract of *Crocus sativus* ameliorated formalin induced rat paw edema due to the presence of flavonoids and tannins. Bader *et al* (2014) also stated that several alkaloids and flavonoids from medicinal plants possessed activities against arthritis induced by formaldehyde in rats and mice.

Further investigation of the application of the extract and fractions for topical use against acute inflammatory conditions was carried out using xylene induced topical ear edema model. In this study, the extract, ethyl acetate, butanol fractions as well as ethyl acetate VLC and Sephadex fractions exhibited significant anti-inflammatory activities against xylene induced topical edema (Tables 6, 12 and 14), suggesting that they prevented irritation of the living tissues, fluid

accumulation and edema formation by the phlogistic agent, xylene, and could be useful in the management of skin related inflammatory disorders. This may be attributed to the presence of terpenoids, flavonoids, saponins and tannins in the extract and selected fractions of *D. filix-mas.* A study by MartinezVazquez *et al* (1999) on the extract of *Euphorbia hirta* showed that terpenoids elicited anti-inflammatory activities against xylene induced topical inflammation in mice. Mei and Jong (2012) also showed that tannins from *Euphorbia hirta* elicited anti-inflammatory activity against xylene induced topical edema. Flavonoids from the extract of *Sophora flavescens* (Jeong *et al.,* 2010) have also been reported to ameliorate ear edema and chronic skin inflammation.

From the result of vascular permeability test (Table 9), administration of extract, ethyl acetate and butanol fractions of *D. filix-mas* resulted in a decrease in vascular permeability as evidenced by reduction in dye concentration of mice peritoneal fliud suggesting that they could inhibit the permeability of blood vessel during inflammatory responses. Phytochemicals such as saponins, alkaloids and flavonoids present in the extract, ethyl acetate and butanol fractions may be responsible for the anti-vascular permeability properties. Küpeli *et al* (2002) reported that alkaloids from various species of Berberis elicited anti-inflammatory activities against acetic acid-induced increase in vascular permeability model in mice. Flavonoids such as apigenin, quercetin, naringenin and luteolin from leaf extract of *Barleria cristata* also reduced increased vascular permeability in mice (Gambhire *et al.,* 2009).

Further mechanistic studies were explored using heat and hypotonic induced haemolysis of human erythrocyte models. Inhibition of heat and hypotonic induced haemolysis of human erythrocyte by extract and fractions, especially ethyl acetate and butanol fractions (Tables 7 and 11) suggests their abilities to stabilize the erythrocyte membrane against inflammatory insults.

This may be attributed to presence of flavonoids, alkaloids and terpenoids. Studies by various authors have shown that these constituents possess membrane stabilizing properties using heat and hypotonic induced haemolysis models. A study by Sinbad and co-workers (2017) showed that flavonoids and alkaloids in the extract of *Sphenocentrum jollyanum* elicited membrane stabilizing properties in human erythrocyte model. Flavonoids and alkaloids from medicinal plants have been reported to modulate inflammation by stabilizing their erythrocyte membranes as well as inhibiting lipid peroxidation in different systems (Middleton *et al.,* 2000). The flavonoid, quercitrin isolated from *Allamanda cathrartica* elicited anti-inflammatory activity through membrane stabilization in hypotonic induced haemolysis model (Hema, 2014). Membranene stabilizing properties of alkaloids from various medicinal plants have also been reported (José *et al.,* 2006). A study by Joseph *et al* (2013) showed that the leaf extracts of *Clerodendrum paniculatum* exhibited human red blood cell membrane stabilization effect due to presence of terpenoids and flavonoids.

There was a reduction in total leukocyte count following treatment with the extract, ethyl acetate and butanol fractions (Table 10). This suggests that *D. filix-mas* could reduce activation and recruitment of leukocytes to the site of inflammation, which could be an additional mechanism of their anti-inflammatory properties. Presence of flavonoids in the extract, ethyl acetate and butanol fractions of *D. filix-mas* may be responsible for the anti-leukocyte migration activity. Studies by Panche *et al* (2016) and Ambriz-Pérez *et al* (2016) revealed that flavonoids from various medicinal plants decrease the number of leukocytes in inflammatory conditions induced by carragenaan in rats and mice. Catechol group of flavonoids was reported to inhibit neutrophils migration (Kanashiro *et al.,* 2007).

It is a known fact that one of the major mechanisms of conventional non-steroidal anti-inflammatory drugs, NSAIDs is by inhibition of prostaglandin formation (Ananya and Sandip, 2014). Also, non-selective inhibition of NSAIDs against Cyclooxygenase -2 (actual anti-inflammatory effects) and Cyclooxygenase - 1 (adverse related effects such as gastrointestinal ulcers and renal bleeding) have been documented (George *et al.,* 2015).

From this study, absence of gastric lesion in groups treated with the extract, ethyl acetate and butanol fractions of *D. filix-mas* when compared with Indomethacin, a reference NSAID, suggests that *D. filix-mas* is not associated with gastrointestinal irritation as well as gastric lesion and could be a better choice in the management of chronic inflammatory disorders than the conventional NSAIDs. This is also substantiated by the selective inhibition in the activity of COX-2 enzymes (Table 13) by the purified ethyl acetate fraction (VLC-E7).

Non-ulcerogenic and selective COX-2 inhibitory activity of the extract and selected fractions of *D. filix-mas* may be attributed to the presence of flavonoids and alkaloids. A study by Hwang *et al* (2012) showed that the extract of *Ailanthus altissima* inhibited COX 2 enzyme as a result of its flavonoids. Mutoh *et al* (2000) also reported that the flavonoid, quercetin, suppressed the activity of cyclooxygenase-2 promoter-dependent transcriptional activity in colon cancer cells. Study by D'Mello *et al* (2011) revealed that flavonones and flavones, are preferential inhibitors of COX-2 enzymes which is associated with inflammation. Studies by Mezui *et al* (2017) on *Eremomatax speciosa* and De Barros *et al* (2016) on *Senocia chilensis* revealed that these plants possess gastroprotective as well as anti-inflammatory properties in mice and rats models due to the presence of flavonoids. The flavonoid, quercetin in some medicinal plants such as *Abrus cantoniensis, Bauhinia curvula Benth* have been reported to exert gastroprotective effects in animal models (Li *et al*., 2015; Beber *et al.,* 2017).

Alkaloids from *Piper nigrum* have been shown to inhibit the activity of cyclooxygenase-2 (Ngo *et al.,* 2017). Study by Mir *et al* (2017) revealed that presence of flavonoids and other polyphenols in the extract of *Tridax procumbens* aerial parts elicited anti-inflammatory activities via inhibition of COX-1 and COX-2 enzyme. COX-1 and COX-2 inhibitory activities of Prenylated flavonoids isolated from the root of *Sophora flavescens* was also reported (Jeong *et al.,* 2010). In this study, presence of flavonoids, quercitrin and kaempferol in the extract, ethyl acetate and butanol fractions of *D. filix-mas* as revealed by HPLC chromatograms (Figures 3 to 13, appendix) could have contributed significantly to its anti-inflammatory and gastroprotective properties. Quercetrin, kaempferol and the isolated compound, Quercetin-3O-αL-rhamnopyranoside present in the ethyl acetate VLC fraction (VLC-E7) of *D. filix-mas* (Figure 14) has also been reported to elicit anti-inflammatory properties in other medicinal plant (Middleton *et al*., 2000; Warren *et al.,* 2009; Dai *et al.,* 2013; Hema, 2014; Abdulatif*et al.*, 2016).

In this study, we also investigated the antioxidant properties of the extract and fractions of *D. filix-mas*. The antioxidant studies were motivated by the fact that free radicals are linked to the pathogenesis of inflammatory disorders such as rheumatoid arthritis (Khatoon *et al.,* 2013; Pandey *et al.,* 2017).

From the antioxidant results, inhibition of nitric oxide generation (Table 18) and scavenging of DPPH (Tables 15, 16 and 19) by the extract, ethyl acetate and butanol fractions of *D. filix-mas* suggests that they could prevent oxidative stress mediated by free radicals. Ferric reducing capacity of the extract, ethyl acetate and butanol fractions of *D. filix-mas* (Table 18) suggests that this medicinal plant possesses reducing agents that could mop up free radical by donating hydrogen atoms. Higher antioxidant activities elicited by ethyl acetate fraction (VLC-E7) could be attributed to its higher phenolic content. Flavonoids which are phenolic compounds in various

medicinal plants have capacities to neutralize several forms of free radicals due to their electron donating abilities (Habibur *et al.,* 2013; Ajaghaku *et al.,* 2017). Mir *et al* (2017) also reported that flavonoids from *Tridax procumbens* possess antioxidant properties in DPPH, ferric reducing and other antioxidant models. Phenolic compounds, catechin, epicathechin, dihydroquercetin isolated from *Alchornea floribunda leaf* were also found to elicit antioxidant properties in DPPH and ferric reducing antioxidant models (Ajaghaku *et al.,* 2017). Chikezie *et al* (2015) have also shown that condensed tannins from leaves, twigs and stem bark of *Canarium album* elicited DPPH scavenging activity as well as ferric reducing properties.

Anti-oxidant properties elicited by extract, ethyl acetate and butanol fractions suggest that inhibition of free radical generation could also be an additional anti-inflammatory mechanism of *D. filix-mas.* Studies have shown that inhibition of free radicals by antioxidants in the course of inflammation could block arachidonic acid metabolism generated free radicals (Pandey *et al.,* 2017).

Higher anti-inflammatory and anti-oxidant activities elicited by ethyl acetate and butanol fractions suggest that they contain moderate polar bioactive compounds. A study by Dehkharghanian *et al* (2010) revealed that solvents of various polarities have abilities to extract different phytochemical constituents from medicinal plants. Medium polar solvent such as ethyl acetate has the ability to extract semi-polar and polar flavonoids, tannins and terpenoids (Paini *et al.,* 2014; Chikezie *et al.,* 2015).

Purification of the ethyl acetate fraction (VLC-E7, which demonostrated better anti-inflammatory and antioxidant properties compared to the extract and other fractions) led to the isolation of the

flavonoid, quercetin-3O-αL-rhamnopyranoside (Figure 14 and table 21), which may be responsible for the observed properties.

The belief that medicinal plants are natural does not guarantee their safety until they are subjected to scientific validations (Hwan *et al.,* 2013; Uma *et al.,* 2013). Despite the popular use of *D. filix-mas,* there is paucity of information on its toxicological profile. This prompted the investigation of its acute and repeated systemic toxicity tests in this study.

Chronic disorders such as rheumatoid arthritis demand repeated administration of the extract for a longer duration of time. Thus, a 14-day repeated toxicity, sub-chronic and chronic toxicity tests were carried out in this study.

Haematological parameters, as markers of toxicity are vital in knowing how test substances affect the haematopoietic system (Uma *et al.,* 2013; Otunola and Afolayan, 2017). From the sub-acute toxicity study, non-significant changes in haematological parameters (Tables 22 and 23) suggest that the extract does not have the potential to alter red blood cell production or cause bone marrow toxicity.

Most repeated toxicity studies on medicinal plants and other xenobiotic revealed that the liver and kidneys are major targets to toxic agents (Muhammad *et al.,* 2015). The liver is meant to metabolize foreign toxic agents while the kidneys aid in eliminating such metabolites from the system in active or inactive forms (OECD, 2010; Obidike *et al.,* 2011; Obidike and Salawu, 2013). This prompted the selection of the liver and kidneys as target organs for this study.

Significant increase in renal biomarkers, urea and creatinine in 1000 and 2000 mg/kg groups (Table 26) suggests that these doses may be nephrotoxic. This is corroborated by the kidney histopathology results characterized by proliferative glomerulopathy with vascular congestion (Figure 16). This toxicity may be attributed to the presence of secondary metabolites in the extract

as depicted in the phytochemistry results (Table 3). High intake of cardiac glycosides have been reported to cause renal toxicity in animals (Haden *et al.,* 2011; Chikezie *et al.,* 2015). Olivoto *et al* (2017) reported that nearly all alkaloids from plants which are responsible for nitrogen, urea and uric acid secretion in animals are known to be toxic to the kidney when ingested in large quantity. Venkateswar *et al* (2017) have reported that vinca alkaloids cause increase in urea and creatinine levels as well as kidney toxicity. Therefore, presence of cardiac glycosides, alkaloids in the extract may be responsible for its kidney toxicity.

From this study, non-significant alteration in liver biomarkers (Table 25) and non-distortion in liver cytoarchitectures (Figure 15) suggests that the liver integrity was not distorted following administration of 250, 500, 1000 and 2000 mg/kg doses of the extract for 14 days. The observed toxicity in the kidney may be due to the kidney's incapability to eliminate toxic metabolites of the extract produced by the liver. Various studies (Kristine, 2010; Suzery *et al*., 2017; Irina and Konstantin, 2017) on the mechanism of tissues regeneration following exposure to toxic agents revealed that the liver is a stable organ with high continuous regenerative and proliferative capacity as well as high resistance to toxic agents, which the kidney cannot do.

Non-significant alteration in lipid profile parameters (Table 24) indicates that the extract may not cause disorder in lipid metabolism when used for 14 days at the tested doses.

Studies by Moke *et al* (2015) revealed that evaluation of ulcerogenic properties of medicinal plants could aid in validating their safety profile relating to their long term use. Absence of distortions in gastric mucosa suggests that the extract does not have the potential to induce ulcer when used for 14 days at the tested doses.

Non-significant difference in body weight gain (Table 27) suggests that the extract does not possess appetite suppressive and weight reduction properties. Ekpenyong *et al* (2012) reported

that high level of hydrolyzable tannins (non-digestible form of tannin) could cause appetite suppression and body weight reduction when compared to condensed tannins. Thus, the extract may not contain high amount of hydrolysable tannins that could result in weight reduction.

Changes in organ weight serve as a biomarker in toxicity assessment (Muhammad *et al.,* 2015). Organ toxicity such as hypertrophy or hyperplasia may occur when certain toxicants cause serious damage to the functionality of vital organs (Otunola and Afolayan, 2017; Qu *et al.,* 2017). The non-alteration in organs weights (Tables 28) is an indication that the extract may not be associated with significant organ toxicity effect.

From the haematology results of 90 day sub-chronic toxicity test, significant increases in PCV, hemoglobin and RBC counts on the 31st day in groups treated with 250 and 500 mg/kg (Table 29) suggest that the extract could promote red blood cell production at 250 and 500 mg/kg doses following 30 days exposure. Plants secondary metabolites such as flavonoids and terpenoids have been reported to promote erythropoiesis (Osano *et al.,* 2016) and may account for the increase in PCV, RBC and hemoglobin on day 31. Non-significant alterations in PCV, hemoglobin and RBC levels on day 61 and 91 suggests that longer duration exposure to the extract may hinder further erythropoisis at 250 and 500 mg/kg doses. Long term intake of medicinal plants rich in saponins have been reported to cause hemolysis of red blood cells (Ekpenyong *et al.,* 2012). Yadav and Agarwala (2011) also revealed that the presence of high level of saponins can result in haemolysis of red blood cells.

Debelo *et al* (2016) stated that reduction in WBC level is associated with immune suppression. Thus, significant reduction in WBC and lymphocyte counts as well as significant increase in medium size cell (MID) and granulocyte counts observed at 250 and 500 mg/kg on the day 91 (Table 30) suggests that long term exposure of the animals for 90 days may result in insufficient

production of leukocytes due to bone marrow toxicity of the extract. Haemolytic effects of saponins present in the extract may contribute to the reduced leukocyte counts. Usually, reduction in total leukocyte and lymphocyte count correlates with an increase in MID and granulocyte count (Neha *et al.,* 2010). However, reduction in WBC and lymphocyte counts as well as increase in granulocyte and MID levels observed on day 91 were not observed following 28 days recovery studies, suggesting that immune suppressing effect of the extract was reversible.

Non-significant changes in liver and kidney biomarkers on days 31 and 61 (Tables 32 and 33) suggests that administration of the extract up to 500 mg/kg would not be associated with liver and kidney toxicities. This is substantiated by the photo-micrographs of liver and kidney histo-architecture on days 31 and 61(Figures 17 to 20).

From table 32, significant increase in liver enzymes (ALT, AST and ALP) on day 91 in 250 and 500 mg/kg groups suggest that the extract could be hepatotoxic following long duration of exposure. This is substantiated by the liver histopathology results characterized by congestion of the hepatic triad leading to displacement of red cells from the intravascular space to the loose connective tissues in dose dependent manner (Figure 21).

In recovery studies, significant increase in liver enzymes, as well as histopathological changes observed on day 91were not observed following withdrawal of the extract from animals (Table 32 and figure 23), suggesting that the injuries attributed to long term exposure of the extract for 90 days was reversible.

Significant increase in kidney function parameters, urea and creatinine (Table 34) in 250 and 500 mg/kg on day 91 suggests that longer duration of exposure to the extract could be associated with kidney toxicity. These changes was corroborated by the kidney histopathology results (Figure. 22) characterized by predominant glumeruli proliferation with loose Bowmans' space in the kidney in

dose dependent manner. Also, this suggests that doses below 250 mg/kg may be tolerated and safe even beyond 90 days exposure. Significant increase in kidney function parameters and kidney histopathological changes detected on the day 91 were not observed in the recovery studies (Table 34 and Figure 24), suggesting that the injuries attributed to long term exposure of the extract for 90 days was reversible.

Liver and kidney toxicities observed on day 91 of this study may be attributed to the presence of some secondary metabolites in the extract of *D. filix-mas* which have been reported to be associated with liver and kidney toxicities.

Studies by several authors have revealed that exposure to high levels of plant secondary metabolites such as tannins, saponins, glycosides and alkaloids could cause hepatorenal toxicity (Hassan *et al.,* 2007; Netala *et al.,* 2014; Louis *et al.,* 2014; Mariangela *et al.,* 2016). Builders *et al* (2012) revealed that presence of tannins in the stem bark extract of *Parkia biglobosa* may be responsible for its liver and kidney damage. Osano *et al* (2016) also revealed that saponins and tannins in methanol leaf extracts of *Prosopis juliflora* may be associated with its hepatorenal toxicity. Report has also shown that long term consumption of medicinal plant rich in flavonoids could result to auto-oxidation of reactive oxygen species, thereby causing liver and kidney toxicities (Eweka and Enogieru 2011; Namjoo *et al.,* 2013). Kidney toxicities by alkaloids have been reported due to their abilities to cause increase in urea and creatinine levels (Olivoto *et al.,* 2017; Venkateswar *et al.,* 2017). Alkaloids, especially Pyrrolizidine alkaloids have been reported to be hepatotoxic due to their interference with protein synthesis, thereby causing biochemical lesion of the hepatocytes leading to necrosis (Aksel, 2010). From this study, the presence of tannins, saponins, glycosides, flavonoids and alkaloids in *D. filix-mas* leaf extract may be responsible for the liver and kidney toxicities observed on day 91.

Reports have shown that increase in cholesterol and triglyceride levels could be associated with heart disease (Kayode *et al.,* 2016). From this study, significant reduction in total cholesterol and triglyceride levels at 250 mg/kg dose on day 61 (Table 31) suggests that 250 mg/kg dose could promote lipid lowering. Presence of saponins and flavonoids in medicinal plants have been documented to play significant roles in cholesterol and lipid lowering (Yadav and Agarwala, 2011; Builders *et al.,* 2012).

Non-significant difference in body weight gain of animals treated with various doses of the extract for 90 days (Table 35) suggests that the extract does not possess high level of hydrolysable tannins that may suppress appetite and body weight gain.

Non-significant changes in organ weight in day 91 (Tables 36) indicate that the extract may not be associated with hypertrophy or hyperplasia. Absence of ulcerations in the stomach mucosa of rats exposed to the extract for 90 days suggests that the extract may not be ulcerogenic when it is used for longer duration of 90 days.

From the results of chronic toxicity test, non-significant difference in haematology parameters (Tables 37) suggests the extract at the tested doses did not distort red blood cell production as well as immune function following its 6 months exposure to animals.

The observed significant ($p<0.05$) reduction in liver biomarkers, ALT and AST (Table 39) suggests that the extract may not be hepatoxic at the doses administered. This is supported by the absence of distortion in liver architecture (Figure 25).

Significant increase in renal function parameters, urea and creatinine (Table 41) as well as toxicity in kidney architecture characterized by glomerulonephritis of the glomerulus with acute tubular necrosis of the renal tubules in dose dependent manner (Figures 26) suggests that the extract is nephrotoxic at the tested doses following long term exposure. This may be due to

inability of animals' kidney to effectively eliminate bioacummulated waste products metabolized by the liver. Absence of kidney toxicities observed in recovery studies (Table 41 and figure 28) suggests that the toxicity posed by the extract was reversible.

These could also be attributed to the presence of secondary metabolites, saponins, tannins, flavonoids, alkaloids, cardiac glycosides which have organ related toxicities in 90 days sub-chronic toxicity studies. Hassan *et al* (2007) also revealed that some organ toxicities could occur due to the interaction of various plant secondary metabolites

Non-significant alteration in lipid profile parameters (Table 38) suggests that the extract may not alter lipid function at the tested doses and duration.

The non-significant alteration in body weight gain (Table 42) among extract treated groups compared to control group suggests that water and feed intake were not affected by the extract. Non-significant difference in organ weights (Table 43) following 6 months exposure of animals to the extract suggest the toxicity observed did not cause severe organ deformity. Also, absence of lesion on the stomach mucosa of animals suggests that the extract does not have the potential to cause ulcer at the tested doses and duration.

In this study, the teratogenic effect of *D. filix-mas* leaf extract was evaluated in order to validate its safety in pregnancy.

Progressive increase in the body weights of the pregnant mice (Table 44) could be as a result of organogenesis which resulted in weight gain. This is substantiated by reduced body weights which occurred after parturition in all groups. Study by Saleem *et al* (2014) revealed that body weight of pregnant mice increased progressively during the period of organogenesis. A non-significant difference in body weight of mice in treatment groups compared to the control group

suggests the extract may not contain anti-nutritive constituents which may contribute to nutrient malabsorption, loss of appetite and body weight reduction in pregnancy.

Non-significant change in number of littered pups, body weight of pups, tail length of pups as well as crown rump length of pups in the treatment groups (Table 45) suggests that the extract does not have deleterious effect on the development of functional parts of these organs. This is corroborated by absence of middle ear disease, polydactyl limbs, open eyes and normal general appearance observed in control and extract treated groups (Table 46).

Absence of deformity in liver, kidneys, heart and lungs architectures (Figures 29 - 32) suggests that normal development (organogenesis) of the these organs (liver, kidneys, lungs and heart) in pups were not adversly affected by the extract.

During fetal development, long bone/skeletal formation is known to start as proliferation of cartilaginous scaffold that is progressively resorbed and replaced by new bone in the epiphyseal areas, thereby undergoing mineralization to form primary new bone (Kovacs, 2011). From the histology results, photomicrograph of femur sections from all groups were actively proliferating and had mineralising cartilages, except in high dose, 1000 mg/kg (Figure 33) that showed poor mineralization, suggesting that high doses of the extract may cause less calcium depositions thereby resulting in reduced ossification rate. Thus, high doses of *D. filix-mas*, up to 1000 mg/kg may be teratogenic to the femur. Studies have revealed that phytochemicals such as flavonoids could act as agonists and antagonists of the human estrogen receptors thereby causing poor fetal bone development or bone retardation (Collins-Burrow *et al.,* 2012; Nuning *et al.,* 2017). Also, high level of flavonoids have been reported to posses several biological properties such as antiapoptosis, anti-inflammatory, antioxidant, protection and improvement of endothelial

function, antiaging, anticarcinogen, as well as protection of vital organs against damage by toxicants (Yadav and Agarwala, 2011).

CHAPTER FIVE

CONCLUSION, RECOMMENDATIONS AND CONTRIBUTION TO KNOWLEDGE

5.1 CONCLUSION

The present study evaluated the anti-inflammatory and toxicological effects of *D. filix-mas.*

Acute toxicity test revealed that the extract was non-toxic at 5000 mg/kg.

Phytochemical screening revealed the presence of tannins, phenols, flavonoids, saponins, steroids, alkaloids, terpenoids, reducing sugar and cardic glycosides in various proportions in the extract and fractions of *D. filix-mas.*

The extract, butanol and ethyl acetate fractions of *D. filix-mas* produced significant anti-inflammatory properties in egg-albumin induced paw edema, formaldehyde induced arthritis, xylene induced mouse ear topical edema, heat and hypotonic induced erythrocyte hemolysis, carragenaan induced leukocyte migration and acetic acid induced peritoneal capillary permeability. Ethyl ecetate VLC and Sephadex fractions also elicited significant anti-inflammatory properties against xylene induced mouse ear topical edema. The purified fractions, VLC-E7 selectively inhibited COX-2 enzyme.

Ethyl acetate fraction had higher total phenolic and flavonoid contents and also produced a better antioxidant activity compared to other fractions.

HPLC analysis revealed Quercetrin as the major compound in the extract and various fractions of *D. filix-mas.*

Structural elucidation of the purified ethyl acetate fraction (VLC-E7) using High Performance Liquid Chromatography mass spectrometry (HPLC-MS) and nuclear magnetic resonance (NMR) resulted to the compound, Quercetin-3O-αL-rhamnopyranoside, which may account for the anti-inflammatory activity of *D. filix-mas* in tradional medicine.

The 14 days repeated toxicity study revealed that 1000 and 2000 mg/kg doses of the extract were nephrotoxic. The 90-days sub-chronic toxicity studies revealed that 250 and 500 mg/kg doses of crude extract were hepatotoxic and nephrotoxic, but the toxicities were reversible following recovery studies. Six months chronic toxicity study also revealed that 31.23, 62.5 and 125 mg/kg doses of the extract were nephrotoxic, but the nephrotoxicity was reversible following recovery studies.

Teratogenicity study showed that high dose (1000 mg/kg) of the ethanol leaf extract of *D. filix-mas* resulted to poor bone mineralization of the pups of treated mice.

Generally, the leaf extract of *D. filix-mas* possesses anti-inflammatory and anti-oxidant properties which maybe due to its isolated compound (Quercetin-3O-αL-rhamnopyranoside) which justifies its folkloric use in the management of rheumatoid arthritis and other diseases. However, its long term use may be nephrotoxic and teratogenic.

5.2 RECOMMENDATIONS

The use of high doses of *D. filix-mas* leaf extract over time should be discouraged among populace due to it nephrotoxic potential. Caution should be exercised by pregnant women who use high doses of the extract for various ailments due to its teratogenic potential on the femur. There are some areas that this work could not explore due to lack of resources. These include;

carcinogenicity, mutagenicity and reproductive toxicity of the extract, possible mechanism(s) by which *D. filix-mas* exert liver and kidney toxicities, isolation of the bioactive compound (Quercetin-3O-αL-rhamnopyranoside) in large quantity from *D. filix-mas* for the development of a COX-2 selective anti-inflammatory drug. Studies to evaluate the pharmacokinetics and pharmacodynamics mechanism(s) which could have been involved in the observed anti-inflammatory properties of *D. filix-mas* leaf extract should be carried out.

5.3 CONTRIBUTION TO KNOWLEDGE

This study has validated the ethnomedicinal use of *D. filix-mas* in the management of inflammatory conditions.

Anti-inflammatory properties of *D. filix-mas* has been reported for the first time in this study using various models.

This plant could also be of importance for the development of anti-inflammatory agents with COX- 2 inhibitory mechanism.

The active compound, Quercetin-3O-αL-rhamnopyranoside responsible for its bioactivities was elucidated and reported for the first time in this study.

Safety profile (acute and long term toxicity and teratogenicity profiles) was also established on this plant for the first time.

REFERENCES

Abdullatif, A., Ahmad, N., and Abed, N.A (2016). Anti-Inflammatory activity of natural products. *Molecules*. 21, 1321: 2-19.

Adi, A.H. and Alturkmani, H.J. (2013). Physiologically lucky: the role of medical physiology in modern medical education: an interview. *Perspectives* on *Medical Education*; 2: 99–103.

Ahlem, R., Souad, I.B., Béatrice, B., Valérie, M.L., Fathi, M., Evelyne, O., Jamila, K.C. and Malika, T.A. (2015). Total phenolic, total flavonoid, tannin content, and antioxidant capacity of *Halimium halimifolium* (Cistaceae). *Journal of Applied Pharmaceutical Science*. 5 (1): 52-57.

Ahmed, M. (2015). Acute toxicity (lethal dose 50 calculation) of herbal drug, Somina in rats and mice. *Pharmacology and Pharmacy.* 6: 185-189.

Ajaghaku, D.L., Ilodigwe, E.E., Okonta, M.J., Ogue, C.O., Okafor, M.A. and Igboeme, S.O. (2013). Mechanism of anti-inflammatory activity of the leaf extract and fractions of *Millettia aboensis. International Research Journal of Pharmacy*. 4(9):54-59.

Ajaghaku, D.L., Obasi, O., Umeokoli, B.O., Ogbuatu, P., Nworu, C.S., Ilodigwe, E.E., and Okoye, F.B.C. (2017). *In vitro* and *in vivo* antioxidant potentials of *Alchornea floribunda* leaf extract, fractions and isolated bioactive compounds. *Avicenna Journal* of *Phytomedicine*; 7 (1): 80-92.

Aksel, B. (2010). Bioactive compounds in plants – benefits and risks for man and animals. Proceedings from a symposium held at The Norwegian Academy of Science and Letters, Oslo, 13 – 14 November 2008 Novus forlag, Oslo 2010. (http://www.dnva.no/geomed); http://www.dnva.no/binfil/download.php?tid=48677. Accessed on 21 December, 2017.

Ambriz-Pérez, D.L., Leyva-López, N., Gutierrez-Grijalva, E.P., and Heredia J.B. (2016). Phenolic compounds: Natural alternative in inflammation treatment. A Review *Cogent Food and Agriculture.* 2: 1131412.

Anamika, P., Naveen, G., Anoop, S., Arun, K. and Singh, A.P. (2017). A review on ethno-medicinally important species of Pteridophytes. *International Journal of Current Science Research*. 3(3):1186-1201.

Ananya, C. and Sandip, K.B (2014). Herbal remedy: An alternate therapy of nonsteroidal anti-inflammatory drug induced gastric ulcer healing. Ulcer. Hindawi Publishing Corporation. Volume 2014, Article ID 361586, 13 pages.

Anoop, M.V. and Bindu, A.R. (2015). *In-vitro* anti-inflammatory activity studies on *Syzygium zeylanicum* (L.) DC leaves. *International Journal of Pharma Research & Review*; 4(8):18-27.

Anosike, C.A. and Obidoa, O. (2010). Anti-inflammatory and anti-ulcerogenic effect of ethanol extract of Coconut (*Cocos Nucifera*) on experimental rats**.** *African Journal of Food, Agriculture, Nutrition and Development.* 10 (10):4286-4300.

Arowora, K.A, Imo, C., Ezeonu, C.S, and Muhammad, Z.I. (2016). Effects of ethanolic extracts of *Datura metel* on blood lipid profile of male albino rats. *International Journal of Scientific Reports*; 2(10):248-52.

Arsad, S.S, Mohd, E.N., Hamzah, H., and Othman, F. (2013). Evaluation of acute, subacute and subchronic oral toxicity of *Rhaphidophora decursiva* (Roxb.) Schott extract in male Sprague Dawley rats. *Journal of Medicinal Plant Research*; 7: 3030-40.

Arya, V. and Arya, M.L. (2011). A review on anti-inflammatory plant barks. *International Journal of Pharm Tech Research. 3*, 899–908.

Asma, R., Majid, M., Ghader, J., Shahram, J., Masoud, M., Daryosh, M. and Mohammand, R.R. (2016). Acute and subchronic toxicity study of the median septum of *Juglans regia* in Wistar rats. *Advanced* pharmaceutical bulletin. 6(4): 541–549.

Auriel, E., Regev, K. and Korczyn, A.D. (2014). Nonsteroidal anti-inflammatory drugs exposure and the central nervous system. *Handbook of Clinical Neurology.* 119, 577–584.

Azubike, N.C., Okwuosa, C.N., Achukwu, P.U., Maduka, T.C., and Chike, O. (2015). Acute toxicity and histopathological effects of crude aqueous extract of *Jatropha curcas* leaves in mice. *Research Journal of Medicinal Plant.* 9 (7): 340-346.

Bader, G.N., Mir, P.A., and Bhat, Z.A. (2014). Present status of antiinflammatory and antirheumatic phytoconstituents: A review. *World Journal of Pharmacy and Pharmaceutical Sciences*. 3 (11): 272-310.

Bafor, E.E., Omokaro, W.O., Uwumarongie, O.H., Elvis-Offiah, U.B., Omoruyi, O, Viegelmann, C.V. and Edrada-Ebel, R. (2017). *D. filix-mas* (Dryopteridaceae) leaves inhibit mouse uterine activity. *Journal of Medicinal Plants for Economic Development*. 1(1):1-12. https://www.jomped.org/index.php/jomped/article/view/25/57. Accessed January 21st, 2018.

Bancroft, J.D. and Gamble, M. (2002). Theory and practice of histological techniques. Churchill Livingstone, Edinburgh. 16-64.

Bamgbose S.O.A, Noamesi B.K (1981). Studies on cryptolepine II: inhibition of carrageenan-induced oedema by cryptolepine. Planta Medica. 42: 392-396.

Bashir, L., Oluwatosin, K.S., Agboola, A.R., Ibrahim, A.O., Adeniyi, A.K and Prince C.O. (2015). Effect of methanol extract of *Telfairia occcidentalis* on haematological parameters in Wister rats. *Journal of Medical Science*. 15 (5): 246-250.

Batsatsashvili K., Mehdiyeva N., Kikvidze Z., Khutsishvili M., Maisaia, Sikharulidze S., Tchelidze, D., Alizade V., Narel Y. Zambrana, P. and Rainer W. (2016). *Dryopteris filix – mas* (L) Schott Dryopteridaceae. Bussmann Springer International Publishing AG. R.W. Bussmann (ed.), Ethnobotany of the Caucasus, European Ethnobotany, DOI 10.1007/978-3-319-50009-6_87-1. Accessed on July 5th, 2018.

Beber, A.P., de Souza, P., Boeing, T., Somensi, L.B., Mariano, L.N., Cury, B.J., Burci, L.M., da Silva, C.B., Simionatto, E., de Andrade, S.F, and da Silva, L.M. (2017). Constituents of leaves from *Bauhinia curvula* Benth. exert gastroprotective activity in rodents: role of quercitrin and kaempferol. Inflammopharmacology.

https://www.ncbi.nlm.nih.gov/m/pubmed/28176198/#. doi: 10.1007/s10787-017-0313-8. [Epub ahead of print]. Accessed on December 22nd, 2017.

Berinyuy, E.B, Lawal, B., Olalekan, I.A., Olalekan, A.A., Yusuf, S.S. and Ossai, P.C. (2015). Hematological status and organs/body-weight parameters in Wister rats during chronic administration of *Cassia occidentalis*. *Inter. Blood Res. Rev*. 4: 1-7.

Brender, J.D., Felkner, M., Suarez, L., Canfield, M.A., and Henry, J.P. (2010). Maternal pesticide exposure and neural tube defects in Mexican Americans. *Annals of Epidemiology*. 20 (1):16-22.

Builders, M.I., Isichie, C.O., and Aguiyi, J.C. (2012). Toxicity studies of the extracts of *Parkia biglobosa* stem bark in rats. *British Journal of Pharmaceutical Research.* 2(1): 1-16.

Chang, X., Li, W., Koike, K., Wu, L. and Nikaido, T. (2006). Phenolic constituents from the rhizomes of *Dryopteris crassirhizoma*. *Chemical and Pharmaceutical Bulletin*. 54, 748–750.

Chatchai, C., Songpol, C., Anan, C. and Wanchai, D. (2013). Acute and chronic oral toxicity of a partially purified Plaunotol extract from *Croton stellatopilosus* Ohba. Hindawi publishing Corporation. *Biomed Research International.* Vol 2013. Article ID 3 03162, 12 pages.

Chikezie, P.C., Ibegbulem, C.O. and Mbagwu, F.N. (2015). Medicinal potentials and toxicity concerns of bioactive principles. *Med Aromat Plants.* 4 (3):1-15.

Chirumbolo, S. (2010). "The role of quercetin, flavonols and flavones in modulating inflammatory cell function," *Inflammation and Allergy Drug Targets*. 9(4):263–285.

Chivapat, S., Chavalittumrong, P., Phisalpong, P.W.C. and Rungsipipa, A. (2011). Chronic Toxicity Study of *Garcinia mangostana* Linn. pericarp Extract. *Thai Journal* of *Veterinary Medicine.* 41(1): 45-53.

Chukwuma, B.D., Kenechi, A.U., Nnebue, C.C., Ikechukwu, I.M., Kevin, C.D., Chuka, C.A., Anthony, C.I., and Irene, A.M. (2016). "Socio-demographic determinants of herbal

medicines use in pregnancy among Nigerian women attending clinics in a tertiary hospital in Imo State, South-East, Nigeria". *American Journal of Medicine Studies*. 4(1): 1-10.

Collins-Burow, B.M., Antoon, J.W., Frigo, D.E., Elliott, S., Weldon, C.B., Boue, S.M., Beckman, B.S., Curie, T.J., Alam, J., McLachlan, J.A. and Burow, M.E. (2012). Antiestrogenic activity of flavonoid phytochemicals mediated via the c-Jun N-terminal protein kinase pathway: Cell-type specific regulation of estrogen receptor alpha. *Journal* of *Steroid Biochemistry* and *Molecular Biology*. 132(0): 186–193.

Costa, K.C., Bezerra, S.B., Norte, C.M., Nunes, L.M.N. and Olinda, T.M. (2012). Medicinal plants with teratogenic potential: current considerations. *Brazilian Journal of Pharmaceutical Sciences*. 48 (3):427-433.

Cruz, M.P., Andrade, C.M.F., Silva, K.O., de Souza, E.P., Yatsuda, R., Marques, L.M., Juceni, P., David, J.P., David, J.M., Napimoga, M.H. and Clemente-Napimoga, J.T. (2016). Antinoceptive and anti-inflammatory activities of the ethanolic extract, fractions and flavones isolated from *Mimosa tenuiflora* (Willd.) Poir (Leguminosae). PLoS ONE 11(3): 1-29.

D'Mello, P., Gadhwal, M. and Joshi, U. (2011). Modeling of COX-2 inhibitory activity of flavonoids. *International Journal of Pharmacy and Pharmaceutical Sciences.* 3, 33–40.

Dai, X., Ding, Y., Zhang, Z., Cai, X. and Li, Y. (2013). Quercetin and quercitrin protect against cytokine-induced injuries in RINm5F β-cells via the mitochondrial pathway and NF-κB signaling. *International Journal of Molecular Medicine*. 31(1):265-71.

De Barros, D.M., Mota da, S.L., Boeing, T., Somensis, L.B., Cury, B.J., De Moura, B.L., Santin, J.R., De Andrade, S.F., Monache, F.D. and Cechinel-Filho, V. (2016). Pharmacological reports about gastroprotective effects of methanolic extract from leaves of *Solidago chilensis* (Brazilian arnica) and its components quercitrin and afzelin in rodents. *Naunyn Schmiedeberges Arch Pharmaco*l. 389 (4): 403-417.

Debelo, N., Afework, M., Debella, A., Makonnen, E., and Ergete, W. (2016). Assessment of hematological, biochemical and histopathological effects of acute and sub-chronic

administration of the aqueous leaves extract of *Thymus schimperi* in rats. *Journal of Clinical Toxicology*. 6(2); 1-9.

Deepa, M. and Renuka, D. (2014).Potent anti-inflammatory medical plant-a review. *International Journal of Pharmacy and Pharmaceutical Sciences*. 6 (4):43-49.

Dehkharghanian M, Adenier H, and Vijayalakshmi MA. (2010). Analytical methods study of flavonoids in aqueous spinach extract using positive electrospray ionization tandem quadrupole mass spectrometry. *Food Chemistry*; 121: 863–870.

Doughari, J.H. (2012). Phytochemicals: extraction methods, basic structures and mode of action as potential chemotherapeutic agents, phytochemicals - A global perspective of their role in nutrition and health, Dr Venketeshwer Rao (Ed.), ISBN: 978-953-51-0296-0, InTech, Available from on www.intechopen.com. Acceessed January 15th, 2018.

Doumas, B.T., and Biggs, H.G. (1976). Standard methods of clinical chemistry, Academic Press, N.Y. 7:175.

Ekpenyong, C.E., Akpan, E.E. and Udoh, N. S. (2012). Phytochemistry and toxicity studies of *Telfairia Occidentalis* aqueous leaves extract on liver biochemical indices in Wistar rats. *American Journal of Medicine and Medical Sciences*. 2(5): 103-110.

Ekwere, O.E., Tamunotonye, W.J., Iornumbe, U., Ikoni, O. and Francis, K.O. (2011). Possible embryotoxic and teratogenic effect of a phytodrug (RICOM 13-J) on pregnant female Wistar rats. *Journal of Physics*: *Conference Series* (JPCS). 2:16-19.

Elaine, C. and Stanley, M. (2016). Evaluation of in vitro anti-inflammatory and antioxidant activity of selected Zimbabwean plant extracts. *Journal of herbs, spices and medicinal plants*. 22 (2): 157–172.

Eluwa, M.A., Udoaffah, M.T., Vulley, M.B.G., Ekanem, T.B., Akpantah, A.O., Asuquo, O.A., and Ekong, M.B. (2010). Comparative study of teratogenic potentials of crude ethanolic root bark and leaf extract of *Rauwolfia vomitoria* (apocynaceae) on the fetal heart. *North American Journal of Medical Science*. 2: 592-595.

Enegide C, David A, Fidelis SA. (2013). A new method for determining acute toxicity in animal models. *Toxicology International*. 20(3): 224–226.

Eweka, A.O. and Enogieru, A. (2011). Effects of oral administration of *Phyllanthus amarus* leaf extract on the kidney of adult Wistar rats- A histological study. *African Journal of Traditional, Complementary and Alternative Medicine*. 8(3):307- 311.

Ezeja, M.I., Yusuf, N.O., Samuel, O.O. and Ijeoma, H.U. (2015). Antiinflammatory and antioxidant activities of the methanolic leaf extract of C*issus aralioides*. *American Journal of Pharmacological Sciences*. 3 (1): 1 -6.

Friedewald, WT., Levy, RI. and Fredrickson, DS. (1972). Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative centrifuge. *Clinical Chemistry*. 18:499—500.

Fulda, S. (2012). "Regulation of cell death and survival by resveratrol: implications for cancer therapy," *Anti-Cancer Agents in Medicinal Chemistry*. 12(8): 874–879.

Gambhire MN, Wankhede SS, Juvekar AR. (2009). Antiinflammatory activity of aqueous extract of *Barleria cristata* leaves. *Journal of Young Pharmacists*. 1(3): 220-224.

Genesh, G., Saurabhm M. and Saradam N.C. (2013). Antioxidant and anti-inflammatory activities of the methanolic leaf extract of traditionally used medicinal plants *Mimusops elengi L*. *Journal of Pharmaceutical Science and Research*. 5(6); 125-130.

George, A., Chinnappan, S., Chintamaneni, M., Kotak, V.C., Choudhary, Y., Kueper, T. and Radhakrishnan, A.K. (2015). Anti-inflammatory effects of *Polygonum minus (Huds)* extract (Lineminus™) in in-vitro enzyme assays and carrageenan induced paw edema. *BMC Complementary and Alternative Medicine*. 14: 355:1-7.

Gepdireman, A., Mshvildadze, V., Suleyman, H. and Elias, R. (2005). Acute anti-inflammatory activity of four saponins isolated from ivy: Alphahederin, hederasaponin-C, hederacolchiside-E and hederacolchisideF in carrageenan-induced rat paw edema. *Phytomedicine*. 12(6-7): 440-444.

Ghaly NS, Melek FR, and Abdelwahed NAM. (2010). Flavonoids from *Albizia chinensis of* Egypt. *Revista. Latinoamericana. Química.* 38(3): 153-158.

Gholami, M., Etemad, L., Moallem, S.A., Afshar, M., and Karimi, GH. (2016). Teratogenic effects of Silymarin on mouse fetuses. *Avicenna Journal of Phytomedicine*, 6 (5): 542-549.

Gitelman, H.J. (1976). An improved automatic procedure for the determination of calcium in biologic specimens. *Anal of Biochemistry.* 18: 521-31.

Goodman, L., Gilman, A., Brunton, L., Lazo, J. and Parker, K. (2006). *Goodman & Gilman's the Pharmacological basis of therapeutics*. New York: McGraw-Hill. Chapter 64, pg. 1743-1745.

Golalipour, M.J., Ghafari, S., Maleki, A., Kiani, M., Asadi, E. and Farsi, M. (2011). Study of embryotoxicity of *Mentha piperita* L. during organogenesis in Balb/c Mice. *International Journal of Morphology.* 29(3):862-867.

Habibur, R., Manjula, K., Anoosha, T., Nagaveni, K., Chinna, M.E. and Dipankar, B. (2013). *In-vitro* antioxidant activity of *Citrulus lanatus* seed extracts. *Asian Journal of Pharmaceutical and Clinical Research.* 6 (3): 152-157.

Harborne J.B (1973). Phytochemicals methods. London: Chapman and Hall; 1973. p.49-188.

Haden, M., Marshall, D.A.S. and Murphy, B. (2011). Toxic levels of glycosides in herbal medication: A potential cause of hyperkalaemia. *Scottish Medical Journal*. 56: 236-236.

Han, X., Lia, Z., Lic, C, Jiaa, W., Wangd, H. and Wang, C. (2015). Phytochemical constituents and biological activities of plants from the genus Dryopteris. *Chemistry and Biodiversity*. 12:1131-1162.

Hassan, S.W., Ladan, M.J., Dogondaji, R.A., Umar, R.A., Bilbis, L.S., Hassan, L.G., Ebbo, A.A. and Matazu, I.K. (2007). Phytochemical and toxicological studies of aqueous leaves extracts of *Erythrophyleum africanum. Pakistan Journal of Biological Science.* 10 (21): 3815-3821.

Heinegard, D. and Tiderstrom, G. (1973). Determination of serum creatinine by a direct colorimetric method Clinica *Chimica Acta.* 43: 305.

Hema, K. (2014). *In-vitro* anti-inflammatory activity of Quercitrin isolated from *Allamanda cathrartica Linn. International Journal of Pharma and Bioscience*. 5(4):440-445.

Herrera, A.A., King, R.E.C. and Ipulan, L.A.D.G. (2011). Effects of oral administration of crude leaf extracts of *Aglaia loheri* Blanco and *Ardisia pyramidalis* (Cav.) Pers on mouse embryo morphology and maternal reproductive performance. *Journal of Medicinal Plants Research.* 5(16):3904-3916, 18.

Hill, M.A. (2018). Embryology timeline human development. Retrieved from https://embryology.med.unsw.edu.au/embryology/index.php/Timeline_human_development. Accessed on May 22nd, 2018.

Hosseinzadeh, H. and Younesi, H.M. (2002). Antinociceptive and anti-inflammatory effects of *Crocus sativus* L. stigma and petal extracts in mice. *BMC Pharmacology* 2(1):7:1-8.

Hwang, S.W., Lee, J., Shin, J., Lee, J.Y., Lee, K. and Jang, D.S. (2012). Inhibitory effects of phenylpropanoids isolated from the bark of *Ailanthus altissima* on COX-2 activity. *Bull Korean Chemical Society.* 33(8): 2759-61.

Hwang, Y.H., Ha, H., and Ma, J.Y (2013). Acute oral toxicity and genotoxicity of *Dryopteris crassirhizoma. Journal of Ethnopharmacology.* 149 (1); 133-139.

Igbe, 1., Innih, S., Ogedengbe, V. and Amamina, L. (2015). Chronic toxicity studies of squeous leaf extract of *Voacanga africana* in Wistar rats. *Journal of Applied Science and Environmental Management*. 19 (4): 639-646.

Ihekwereme CP, Melidem CO, Maduka IC, and Okoyeh JN (2018). *In Vivo* antiplasmodial and toxicological effects of extracts of fruit pulp of *Chrysophyllum albidium* G. Don (Sapotaceae). *Tropical Journal of Natural Product Research.* 2(3):126-131.

Ikonone, C.E.O., Akinloye, O.A, Ugbaja, R.N., Omotainse, S.O., Ajayi, O.L and Shopenin, T.M. (2017). Effect of sub-acute exposure to Bonny light crude oil on plasma biochemistry and liver histopatholgy of albino rats. *Animal Research International*. 14(1): 2652 – 2659.

Ilavarasana, R., Mallikab, M., and Venkataramanc, S. (2005). Anti-inflammatory and antioxidant activities of *Cassia fistula linn bark extract. African Journal of Traditional Complementary and alternative medicine.* 2 (1): 70 – 85.

Imazio, M., Brucato, A., and Cemin, R. (2013). "A randomized trial of colchicine for acute pericarditis," *The New England Journal of Medicine*. 369(16):1522–1528.

Irina, V.K and Konstantin, N.Y. (2017). Cellular mechanisms of liver regeneration and cell-based therapies of liver diseases. Hindawi. *BioMed Research International*. Volume 2017, Article ID 8910821, 17 pages.

Islam, T., Das, A., Shill, K.B., Karmakar, P., Islam, S., and Sattar, M.M. (2015). Evaluation of membrane stabilizing, anthelmintic, antioxidant activity with phytochemical screening of methanolic extract of *Neolamarckia cadamba* fruits. *Journal of Medicinal Plant Research*. 9 (5): 151-158.

Jacobs, A. C. and Hatfield, K. P. (2012). History of chronic toxicity and animal carcinogenicity studies for pharmaceuticals. *Veterinary Pathology.* 50(2) 324-333.

Jaijoy, K., Soonthornchareonnon, N., Lertprasertsuke, N., Panthong, A., and Sireeratawong, S. (2010). Acute and chronic oral toxicity of standardized water extract from the fruit of *Phyllanthus emblica* Linn. *International Journal of Applied Research in Natural Products*. 3:48–58.

Jeong, H.J., Ju, S.K., Sam, S.K., Kun, H.S., Hyun, W.C. and Hyun, P.K. (2010). Anti-inflammatory and anti-arthritic activity of total flavonoids of the roots of *Sophora flavescens. Ethnopharmacology*. 127: 589–595.

Jigam, A.A, Muhammad, H.L, Adefolalu, F.S, Abdulkadir, A. and Jimoh, T. (2011). Effects of crude root extract of *Acacia nilotica* in mice. *International Journal* of *Applied Biological Research*. 3: 56 – 68.

José, M.B., Márcia, R.P., Marcelo, D.M., Marcelo, S.S., Karla, V.B.L., Emídio, V.L., Ivana, M.F. and Orlando, S.T. (2006). Anti-inflammatory activity of alkaloids: A twenty-century review. *Brazilian Journal of Pharmacognosy*. 16(1): 109-139.

Joseph, J Bindhu, A.R. Aleykutty, N.A. (2013). *In vitro* and *in vivo* antiinflammatory activity of *Clerodendrum paniculatum* Linn. leaves *Indian Journal of Pharmaceutical Science*. 75(3): 376–379.

Kanashiro, A., Souza, J.G., Kabeya, L.M., Azzolini, A.E. and Lucisano-Valim, Y.M. (2007). Stimulet neutrophils inhibited by flavonoids: Importance of the catechol group. *Z. Naturforsch*. 62:357–361.

Kayode, A.A., Chinedu, I., Chukwuma, S.E., and Zuhairah, I.M. (2016). Effects of ethanolic extracts of *Datura metel* on blood lipid profile of male albino rats. *International Journal of Scientific Reports*. 2 (10): 248-252.

Khalida, P., Muhammad, R., Nighat, R. and Naheed, K. (2012). Comparison of body weight, absolute and relative weight of pituitary gland in Carbimazole and Carbimazole thyroxin treated male albino rats. *Pakistan Journal of Pharmacology*. 29 (1):17-23.

Khatoon, M., Islam, E., Islam, R., Rahman, A.A, Alam, A.H.M.K., Khondkar, P., Rashid, M. and Parvin, S. (2013). Estimation of total phenol and in vitro antioxidant activity of *Albizia procera* leaves. *BMC Research Notes*. 6:121. 1-7.

Kovacs, C.S. (2011). Bone development in the fetus and neonate: role of the calciotropic hormones. *Current Osteoporosis Reports*. 9(4): 274-283.

Kristine, P.K. (2010). Tissue repair. The hidden drama. *Organogenesis* 6:4, 225-233.

Kumar, S., Bajwa., B.S., Kuldeep, S., and Kalia, A.N. (2013). Anti-inflammatory activity of herbal plants: A review. *International Journal of advances in Pharmacy*, *Biology* and *Chemistry*, 2, 272–281.

Kumar, S., Sandhir, R. and Ojh, S. (2014). Evaluation of antioxidant activity and total phenol in different varieties of *Lantana camara* leaves. *BMC Research Notes*. 7:560.1-7.

Kumar, V., Abbas, A.K., Fausto, N., and Aster, J.C. (2010). Tissue renewal, regeneration and repair. In: Robbins and Cotran, eds. Pathologic Basis of Disease. Eighth ed. Philadelphia: Elsevier; 79-110.

Küpeli E, Koşar M, Yeşilada E, Hüsnü K, and Başer C. (2002). A comparative study on the anti-inflammatory, antinociceptive and antipyretic effects of isoquinoline alkaloids from the roots of Turkish Berberis species. *Life Science*. 72 (6):645-57.

Laudato, M., and Capasso, R. (2013). Useful plants for animal therapy. *OA Alternative Medicine*. 1(1):1-6.

Lee, J.C., Hou, M.F., Huang, H.W., Chang, F.R., Yeh, C.C. Tang, J.Y. and Chang, H.W. (2013). Marine algal natural products with anti-oxidative, anti-inflammatory, and anti-cancer properties. *Cancer cell International.* 13 (1):13-55.

Li, H., Song, Z.J., Dai, Y.P., Zhang, S.L., He, X., Guo, C.R., Zhang, W.J., Wang, J.Y., Zhang, C.F., Wang, C.Z. and Yuan, C.S. (2015). Antioxidative activity of flavonoids from *Abrus cantoniensis*against ethanol-induced gastric ulcer in mice. *Planta Medica*. 81 (10): 784-790.

Louis, A.A., Christian, A.E.K., Patrick, G.A. and Kwesi, B.M (2014). Toxicity assessment of *Erythrophleum ivorense* and *Parquetina nigrescens. Toxicology Reports*. 1: 411-420.

Lu, C., Zhang, H.Y., Ji, J., and Wang, G.X., (2012). In vivo anthelmintic activity of *Dryopteris crassirhizoma*, *Kochia scoparia*, and *Polygala tenuifolia* against *Dactylogyrus intermedius* (Monogenea) in gold*f*ish (Carassius auratus). *Parasitology Research*. 110, 1085–1090.

Luiz LS, Wagner V, and Anne LD. (2013). Characterization of flavonoids and phenolic acids in *Myrcia bella* Cambess. Using FIA-ESI-IT-MSn and HPLC-PAD-ESI-IT-MS combined with NMR. *Molecules*. 18, 8402-8416.

Madhavi D.L., Deshpande, S.S., and Salunkhe D.K. (1995). Food antioxidants: Technological: toxicological and health perspectives. Technology & Engineering - CRC Press, ISBN, 082479351X, 9780824793517, 512 pages.

Madu, E.P. (2015). Teratogenic and embryotoxic effects of orally administered Cypermethrin in pregnant albino rats. *Journal of Toxicology and Environmental*. 7(7), pp. 60-67.

Main I.H.M and Whittle B.J.R (1975). Potency and selectivity of methyl analogues of prostaglandin E2 on rat gastrointestinal function. *British Journal of Pharmacology*. 54(3) 309-317.

Mandal A. and Mondal A.K. (2011). Studies on antimicrobial activities of some selected ferns and lycophytes in Eastern India with special emphasis on ethno-medicinal uses. *African Journal of Plant Science.* 5(7): 412-420.

Marc, E.B, Nelly, A., Annick, D.D. and Frederic, D. (2008). Plants used as remedies antirheumatic and antineuralgic in the traditional medicine of Lebanon. *Journal of Ethnopharmacology* 120: 315–334.

Mariangela, M., Filomena, C., Fabrizio, A. and Giancarlo, A.S. (2016). Effects of Saponins on Lipid Metabolism: A review of potential health benefits in the treatment of obesity. *Molecules.* 21 (10):1404; 1-20.

Martinez-Vazquez, M., Ramirez-Apan, T.O., Lazcano, M.E. and Bye, R. (1999) Antiinflammatory active components from n-Hexane extract of Euphorbia hirta. *Journal of the Mexican Chemical Society.* 43:103-105.

Mei, F.S. and Jong, Y.C (2012). Potential Applications of Euphorbia hirta in Pharmacology, Drug Discovery Research in Pharmacognosy, Prof. Omboon Vallisuta (Ed.), ISBN: 978-953-51-0213-7, InTech, Available from: http://www.intechopen.com/books/drug-discovery-

research-in-pharmacognosy/potentialapplications-of-euphorbia-hirta-in-pharmacology. Accessed December 27th, 2017.

Mezui, C., Tchinda, F.S.H., Amang, A.P., Malepa, M.G.B and Tan, P. (2017). Analgesic and non-ulcerogenic activities of the aqueous extract of the aerial parts of *Eremomastax speciosa* Hochst (Acanthaceae) in mice. *Journal of Medicinal Plants Studies*. 5(2): 6-10.

Middleton, E.J.R., Kandaswami, C. and Heoharides, T.C. (2000). The effect of plant flavonoids on mammalian cells, Implication for inflammation, heart disease and cancer. *Pharmacological Reviews*. 52:673-751.

Min, Z., Jianguo, C., Xiling, D., Xuefei, C. and Quanxi, W. (2012). Flavonoid contents and free radical scavenging activity of extracts from leaves, stems, rachis and roots of *Dryopteris erythrosora. Iranian Journal of Pharmaceutical Research*. 11 (3): 991-997.

Mir, S.A., Jan, Z., Mir, S., Dar, A.M. and Chitale, G. (2017). A concise review on biological activity of *Tridax procumbens* Linn. *Organic Chemistry Current Research.* 6:177: 1-4.

Mitul, P.M. and Shivalinge, G.K.P. (2012). "*In Vivo* animal models in preclinical evaluation of anti-Inflammatory activity- A Review". *International Journal of Pharmaceutical Research and Allied Sciences*.1(2):1-5.

Moein, S., Moein, M., Khoshnoud, M.J., and Kalanteri, T. (2012). *In vitro* antioxidant properties evaluation of 10 Iranian medicinal plants by different methods. *Iran Red Crescent Medical Journal*. 14 (12):771-775.

Mohini, A.P., Manohar, J.P, Konde, A.P.D.C. and Bhoomi, P. (2012). *In-vivo* and *in-vitro* screening of medicinal plants for their anti-inflammatory activity: an overview. *Journal of Applied Pharmaceutical Science*. 2 (6): 19-33.

Moke, E.G., Ilodigwe, E.E. and Erhirhie, O.E (2015). Evaluation of the ulcerogenic potential of the aqueous extract of *Spondias mombin* and *Custus afer. International Journal of Advances in Pharmacy, Biology and Chemistry.* 4(2): 282-286.

Mona, S. M., Wadah, J.A.O., Elrashied, A.E.G., Zuheir, O., Bashier, O., Hassan, S.K. and Magdi, A.M. (2014). Secondary metabolites as anti-inflammatory agents. *The Journal of Phytopharmacology*. 3(4): 275-285.

Mondal, S., Ghosh, G., Anusuri, K.C., and Ganapaty, S. (2017). Toxicological studies and assessment of pharmacological activities of *Abrus precatorius* L. (Fabaceae) ethanolic leaves extract in the management of pain, psychiatric and neurological conditions: An *in-vivo* study. *Journal of Applied Pharmaceutical Science*. 7 (2):207-216.

Muhammad, K., Mohd, S.M., Pinaki, S., Moklesur, R.S., Arindam, D. and Sreemoy, K.D. (2015). Evaluation of the acute and sub-acute toxicity of the ethanolic extract of *Pericampylus glaucus* (Lam.) Merr. in BALB/c mice. *Journal of Acute Disease*. 4(4): 309–315.

Munawaroh, H.S.H., Nandiyanto, A.B.D., Gumilar, G.G., Widi, A. and Subangkit, M. (2017). Effect of oral administration of tungsten trioxide (WO3) particles on hispathological feature of liver and kidney in Rat. 1st annual applied science and engineering conference. IOP Conf. Series: Materials Science and Engineering 180 (2017) 012097.

Murugan, R. and Parimelazhagan, T. (2014). Comparative evaluation of different extraction methods for antioxidant and anti-inflammatory properties from *Osbeckia parvifolia* Arn. – An *in vitro* approach. *Journal of King Saud University – Science*. 26, 267–275.

Maruna, R.F.L. (1958). Colorimetric determination of sodium in human serum and plasma. Clinica *Chimica Acta,* 2: 581.

Musila, M.N, Ngai, D.N, Mbiri, J.W., Njagi, S.M, and Mbinda, W.M. (2017). Acute and sub-chronic oral toxicity study of methanolic extract of *Caesalpinia volkensii* (Harms). *Journal of Drug Metabolism and Toxicology*. 8 (1): 1-8. 222.

Mutoh, M., Takahashi, M., Fukuda, K., Komatsu, H. and Enya, T., (2000) Suppresion by flavonoids of cyclooxygenase-2 promoter-dependent transcriptional activity in colon cancer cells: structure-activity relationship. *Jpn J Cancer Res* 91: 686-691.

Namjoo, A.R., MirVakili, M., Shirzad, H. and Faghani, M. (2013). Biochemical, liver and renal toxicities of *Melissa oficinals* hydroalcoholic extract on balb/C mice. *Journal of HerbMed Pharmacology*. 2(2): 35-40.

NCCLS Approved Standard (1979). Specification for National Committee for Clinical Laboratory Standard.771 E Loncaster Ave., Villanova: 19 -85.

National Institute of Health Consensus Development Conference Statement (NIHCDCS), (1992). Triglyceride, high density lipoprotein and coronary heart disease. Washington D.C. Feb 26-28.

Neha, Y., Priyanka, S., Sitara, J., Suruchi, K., Sarita, K., Sushma, K.V., Bharti, S.R. and Sinha, M.P. (2010). Effect of methanolic extract of *Parthenium hysterophorus* L. on haematological parameters in Wistar albino rat. *The Bioscan*. 2: 357 -363.

Netala, V.R., Ghosh, S.B., Bobbu, P., Anitha, D. and Tartte, V (2014). Triterpenoid saponins: A review on biosynthesis, applications and mechanism of their action. *International Journal of Pharmacy and Pharmaceutical Sciences.* 7:24–28.

Ngo, Q.M., Tran, P.T., Tran, M.H., Kim, J.A., Rho, S.S., Lim, C.H., Woo, M.H., Choi, J.S., Lee, J.H. and Min, B.S. (2017). Alkaloids from *Piper nigrum* exhibit antiinflammatory activity via activating the Nrf2/HO-1 pathway. *Phytotherapy Research.* (4):663-670.

Nicholas, S. and Nicole, T. (2016). Non-Steroidal anti-Inflammatory drugs and Aspirin therapy for the treatment of acute and recurrent idiopathic pericarditis. *Pharmaceuticals*. 9(17):2-18.

Nuning, N., Yan, W., Jamsari, A., Djong, H.T and Mohammad, K. (2017). Methanol plant extract of Rumput Teki (*Cyperus rotundus L.*) causing fetal skeletal retardation in mice. *European Journal of Biomedical and Pharmaceutical Sciences*. 4(6): 128-131.

Nwabunike, I. A., Ezike, A. C., Nwodo, N. J., Udegbunam, S. U. and Okoli, C.O. (2014). Bioactivity-guided studies on the anti-inflammatory activity of extract of aerial parts of

Schwenckia americana L. (Solanaceae). *Journal of Medicinal Plant Research*. 8(22):794-801.

Nworu, C.S., Nwuke, H.C., Akah, P.A, Okoye, F.B.C and Esimone, C.O. (2012). Extracts of *Ficus exasperate* leaf inhibit topical and systemic inflammation in rodents and suppress LPS-induced expression of mediators of inflammation in macrophages. *Journal of Immunotoxicology*. Early Online: 1–9.

Nwosu, M.O., (2002). Ethnobotanical studies on some pteridophytes of Southern Nigeria, *Economic Botany*. 56 (3): 255–259.

O'Neill, J., Brock, C., Olesen, A.E., Andresen, T., Nilsson, M., and Dickenson, A.H. (2012). "Unravelling the mystery of capsaicin: a tool to understand and treat pain," *Pharmacological Reviews*. 64(4): 939–971.

Obel, H., Jorum, A.K., Machocho. and Ngugi, M.P. (2016). Effects of dichloromethane-methanolic leaf Extracts of *Carissa edulis* (Forssk) Vahl on serum lipid profiles in normal rat models. *Journal of Hypertension* 5(2): 217.

Obi, H.1., Ilodigwe, E.E., Ajaghaku, D.L., and Okonta, J.M. (2012). An evaluation of acute and subchronic toxicities of a Nigerian polyherbal antidiabetic remedy. *International Journal of Pharmaceutical science and Research*. 3(9): 3131-3135.

Obidike, I.C. and Salawu, O. (2013). Screening of herbal medicines for potential toxicities. New Insights into toxicity and drug testing http://dx.doi.org/10.5772/54493. 64-88.

Obidike, I.C., Shehu, Idris-Usman, M., John-Africa, L.B. and Salawu, O.A. (2011). An Evaluation of acute and sub chronic toxicological effects of *Hymenocardia acida* leaf extract in adult Wistar rats. *Journal of Pharmacology and Toxicology*. 6(4): 400-408.

Odey, M.O., Itam, E.H., Ebong, P.E., Atangwho, I.J., Iwara, I.A., Eyong, U.E., Nnaligu, I.J., Inekwe, V.U., Johnson, J.T., Ochigbo, V., Udiba, U.U. and Gauje, B. (2012). Antihypertensive properties of the root and stem bark of *Nauclea latifoia* on serum electrolyte profile. *International Journal of Science and Technology*. 2(6): 382-385.

OECD (2008). Draft consultant's proposal. Test guideline 452: chronic toxicity studies. V. 8. OECD TG 452. http://www.oecd.org/chemicalsafety/testing/41753317.pdf. Accessed, march 21st, 2018.

OECD (2012), Test No. 443*:* Extended one-generation reproductive toxicity study, OECD guidelines for the testing of chemicals, Section 4, OECD Publishing.

Ogbonnia, S.O., Mbaka, G.O., Anyika, E.N, Emordi, J.E, and Nwakakwa, N. (2011). An evaluation of acute and subchronic toxicities of a Nigerian polyherbal tea remedy. *Pakistan Journal of Nutrition.* 10:1022-1028.

Okigbo, R.N., Anuagasi, C.L. and Amadi J.E. (2009). Advances in selected medicinal and aromatic plants indigenous to Africa. *Journal of Medicinal Plants Research*. 3(2):86-95.

Okoye, F.B.C., Agbo, M.O., Nworu, C.S., Nwodo, N.J., Esimone, C.O., Osadebe, P.O, and Proksch, P. (2015). New neolignan glycoside and an unusual benzoyl maleic acid derivative from *Maytenus senegalensis* leaves. *Natural Product Research*. 29(2):109-115.

Olivoto, T., Maicon, N., Ivan, R.C., Diego, N.F., Vinícius, J.S., Mauricio, F., Alan J.P. and Velci Q.S. (2017). Plant secondary metabolites and its dynamical systems of induction in response to environmental factors: A review. *African Journal of Agricultural Research*. 12 (2).71-84.

Onyebuagu, P.C., Pughikumo, D.T. and Erigbali, P. (2013). Effects of dietary *Xylopia aethiopica* on serum electrolytes and trace elements in male Wistr rats. *International Journal of Basic, Applied and Innovative Research.* 2(4): 100 – 105.

Osano, K.O., Nyamai, D.W., Ogola, P.E., Ouko, R.O., and Arika, W.M. (2016) Evaluation of *In-vivo* toxicity of dichloromethane: methanolic leaf extracts of *Prosopis juliflora* in female Wistar albino rats. *Journal of Drug Metabolism and Toxicology*. 7: 4-11

Otunola, G.A. and Afolayan, A.J. (2017). Assessment of oral safety profile of aqueous extract blend of three medicinal spices in Wistar rats. *Tropical Journal of Pharmaceutical Research.* 16 (1): 91-99.

Oyedeji, K.O. and Bolarinwa, A.F. (2013). Evaluation of antifertility and teratogenic effects of crude extracts of *Portulaca oleracea* in male and female albino rats. *Asian Journal of Pharmaceutical and Clinical Research*. 6 (2): 217 – 220.

Paini S.W., Tarsisius, D.W., Budianta, F.A.K., Wijaya, E.L. (2014). Difference of solvent polarity to phytochemical content and antioxidant activity of *Pluchea indicia Less* leaves extracts. *International Journal of Pharmacognosy and Phytochemical Research*. 6(4); 850-855.

Panche, A.N., Diwan, A.D., and Chandra, S.R. (2016). Flavonoids: an overview. *Journal of Nutritional Science*.5, e47:1-15.

Pandey, A., Kaushik, A., Wanjari, M., Nandan, Y.D., Jaiswal, B.S. and Dhodi, A. (2017) Antioxidant and anti-inflammatory activities of *Aerva pseudotomentosa* leaves, *Pharmaceutical Biology*. 55(1): 1688-1697.

Parasuraman, S. (2011). Toxicological screening. *Journal of Pharmacology and Pharmacotherapeutics*. 2(2): 74–79.

Patel, A., Patel, A. and Patel, N.M. (2010). Determination of polyphenols and free radical scavenging activity of *Tephrosia purpurea* Linn. leaves (Leguminosae). *Pharmacognosy Research*. 2:152–158.

Prasanth, K.M., Suba, V., Ramireddy, B., and Srinivas, B.P. (2014). Acute and sub-acute (28-Day) oral toxicity studies of ethanolic extract of *Celtis timorensis* leaves in rodents. *Global Journal of Medical Research*: *B Pharma, Drug Discovery, Toxicology and Medicine*. 14 (3)1:7-14.

Qnais, E., Raad, D., and Bseiso, Y. (2014). Analgesic and anti-Inflammatory effects of an extract and flavonoids from *Artemisia Herba-Alba* and their mechanisms of action. *Neurophysiology*. 46 (3):238-246.

Qu, X., Xu, K., Zhao, C., Song, X., Li, J., Li, L., Nie, W., Bao, H., Wang, J. Niu, F., and Li, J. (2017). Genotoxicity and acute and subchronic toxicity studies of a bioactive polyoxometalate in Wistar rats. *BMC Pharmacology and Toxicology*. 18:(26): 1-9.

Rafik, S., Mahesh, P., Ashwini, D. and Sayyed, I. (2014). Evaluation of anticancer, antioxidant, and possible anti-inflammatory properties of selected medicinal plants used in Indian traditional medication. *Journal of Traditional and Complementary Medicine*.4 (4):253-257.

Randhawa, M.A. (2009). Calculation of LD_{50} value from the method of Miller and Tainter, 1944. *Journal* of *Ayub Medical College.* 21(3): 1-3.

Rang, H.P., Dale, M.M., Ritter, J.M. and Moore, P.K. (2003).*Pharmacology*, Elservier Science Ltd, New Delhi, 368-378.

Reitman, S. and Frankel, S.E. (1957). A colorimetric method for the determination of serum glutamic oxaloacetic transaminase and serum glutamic pyruvic transaminase. *American. Journal of Clinical Pathology*. 28:56-63.

Rekha, B., Bhattacharya, S. and Yusuf, A.J. (2014). COX and LOX inhibitory potential of *Abroma augusta* and *Desmodium gangeticum The Journal of Phytopharmacology.* 3(3): 168-175.

Reuter, S., Gupta, S.C., Chaturvedi, M.M. and Aggarwal, B.B (2010). Oxidative stress, inflammation, and cancer, How are they linked? *Free Radical Biology* and *Medicine.* 49: 1603–1616.

Richard, B., Marina, G., Kristina, V.N., and Andras, N. (2016). Selecting female mice in estrus and checking plugs. Cold Spring Harb Protoc; doi:10.1101/pdb.prot092387. 729-731.

Riegsecker, S., Wiczynski, D., Kaplan, M.J, and Ahmed, S. (2013). "Potential benefts of green tea polyphenol EGCG in the prevention and treatment of vascular inflammation in rheumatoid arthritis," *Life Sciences*. 93(8):307–312.

Robert, F. and Ilse, Z. (2014). Plant-Derived Anti-Inflammatory Compounds: Hopes and Disappointments regarding the translation of preclinical knowledge into clinical progress. Hindawi Publishing Corporation. *Mediators of Inflammation*. Volume 2014, Article ID 146832, 9 pages.

Roeschlau, P., E. Bernt and J. W. Gruber. 1974. Determination of cholesterol in the blood. Clinical Chemistry. 12:403.

Roy, A.V. (1970). Rapid method for determining alkaline phosphatase activity in serum with thymolphthalein monophosphate. *Clinical Chemistry* 21: 5.

Saganuwan, S.A. (2016). Toxicity study of drugs and chemicals in animals: An overview. *Bulgarian Journal of Veterinary Medicine*. online first. ISSN 1311-1477; DOI: 10.15547/bjvm.983.

Saleem, U., Ahmad, B., Ahmad, M., Hussain, K., Bukhari, N. and Anjum, A.A. (2014). Teratogenic and embryo-toxic effects of latex and leaved methanol extract of *Euphorbia helioscopia L* in mice and chicken embryos. *The Journal of Animal and Plant Science*. 24 (2):450-454.

Sekendar, A.M., Mostafa, K., Raihan, M.O., Rahman, M.K., Hossain, M.A. and Alam, M.S. (2012). Antioxidant and cytotoxic activities of methanolic extract of *D. filix-mas* (L.) Schott leaves", *International Journal of Drug Development* and *Research*. 4(2): 223-229.

Shah, B.N., Seth, A.K., and Maheshwari, K.M. (2011). A review on medicinal plants as a source of anti-inflammatory agents. *Research Journal of Medicinal Plant.* 5:101–115.

Sheeja, E., Joshi, S.B. and Jain, D.C. (2010). Bioassay-guided isolation of anti-inflammatory and antinociceptive compound from *Plumbago zeylanica* leaf. *Pharmaceutical Biology*. 48(4): 381–387.

Shih, M.F., Cheng, Y.D., Shen, C.R. and Cherng, J.Y. (2010) A molecular pharmacology study into the anti-inflammatory actions of *Euphorbia hirta L*. on the LPS-induced RAW 264.7 cells through selective iNOS protein inhibition. *Journal of Natural Medicines*. 64 (3): 330-335.

Shivarai, G., Prakasg, B.D., Shrutthi, S.K., Vinayak, V.H., Avinash, A.K.M. and Sonai, N.V (2010). Markers of renal function tests. No*rth American Journal of Medical Science*; 2(4): 170–173.

Shrestha, B., Ravi, P. and Talib, H. (2014). Anti-inflammatory activity of ethnobotanical plants used as traditional medicine: A review. *Research and Review: Journal of Botanical Sciences*. 3 (1):8-18.

Shukla, S. and Tiwari, S.K. (2011). Insecticidal activity of *D. filix-mas* (Linn.) Schott ethanolic extract against *Corcyra cephalonica* Staint. (Lepidoptera: Pyralidae). *Journal of Biopesticides* 4 (2): 138 - 143.

Sikora, E., Scapagnini, G., and Barbagallo, M. (2010). Curcumin, inflammation, ageing and age-related diseases. *Immunity and Ageing*. 7:1–4.

Sinbad, O.O., Samuel F.O., Adewale, A., and Adedoyin, O. (2017). Evaluation of membrane stabilizing, proteinase and lipoxygenase inhibitory activities of ethanol extract of root and stem of *Sphenocentrum jollyanum*. *Pierre Journal of Advances in Biology and Biotechnology*. 13(1): 1-8.

Skeggs, LT. and Hochstrasser, HC. (1964). Thiocyanate (colorinmetric) method of chloride estimation. *Clinical Chemistry*, 10: 918.

Soare, LC., Ferdes, M., Stefanov, S., Denkova, Z., Nicolova, R., Denev, P., Bejan., C. and Paunescu, A., (2012). Antioxidant activity, polyphenols content and antimicrobial activity of several native Pteridophytes of Romania. Notulae Botanicae. *Horti Agrobotanici Cluj-Napoca.* 40(1):53-57.

Sofowora A. (1993). Medicinal plants and traditional medicine in Africa. Ibadan: Spectrum Books. p.191-289.

Stone, N.J., Robinson, J.G., Lichtenstein, A.H, Bairey M.C.N., Blum, C.B., Eckel, R.H, Goldberg, A.C., Gordon, D., Levy, D., Lloyd-Jones, D.M., McBride, P., Schwartz, J.S., Shero, S.T,, Smith, S.C., Watson, K., and Wilson, P.W. (2014). American College of Cardiology/American Heart Association Task Force on Practice Guidelines. 2013 ACC/AHA guideline on the treatment of blood cholesterol to reduce atherosclerotic

cardiovascular risk in adults: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines. *Journal* of the *American College* of *Cardiology*. 63(25 Pt B):2889-2934.

Subramanion, L.J., Zuraini, Z., Yeng, C. Yee, L.L., Lachimanan, Y.L. and Sreenivasan, S. (2011). Acute oral toxicity of methanolic seed extract of *Cassia fistula* in mice. *Molecules.* 16, 5268-5282.

Sudip, P., Yousuf, A., Nur-E-Noushin, R., Tanvir, E.M., Sakib, H., Moumoni, S., Nikhil, C.B., Siew, H.G and Ibrahim, K. (2017). Assessment of toxicity and beneficiary effects of *Garcinia pedunculata* on the hematological, biochemical, and histological homeostasis in rats. *Evidence-Based Complementary and Alternative Medicine*. Volume 2017, Article ID 4686104, 11 pages.

Sur, P. Chaudhuri, T. Vedasiromoni, J.R. Gomes, A. and Ganguly, D.K (2001). Anti-inflammatory and antioxidant property of saponin of tea (*Camellia sinensis* (L) O. Kuntze root extract. *Phytotherapy Research*. 15: 174-176.

Suzery, M., Cahyono, B. and Astuti, P. (2017). The acute toxicity test of methanolic extract of *Hyptis pectinata* poit on liver balb/c mice. IOP Conf. Series: *Materials Science and Engineering*. 172 (2017) 012029.

Tae-Seong L, Hee-Jeong M, and Young-Soo B. (2015). Phenolic glycosides from *Cercidiphyllum japonicum* leaves. *Journal of the Korean Wood Science and Technology*. 43(5): 591-599.

Tagarelli, G., Tagarelli, A. and Piro, A. (2010). Folk medicine used to heal malaria in Calabria (southern Italy). *Journal of Ethnobiology and Ethnomedicine*. 6 (27): 1-16.

Tamuno, I., Omole-Ohonsi, A. and Fadare, J, (2010). Use of herbal medicine among pregnant women attending a tertiary hospital in northern Nigeria. *Internet Journal of Gynecology and Obstetrics*. 15(2):1-8.

Terri, A.E. and Sesin, P.G. (1958). Determination of serum potassium by using sodium tetraphenylboron. *American journal of Clinical Pathology.* 29: 86.

Tietz, N.W. (1990). Clinical guide to Laboratory tests, Second edition W.B. Saunders Company, Philadelphia, USA 554-556.

Tobacco A. (1979): Quantitative enzymatic colorimetric determination of urea. *Clinical.Chemisty*.25, 336.

Trease G.E and Evans W.C. (1989). Pharmacognosy. 11 th.ed. London: Bailliere Tindall. p.45-50.

Trinder, P. (1969). Determination of blood glucose using an oxidase-peroxidase system with a non-carcinogenic chromogen. *Annals* of Clinical *Biochemistry* 6: 24.

Ugbogu, A.E., Okezie, E., Uche-Ikonne, C., Duru, M. and Atasie, O.C. (2016). Toxicity evaluation of the aqueous stem extracts of *Senna alata* in Wistar rats." *American Journal of Biomedical Research*. 4 (4): 80-86.

Uma, M., Suresha, M., Thulasiramana, K., Lakshmidevib, E. and Kalaiselvi, P. (2013). Chronic toxicity studies of aqueous leaf extract of Indian traditional medicinal plant *Ocimum tenuiflorum* (Linn.) in rats. *European Journal of Experimental Biology*. 3(5):240-247.

Umapathy, E., Ndebia, E.J., Meeme, A., Adam, B., Menziwa, P., Nkeh-Chungag, B.N and Iputo, J.E. (2010). An experimental evaluation of *Albuca setosa* aqueous extract on membrane stabilization, protein denaturation and white blood cell migration during acute inflammation. *Journal of Medicinal Plants Research.* 4(9):789-795.

Urban, J., Tauchen, J., Langrova, I. and Kokoska L. (2014). *In vitro* motility inhibition effect of Czech medicinal plant extracts on Chabertia ovina adults. *Journal of Animal and Plant Sciences*. 21(2): 3293-3302.

Uwumarongie, H.O., Enike, M.A. and Bafor, E.E (2016). Pharmacognostic evaluation and gastrointestinal activity of *D. filix-mas* (L.) schott (Dryopteridaceae). *Ewemen Journal of Herbal* Chemistry & Pharmacology *Research* 2(1): 19 – 25.

Valentyna, M., Iryna, T., Tetyana, D. and Larysa, M. (2017). A review of the medicinal ferns of Ukraine. *Scripta Scientifica Pharmaceutica*. 4 (1): 46-62.

Van Gelder, M.M.H.J., Van Rooij, I.A.L.M., Miller, R.K., Zielhuis, G.A., De Jong-van den Berg, L.T.W. and Roeleveld, N. (2010). Teratogenic mechanisms of medical drugs. *Human Reproduction Update.* 16 (4):378–394.

Venkateswar, R.V., Das, M.C., Muralidhar, R., Siva, P. and Padma, S. (2017). Evaluation of acute oral toxicity of ethanol leaves extract of *Catharanthus roseus* in Wistar albino rats. *Journal of Clinical and Diagnostic Research*. 11 (3): 1-4.

Vivek, K.B., Rajib, M. and Jae, G.P. (2016). Isolation and purification of plant secondary metabolites using column-chromatographic technique. *Bangladesh Journal of Pharmacology*. 11: 844-848.

Wahyuni, F.S., Arisanty, D., Hayaty, N. and Juwita, D.A. (2017). Sub-acute toxicity study of the ethyl acetate fraction of Asam Kandis Rinds (*Garcinia* cowa Roxb.) on the liver and renal function in mice. *Pharmacognosy Journal*. 9(3):345-349.

Wang, H., Gao, J., Kou, J., Zhu, D., and Yu, B. (2008). Anti-inflammatory activities of triterpenoid saponins from *Polygala japonica*. Phytomedicine: 15: 321–326.

Warren, C.A., Paulhill, K.J. and Davidson, L.A. (2009). Quercetin may suppress rat aberrant crypt foci formation by suppressing inflammatory mediators that influence proliferation and apoptosis. *Journal of Nutrition*; 139(1):101-105.

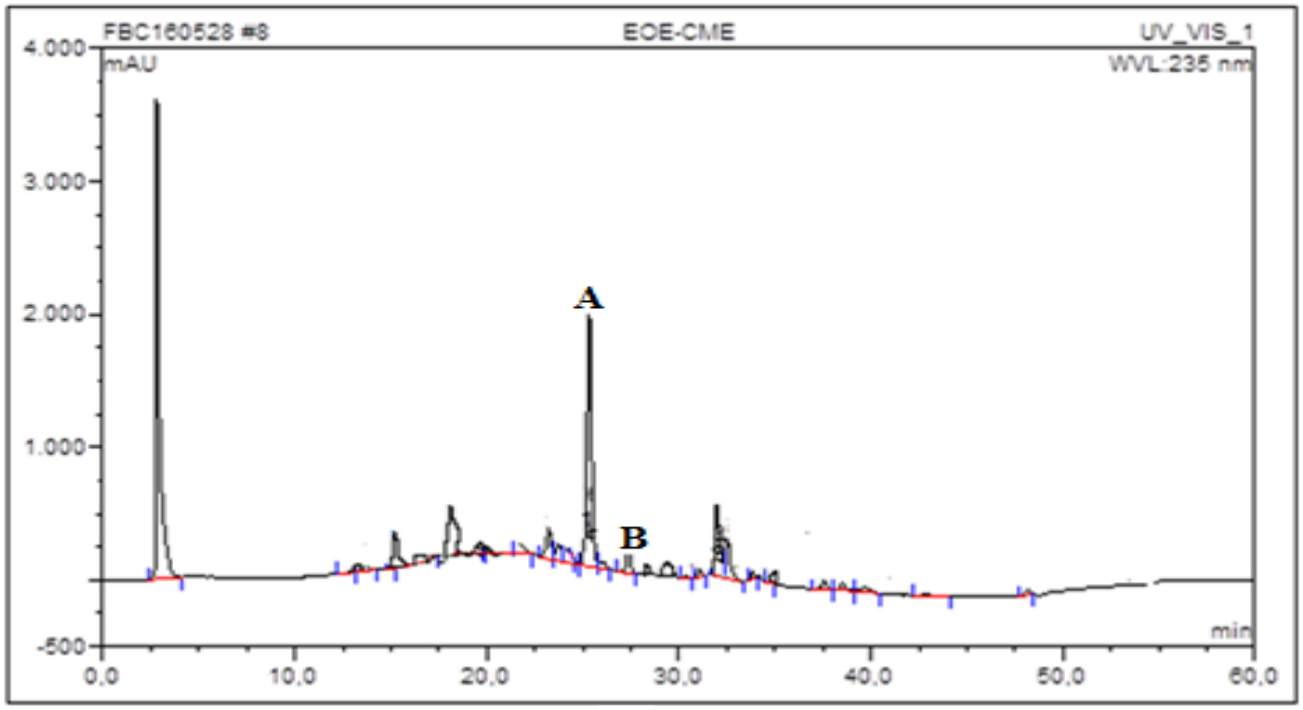
Wu, C. (2010). Overview of developmental and reproductive toxicity research in China: Histroy, funding mechanisms, and frontiers of the research. Birth defects research. Part B, *Developmental and Reproductive Toxicology*. 89(1): 9-17.

Yadav, R.N.S. and Agarwala, M. (2011). Phytochemical analysis of some medicinal plants. *Journal of Phytology*, 3(12): 10-14.

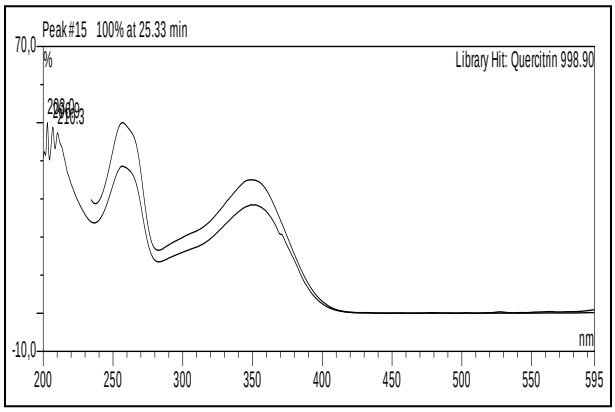
Yan, H., Yuan, Y., Xi, Z., Kun, Z., Shaohua, C. and Zhiyun, D. (2015). Curcumin, Inflammation, and Chronic Diseases: How Are They Linked? *Molecules.* 20: 9183-9213; doi:10.3390/molecules20059183.

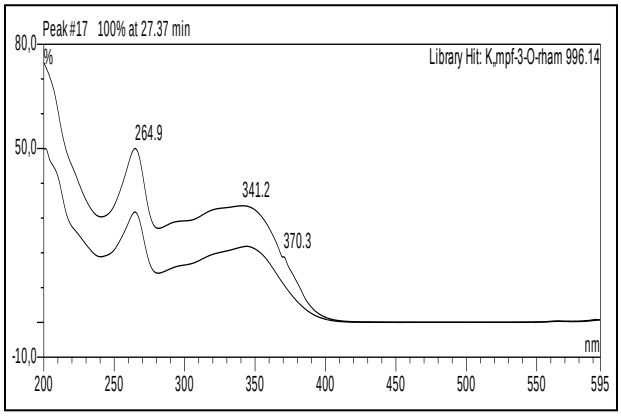
APPENDIX 1

HPLC Chromatograms and UV spectrals of major compound detected in extract and fractions of *D. filix-mas.*



C



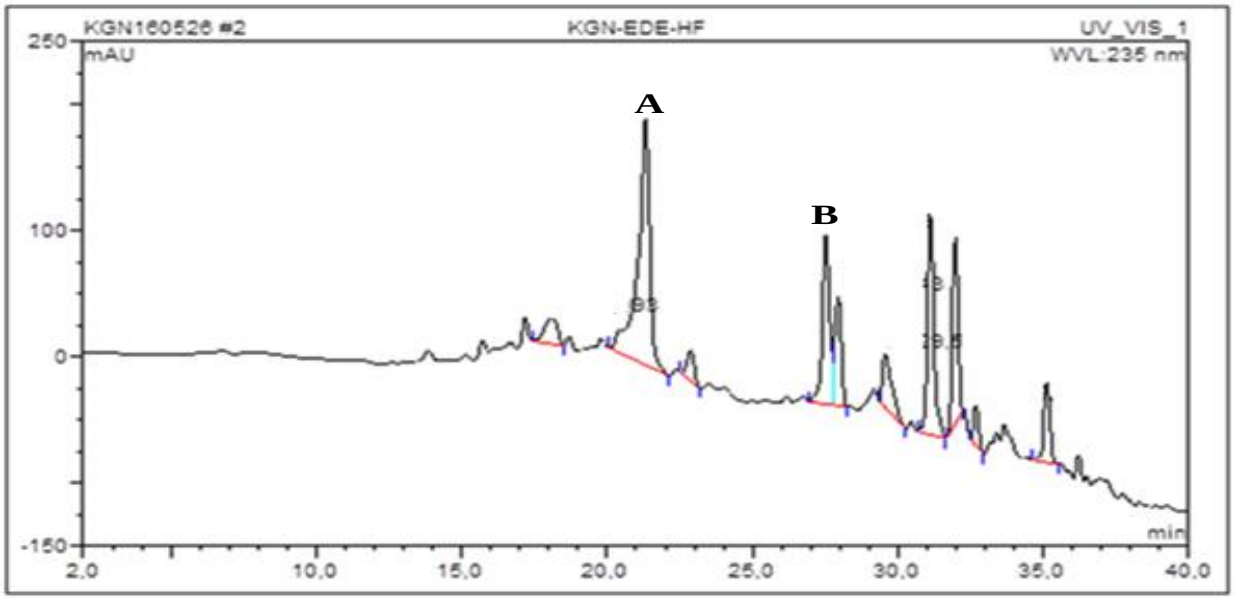


A **B**

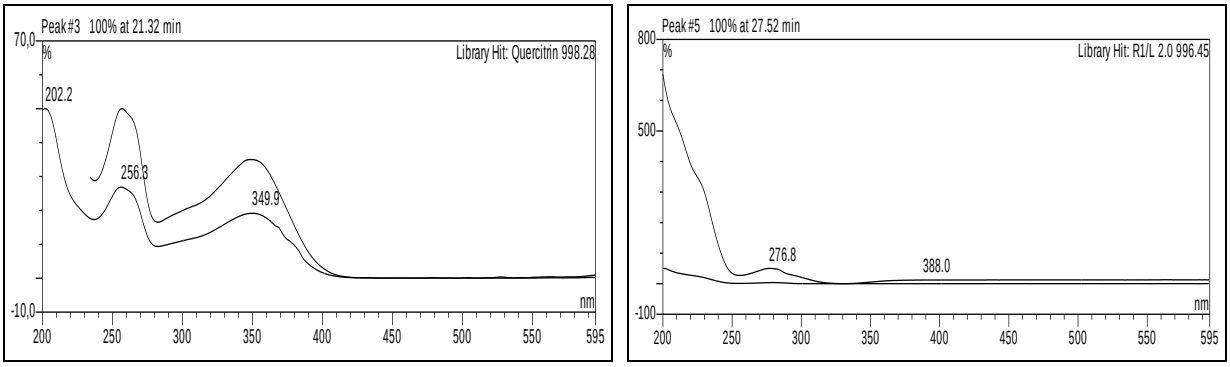
Figure 3: Chromatogram and UV spectral of major compounds detected in extract.

A = Quercitrin (Rt=, 25.33 min, 998.90) and **B** = Kmpf-3-O-rham (Rt = 27.37, 996.14).

C: Chromatogram.



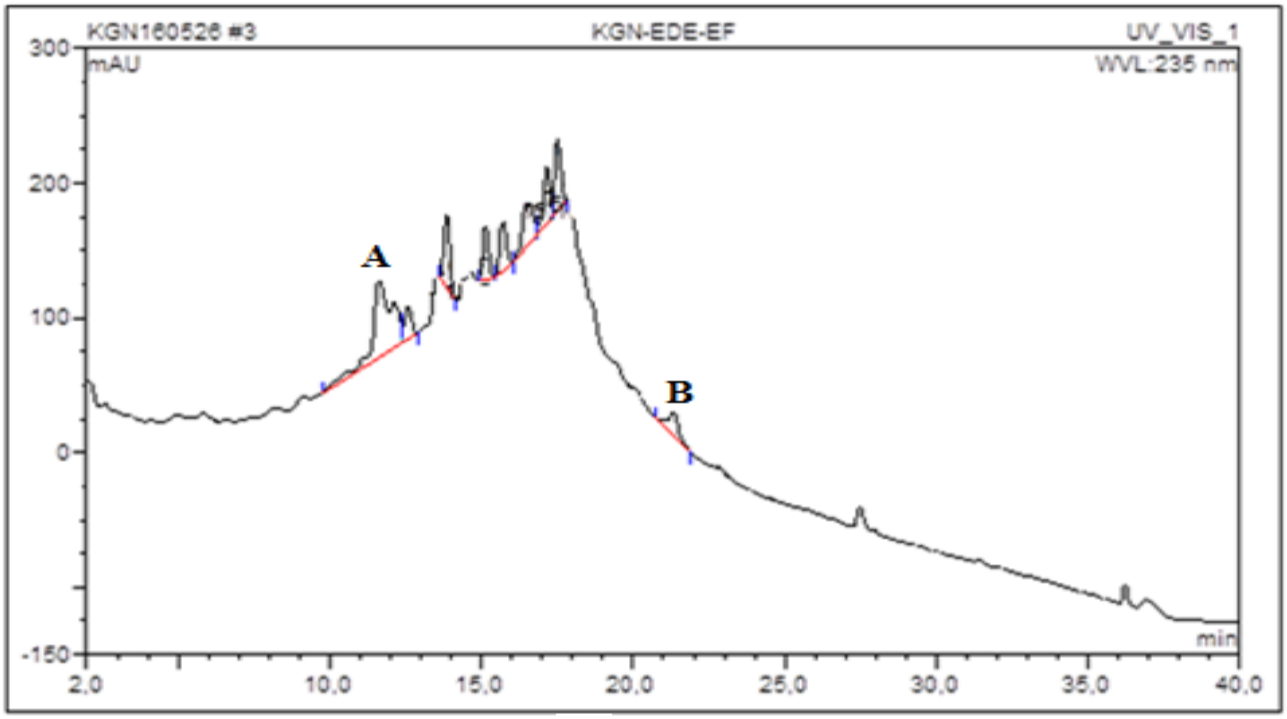
C



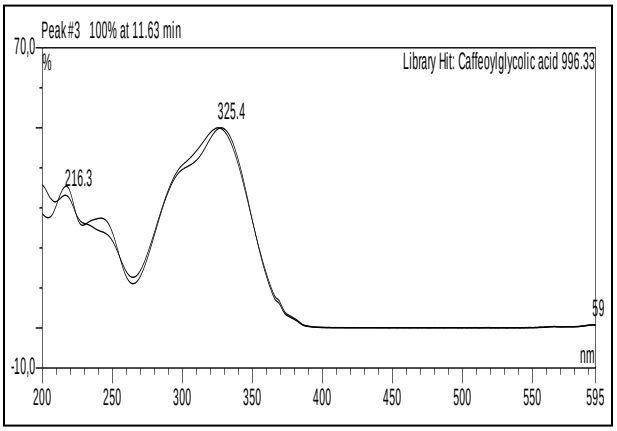
A **B**

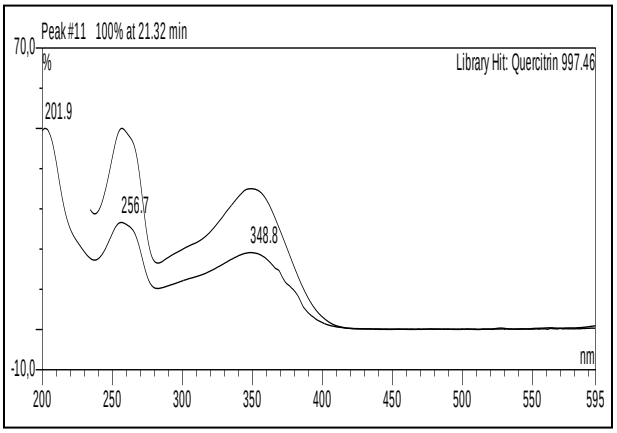
Figure 4: Chromatogram and UV spectral of major compounds detected in n-hexane fraction. **A** = Quercitrin (Rt=, 1.32 min, 998.28) and **B** = R1/L2.0 (Rt=, 27.52 min, 996.45).

C: Chromatogram.



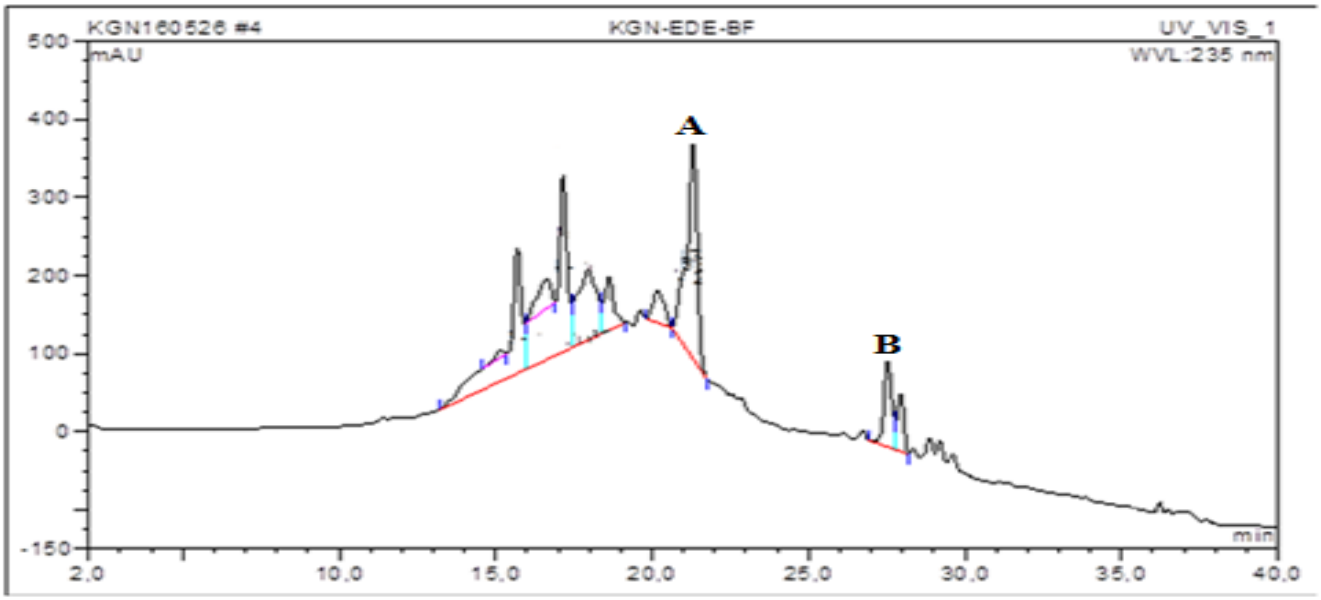
C



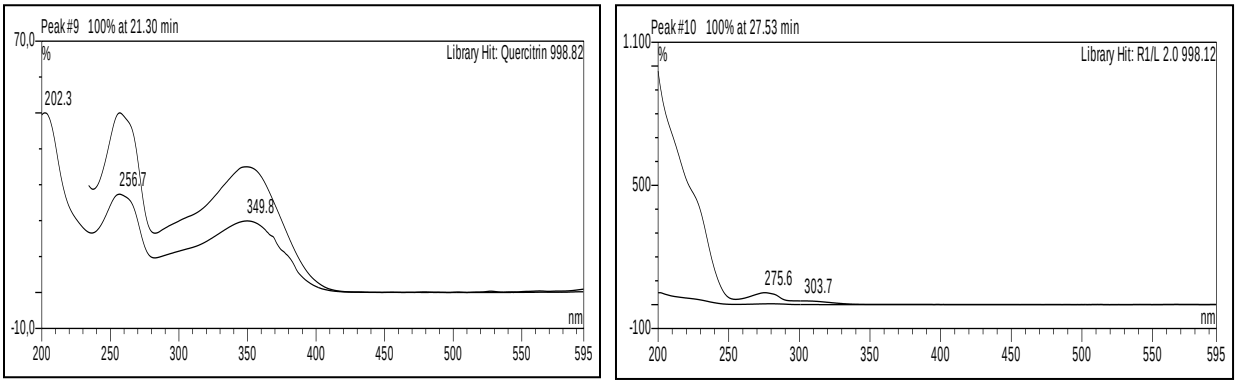


A **B**

Figure 5: Chromatogram and UV spectral of major compounds detected in ethyl acetate fraction. **A** = Caffeoylglycolic acid (Rt=11.63 min, 996.33) and **B** = Quercitrin (Rt=, 21.32 min, 997.46). **C =** Chromatogram.

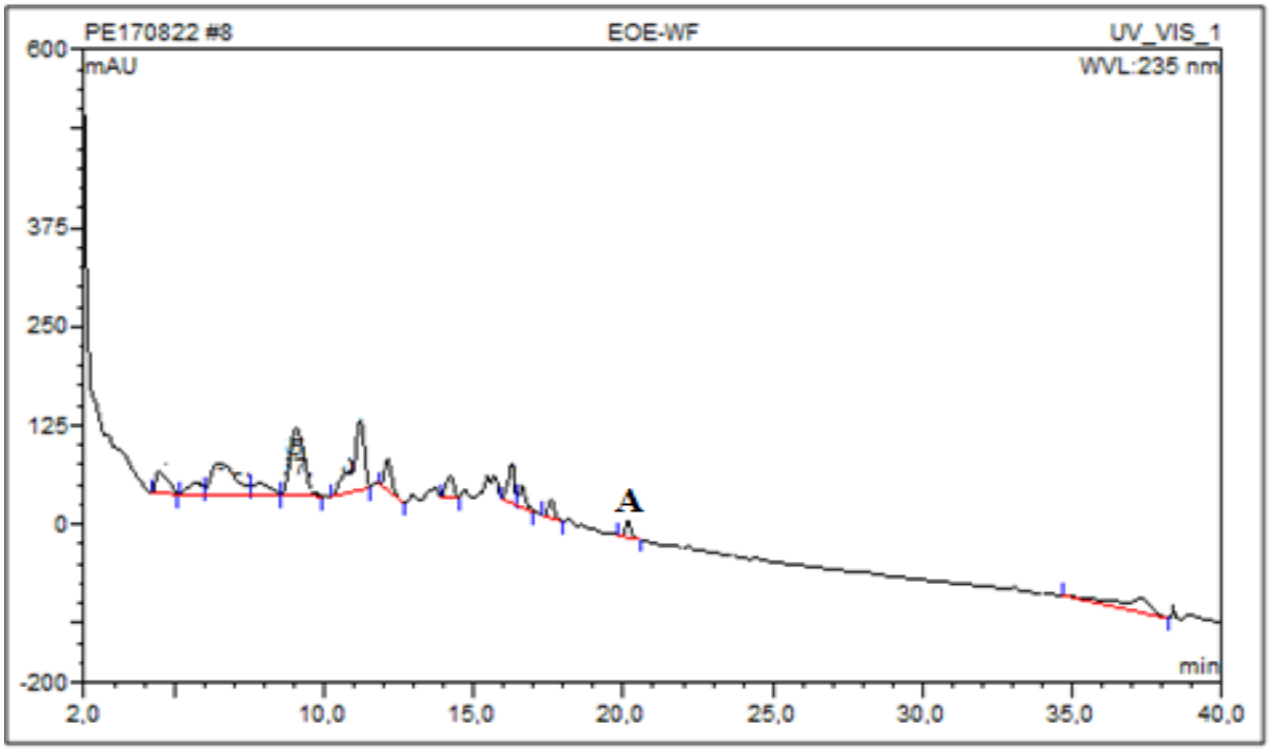


C



A **B**

Figure 6: Chromatogram and UV spectral of major compounds detected in butanol fraction. **A**: Quercitrin, (Rt= 21.30 min, 998.82). **B** =R1/L2.0, (Rt= 27.53, 998.12). **C** = Chromatogram.



B

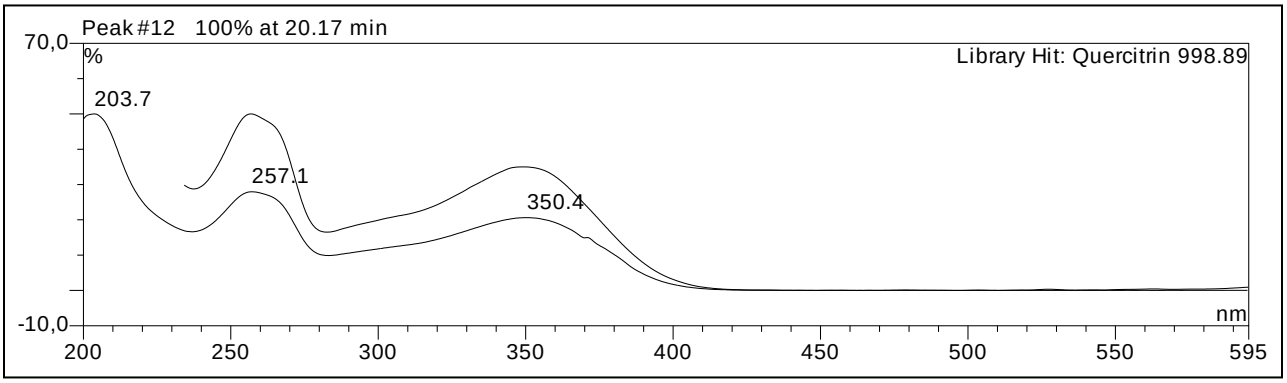
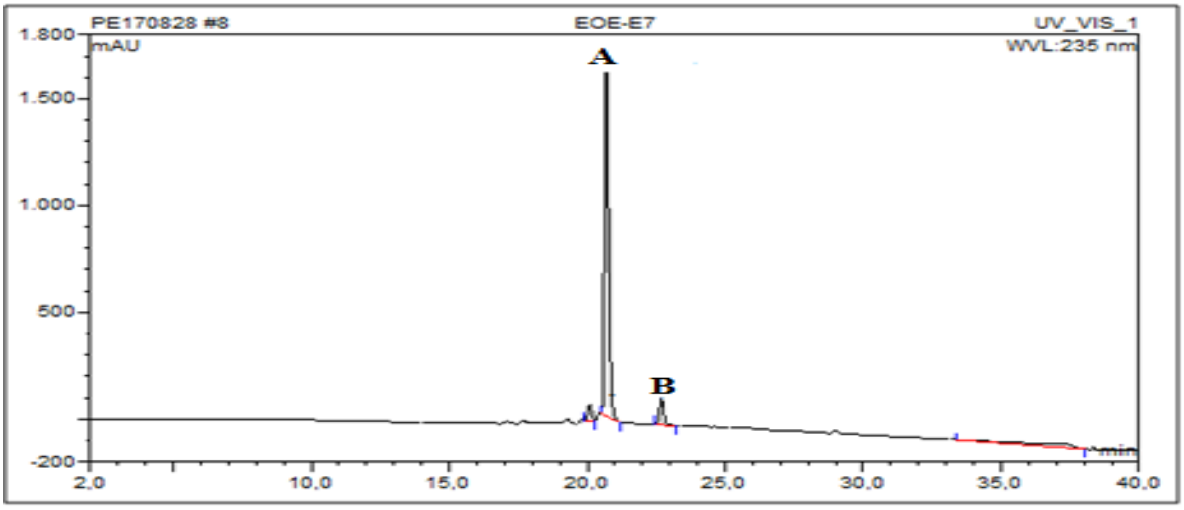
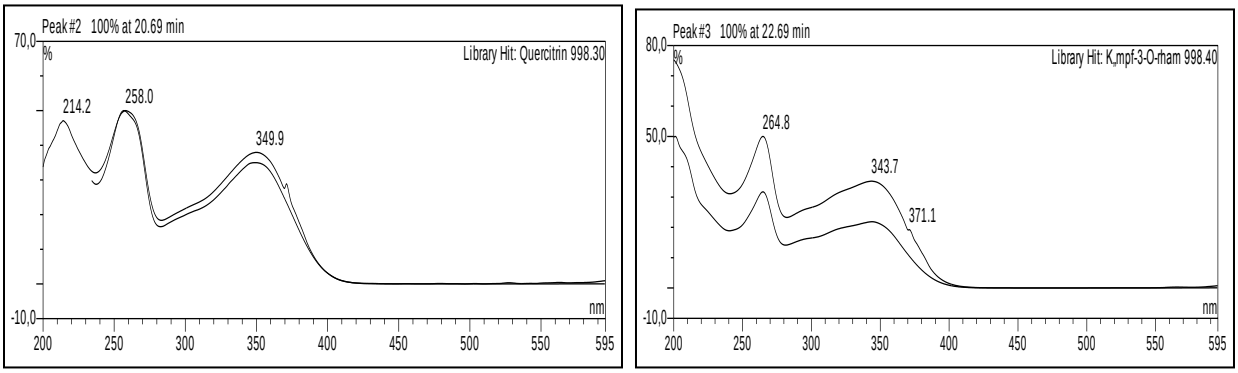


Figure 7: Chromatogram and UV spectra of the major compound detected in Water fraction. **A =** Quercitrin (Rt, = 20.17 min, 998.89). **B =** Chromatogram.



C



A **B**

Figure 8: Chromatogram UV spectral of major compounds detected in VLC-E7 (EOE-E7):

A = Quercitrin (Rt = 20.69 min, 998.30), **B =** K,,mpf-3-O-rham (Rt = 22.69 min, 998.40).

C= Chromatogram.

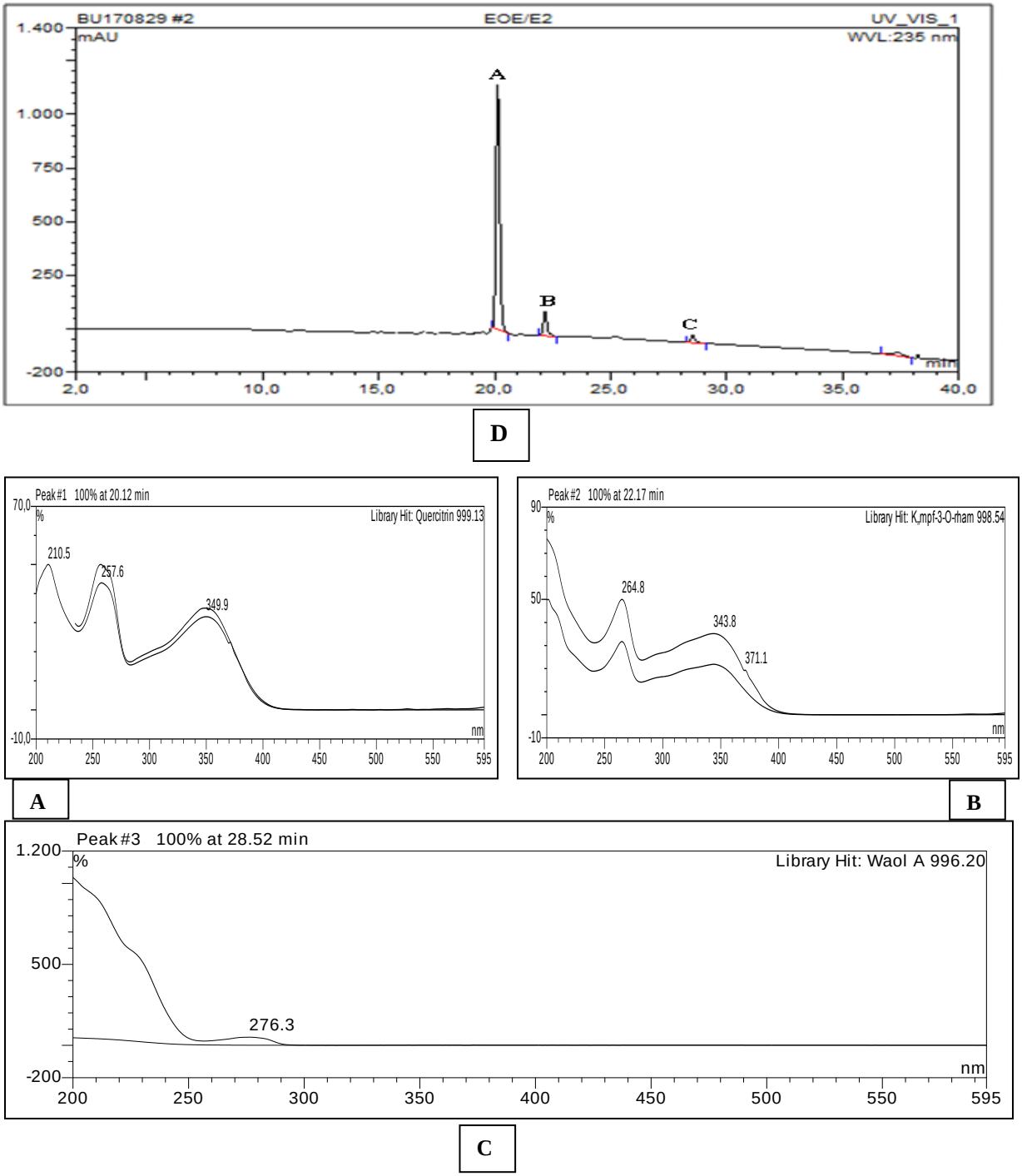


Figure 9: Chromatogram and UV spectral of major compounds detected in SPH-E2 fraction. A = Quercitrin (Rt = 20.12 min, 999.13), **B =** Kmpf-O-rham (Rt = 22.17 min, 998.54) and **C =** Waol A (Rt = 28.52 min, 996.20). **D** = Chromatogram.

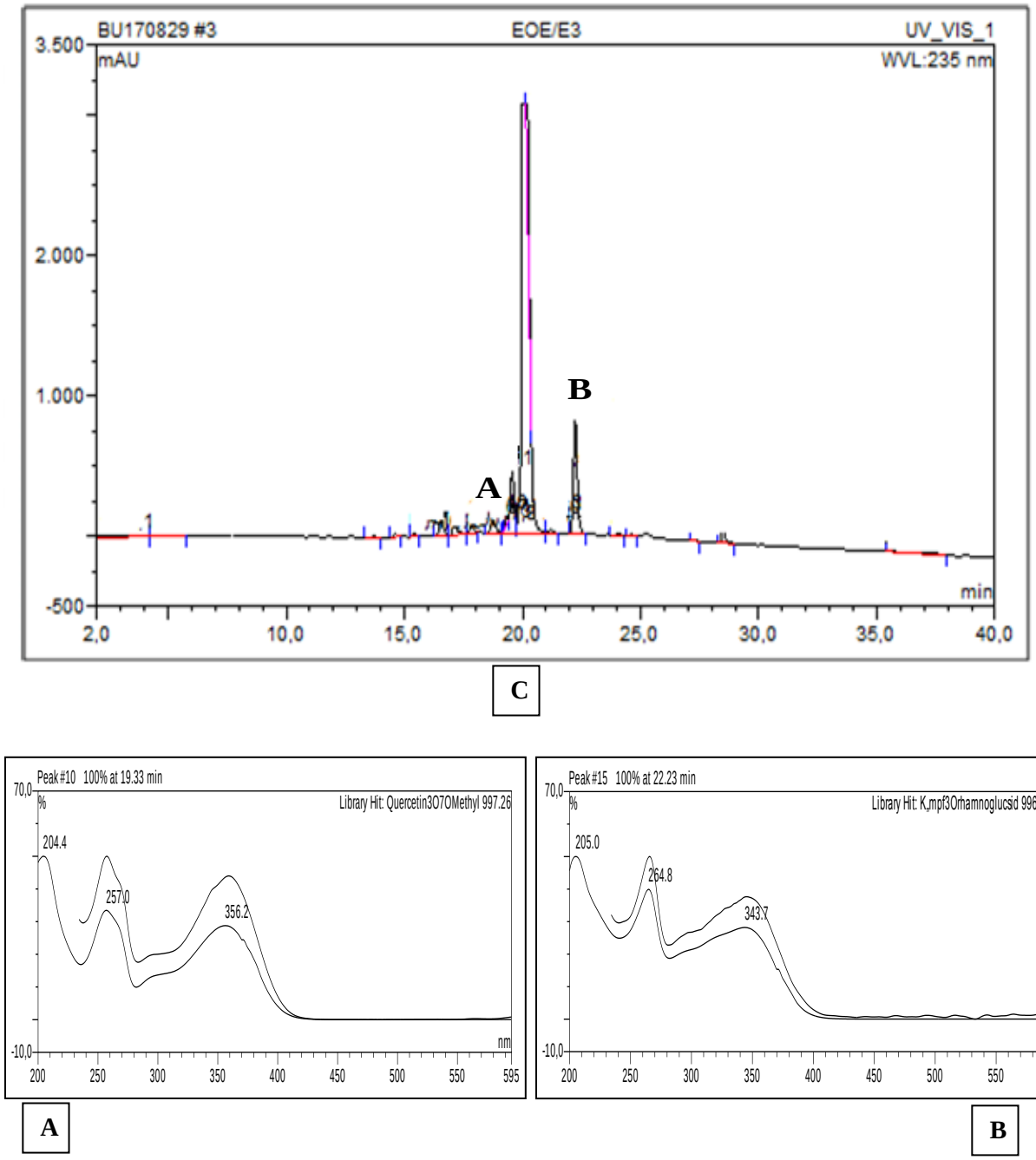


Figure 10: HPLC Chromatogram and UV spectral of major compounds detected in SPH-E3. A = Quercetin3Omethyl (Rt = 19.33 min, 997.26) and **B =** Kampf3Orhamnoglucside (Rt = 22.23 min, 996.29). **C** = Chromatogram.

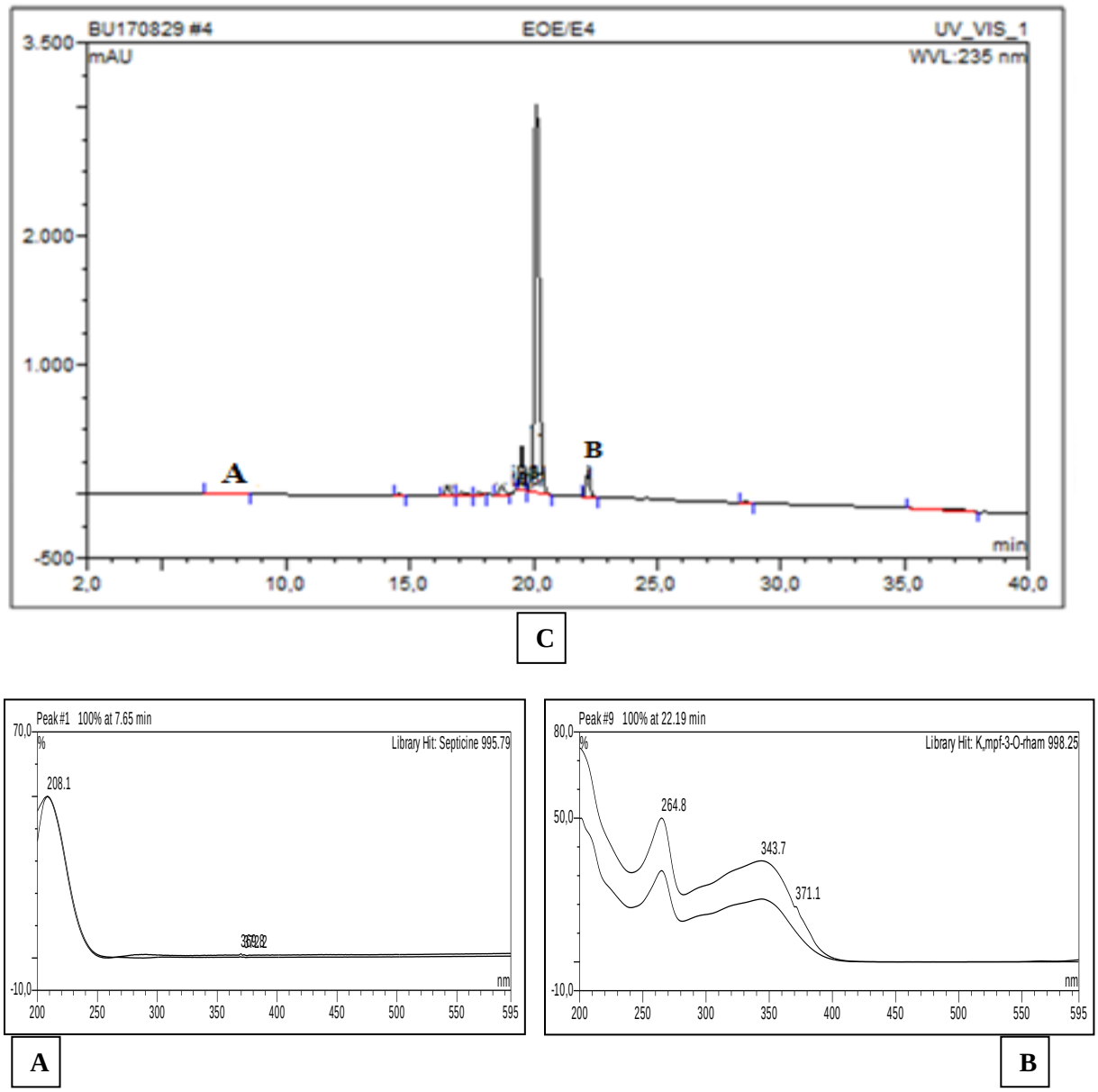
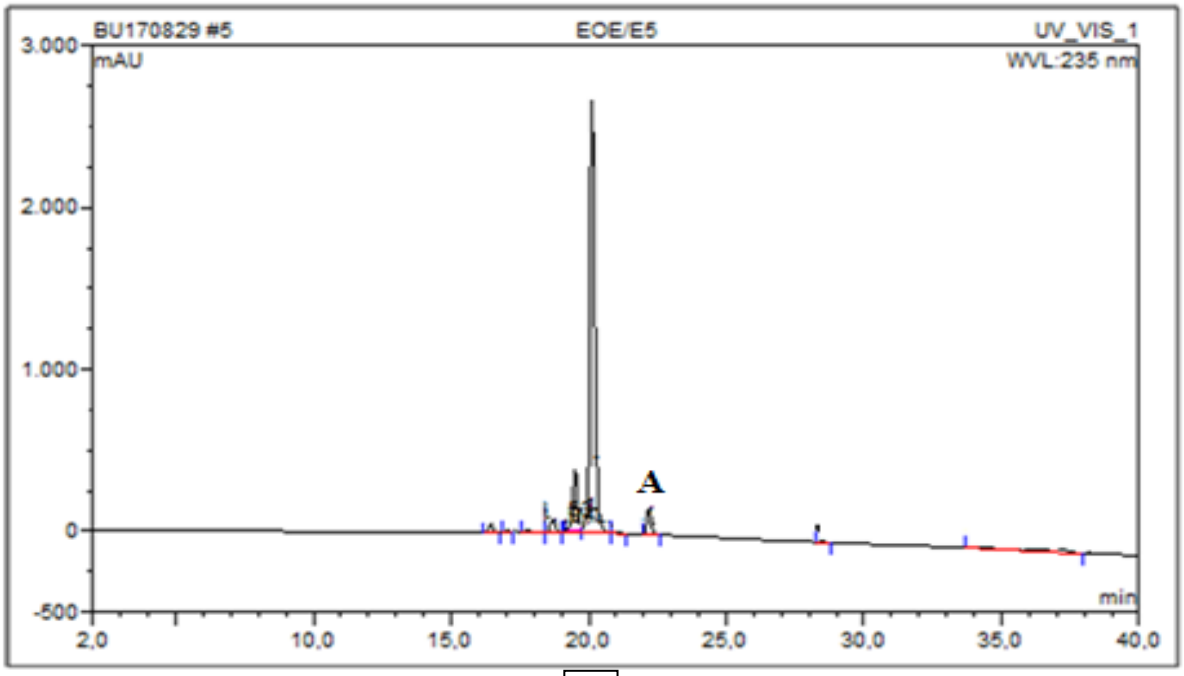
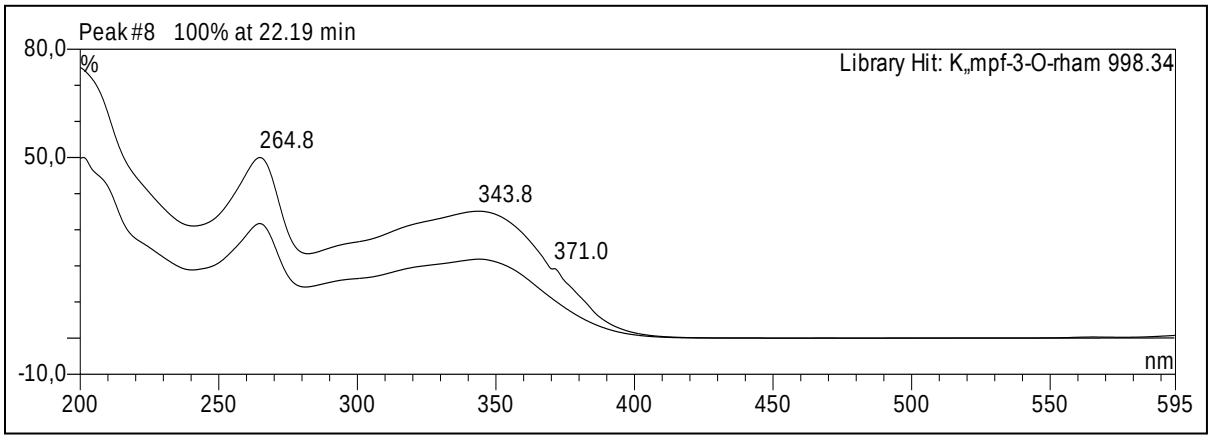


Figure 11: HPLC Chromatogram and UV spectrals of major compounds detected in SPH-E4. A = Septicine (Rt = 7.65 min, 995.79), **B =** Kampf-3-O-rham (Rt = 22.19 min, 998.25). **C** = Chromatogram.



B



A

Figure 12: HPLC Chromatogram and UV spectral of major compounds detected in SPH-E5. **A =** Kmpf-3-O-rahm (Rt = 22.19 min, 998.34), **B=** Chromatogram

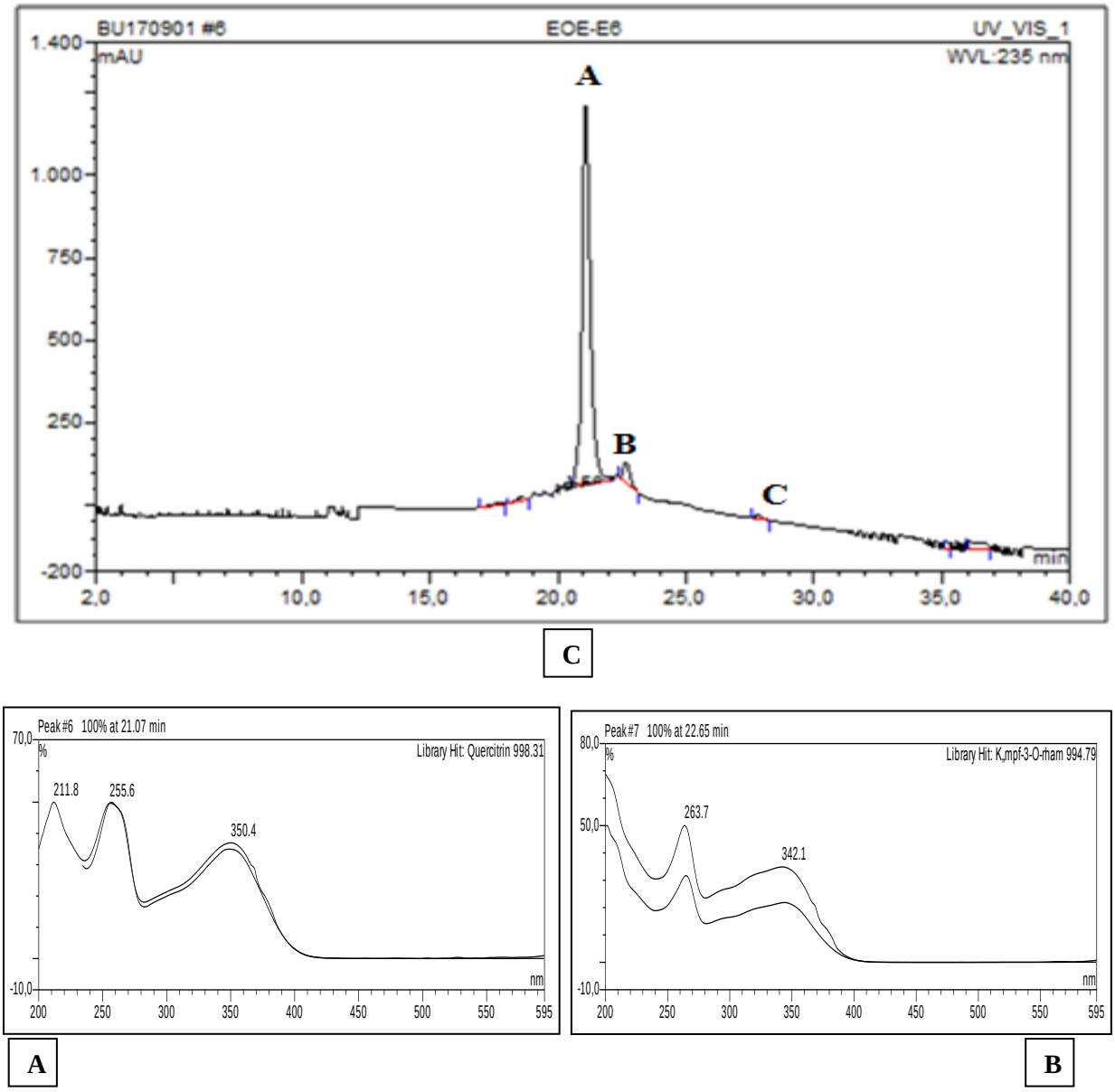
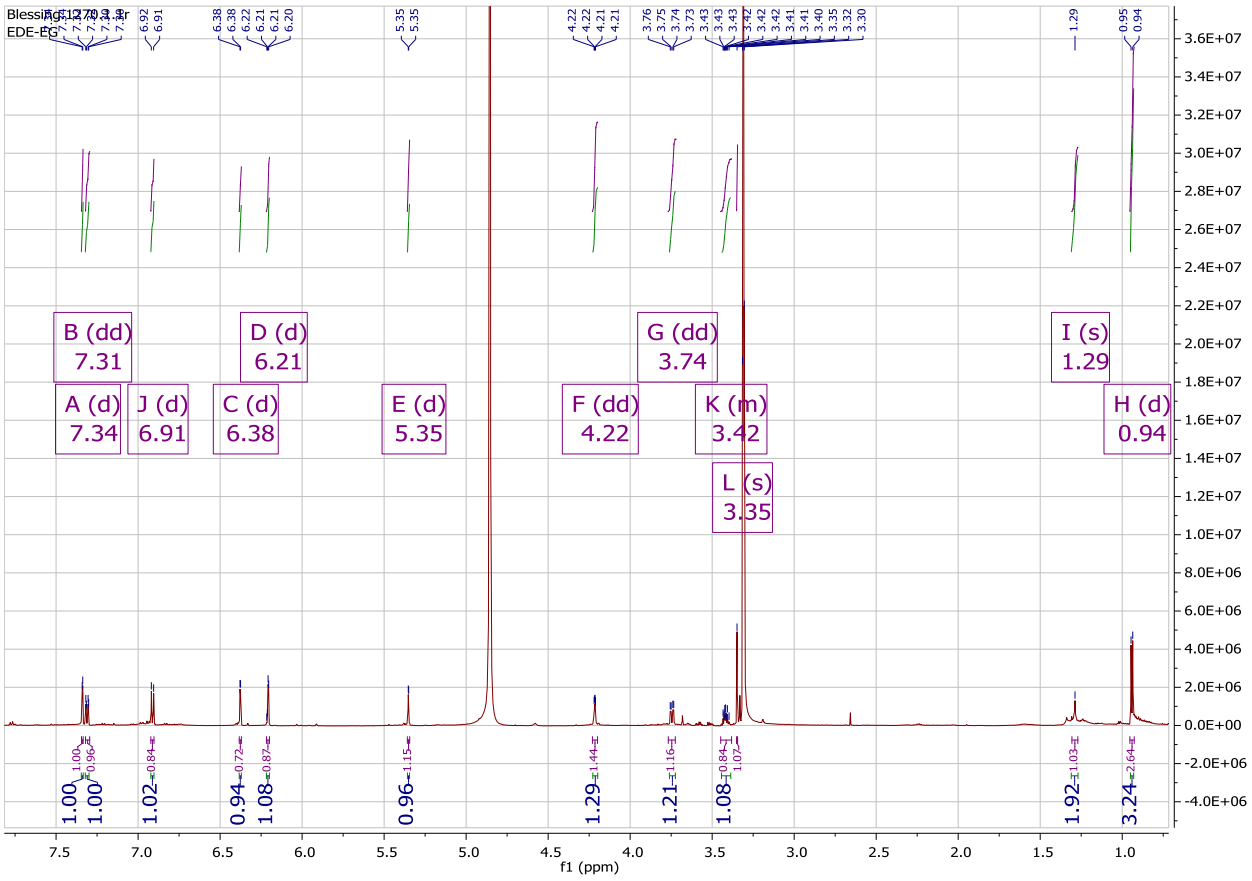


Figure 13: HPLC Chromatogram and UV spectrals of major compounds detected in

SPH-E6. A = Quercitrin (Rt = 21.07 min, 998.31), **B =** Kmpf-3-O-rahm (Rt = 22.65 min, 994.79), **C =** Chromatogram.

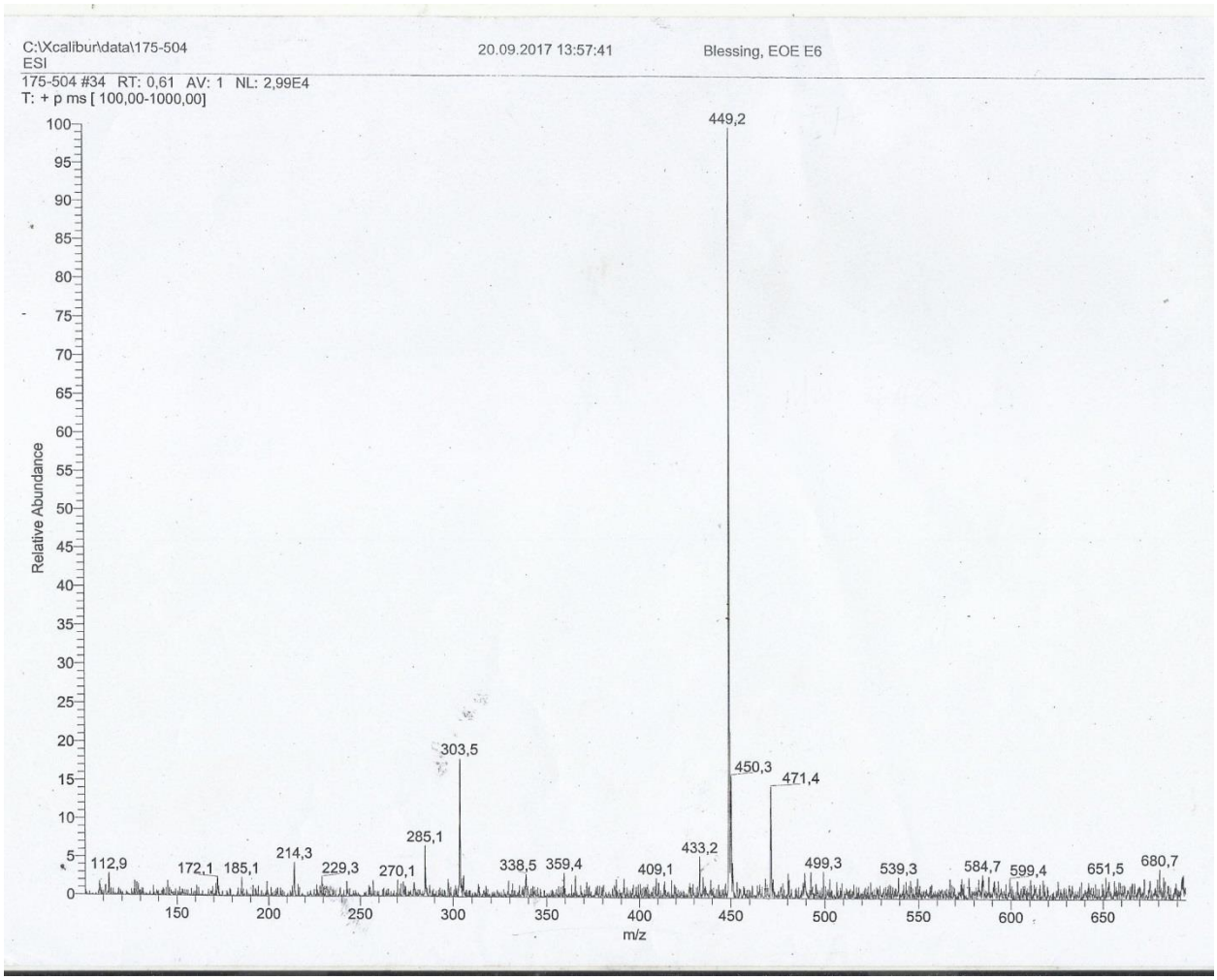
APPENDIX 2

^{1}H NMR of Quercetin-3O-αL-rhamnopyranoside from SPH –E6.



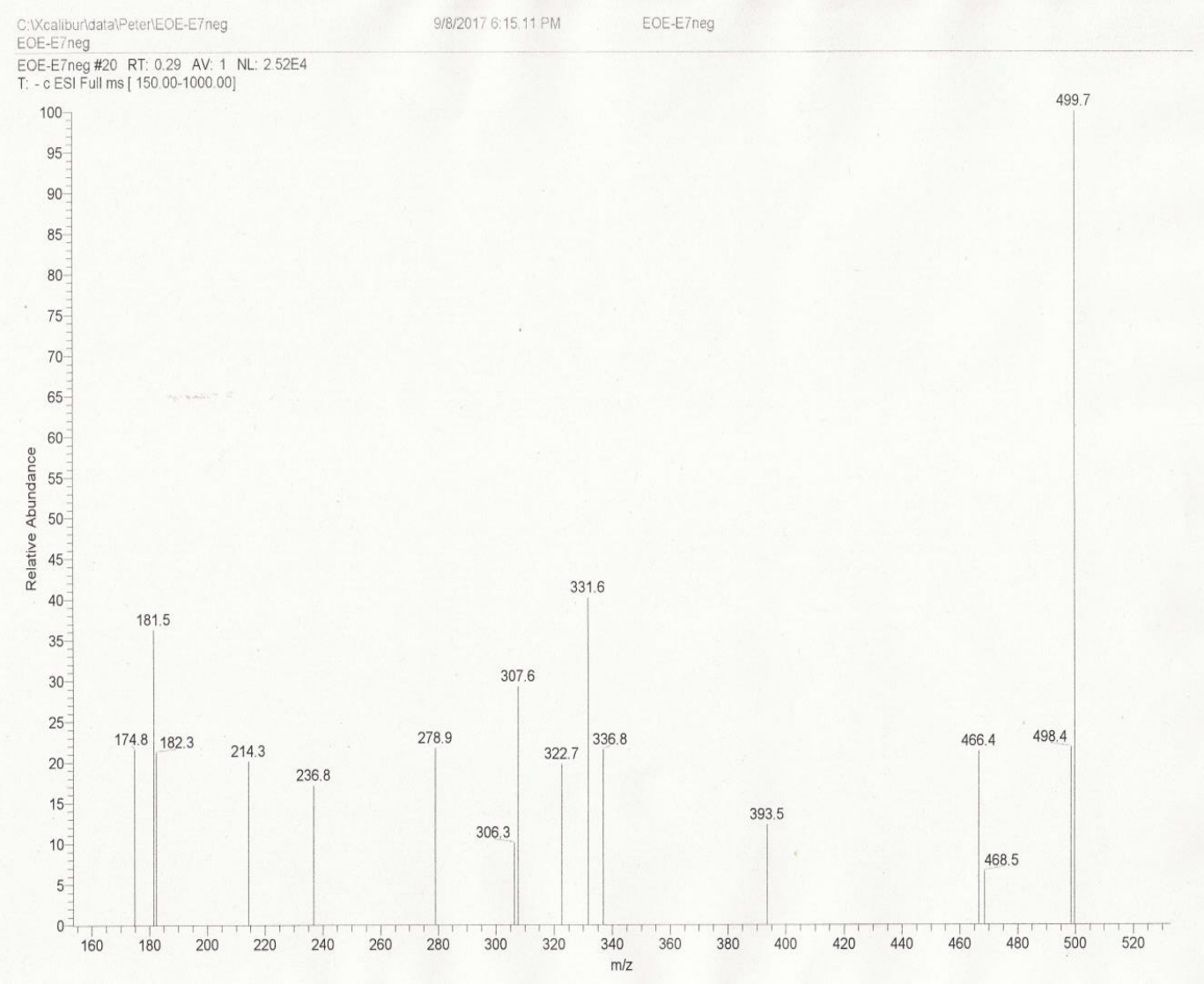
APPENDIX 3

LC-MS SPECTRUM OF Quercetin-3O-αL-rhamnopyranoside from SPH-E6:



APPENDIX 4

NEGATIVE MODE of LC-MS SPECTRUM OF VLC-E7



APPENDIX 5

POSITIVE MODE of LC-MS SPECTRUM OF Quercetin-3O-αL-rhamnopyranoside from VLC-E7: