CHEMICAL CONSTITUENT(S), ANTI-INFLAMMATORY, ANTI-OXIDANT AND ANTI-NOCICEPTIVE ACTIVITIES OF THE ROOTS OF *PALISOTA HIRSUTA* K.SCHUM (COMMELINACEAE).

A THESIS SUBMITTED IN FULFILLMENT OF THE

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Faculty of Pharmacy and Pharmaceutical Sciences

BY

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DECLARATION

I hereby declare that the experimental work described in this thesis is my own work towards the PhD and to the best of my knowledge; it contains no material previously published by another person or material which has been submitted for any other degree of the university, except where due acknowledgement has been made in the text.

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DEDICATION

This work is dedicated to my wife Patience and my children Kwame, Kwabena, Adwoah and Afua.

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Finally, to my family, Patience my wife and my children Kwame, Kwabena, Adwoah and Afua, thank you for your love and support over the years.

ABSTRACT

The roots of *Palisota hirsuta* are used in Ghana and other West African countries where the plant grows for the treatment of various disease conditions such as rheumatoid arthritis (other painful and inflammatory conditions), infertility in females, anaemia, dysentery etc. The current study seeks to evaluate the anti-inflammatory, analgesic and anti-oxidant properties of the ethanolic root extract of *P.hirsuta,* the methanolic and petroleum ether fractions thereof and the constituents isolated from these fractions using animal models.

The carrageenan-induced foot oedema test in 7-day old chicks was used to assess the antiinflammatory effect of the ethanolic extract, methanolic and petroleum ether fractions and all the isolated constituents. Diclofenac and dexamethasone were used as reference drugs for these assessments. The effect before the induction of inflammation (pre-emptive) paradigm was used for the assessment of inflammation. The analgesic (anti-nociceptive) effect of the ethanolic extract methanolic and petroleum ether fractions as well as the isolated constituents was assessed using the formalin-induced nociception test in the mice paw. The *in vitro* antioxidant properties of the isolates PH I, II, III, IV, V and VI were evaluated by using the lipid peroxidation assay, 2,2-diphenyl-1-picrylhydrazylhydrate (DPPH) radical scavenging assay and reducing power test. The standard antioxidant n-propyl gallate was used as the

reference antioxidant in all the assays (the anti-oxidant assays were carried on the isolates following qualitative tests with DPPH). As part of the current study, the structures of the isolated constituents were evaluated using Gas Chromatography coupled with Mass Spectrometer (GC/MS) and Nuclear Magnetic Resonance.

The crude extract PHC, methanolic fraction PHM and the petroleum ether fraction PHE of *P.hirsuta* (30-300mg/kg, *p.o*) dose dependently reduced oedema with maximal effects of 64.01±10.90%, 72.91±4.06%, and 57.86±11.89% (prophylactic) respectively.

The extract PHC (30-300mg kg-^{1}, *p.o*) caused a dose-related inhibition of both phases (i.e. phase 1 and phase 2) of the formalin-induced nociception. The highest dose caused maximal inhibitions of 40.89±24.10% and 66.25±32.08%. The methanolic fraction; PHM (30-300mgkg^{1}, *p.o*), also produced maximal inhibitions of both phases by 72.00±15% and 61.00±41.97%. PHE, which is the petroleum ether fraction dose-dependently, inhibited both phases of the formalin-induced nociception. Maximal inhibitions of 45.02±29.81% and 80.50±4.62% were respectively produced by PHE. For the isolates, PH I, II, III, IV, V and VI, the maximal inhibitions produced are as follows; PH I: 55.08±14.90% and 63.57±25.91%, PH II:

90.86±6.407% and 67.47±20.84%, PH III: 57.87±8.43% and 79.90±7.05%, PH IV: 71.39±9.19% and 89.19±3.81%, PH V: 61.34±11.07% and 82.89±3.97%, PH VI: 70.14±8.60% and 86.18±7.42% of the licking time in the early and late phases. Morphine as the reference drug (positive control) reduced the duration of formalin evoked nociceptive behaviour by a maximum percentage of 92.49±4.679% in the early phase and 95.16±5.49% in the late phase of the test.

The isolates (0.5-3mgml^{-1}) exhibited a lipid peroxidation activity as per the IC_{50} in the following corresponding manner; PH I 0.260, PH II 0.295, PH III 1.545, PH IV 0.007, PH V 1.769 and PHVI 1.453.The n-propyl gallate (0.001-0.3mgml-1) showed a lipid peroxidation activity by an IC_{50} of 0.015.

The relative anti-oxidative activity in the DPPH decolourization assay (DPPH scavenging activity) of isolates as defined by the IC_{50} produced by the isolates (0.5-3mgml^{-1}) were correspondingly PH I—3.304; PH II—3.636; PH III—0.241; PH IV—0.880, PH V--- 4.414 and PH VI—0.163. For n-propyl gallate (0.01-0.3mgml^{-1}) the IC_{50} was found to be 0.058 in the DPPH decolourization assay. The isolates showed reducing power potential by their EC_{50} values corresponding to PH I—5.874; PH II —5.122; PH III—5.238; PHIV—0.524, PH V 4.501 and PH VI—1.723. The reference antioxidant n-propyl gallate showed a reducing power by an EC_{50} of 1.723. The findings in all three antioxidant activity assays show that the isolates have potent antioxidant properties which might partly account for the anti-inflammatory and anti-nociceptive/analgesic activities.

Spectral analyses resolved PH IV to be 20-Hydroxyecdysone, PH I to contain the fatty acids eicosanoic acid and ethyl stearate, PH II to be a mixture of trimethyl benzene, cymene and ethyl hexadecanoate (ethyl palmitate) and PH III to be a mixture of methyl palmitate and octadecanoic acid.

The roots extract PHC, the methanolic fraction PHM and ether fraction PHE exhibited antiinflammatory activity as well as anti-nociceptive activity. The isolates PH I, II, III, IV, V and VI showed both anti-inflammatory and anti-nociceptive activities. The isolates also

showed anti-oxidant action. 20-Hydroxyecdysone was isolated for the first time from the roots of *Palisota hirsuta.*

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ABBREVIATIONS

^{13}C-NMR----- Carbon 13 Nuclear Magnetic Resonance

AAE---------- Ascorbic acid equivalent

AAPH --------2,20-azobis(2-amidinopropane) dihydrochloride

x

ABTS -------- 2,20-azinobis(3-ethylbenzothiazoline-6-sulphate)

ACC ---------Anteriorcingulate cortex

AD ---------- Alzheimer's disease

AIDS ------- Acquired Immunodeficiency Syndrome

ALD ------- Alcohol-induced Liver disease

AUC ------- Area under Curve

BDH-------- British Drug House

BHA -------Butylatedhydroxyanisole

BHT -------Butylatedhydroxytoluene

CAT ------- Catalase

CNS ------- Central Nervous System
COX ---------- Cyclooxygenase

CUD ---------- Cardiovascular Disease

CVD ---------- Cardiovascular disease

DCF ----------Dichlorofluorescein

DCFH-DA ----Dichlorofluorescein-diacetate

DNA ---------- Deoxyribonucleic acid

DNA ---------- Deoxyribonucleic acid

DPN ---------- Diabetic peripheral neuropathy

DPPH --------- 1, 1-Diphenyl-2-picrylhydrazyl

EAA's -------- Excitatory amino acids

EMG --------- Electromyography

FRAP -------- Ferric reducing ability of plasma

GC/MS ----- -Gas Chromatography/Mass Spectrometer

GPx ----------Glutathioneperoxidase

GSH --------- Glutathione

^{1}H-NMR----- Proton Nuclear Magnetic Resonance IASP -------- International Association for the Study of Pain

ICAM ------ Intercellular Adhesion Molecules

IL-1 --------- Interleukin-1

KMBA ----- Alpha-ketoc-methiolbutyric acid

LDL --------Low density lipoprotein

LTO --------Latent Time of Observation

MCI ------- Mild Cognitive Impairment

MDA ------Malondialdehyde

NMDA -----N-Methyl-D-aspartic acid

NMR ------ Nuclear Magnetic Resonance

NTI -------- Number of tentative Intromissions

ORAC ----- Oxygen radical absorbance capacity

PCL --------Photochemiluminescence

PD---------- Parkinson's disease

PE --------- Beta-phycoerythrin

PHN ------- Post-herpetic neuralgia

PMNL -----Polymorphonuclear

leucocyte

PMNL -----Polymorphonuclear leucocyte

PUFA ------Polysaturated fatty acid

RNS ------- Reactive Nitrogen Species

ROS ------- Reactive oxygen species

RT--------- Retention time

SOD ------ Superoxide dismutase

TCA ------ Trichloroacetic acid

TCM ------Traditional Chinese Medicine

TE --------Trolox equivalent

TEAC -----Trolox equivalent antioxidant capacity

TLC -------Thin Layer Chromatography

TM ------- Traditional Medicine

TNF -------- Tumor Necrosis Factor

TOSC -------Total oxyradical Scavenging Capacity

TRAP ------ Total Radical Trapping Parameter VCAM ----- Vascular Cell Adhesion Molecule

WDR------- Wide Dynamic Range

WHO------- World Health Organization

Chapter 1 INTRODUCTION

1.1 GENERAL INTRODUCTION

Traditional medicine is the sum total of knowledge, skills and practices based on the theories, beliefs and experiences indigenous to different cultures that are used to maintain health, as well as to prevent, diagnose, improve or treat physical and mental illnesses (WHO, 2008). Traditional medicine may also incorporate health practices, approaches, knowledge and beliefs incorporating plant, animal and mineral based medicines, spiritual therapies, manual techniques and exercises, applied singularly or in combination to treat, diagnose and prevent illnesses or maintain well-being"(WHO, 2008).

Alternative or complementary medicine is traditional medicine that has been adopted by other populations (outside its indigenous culture).Herbal medicines include herbs, herbal materials, herbal preparations, and finished herbal products that contain parts of plants or other plant materials as active ingredient. Countries in Africa, Asia and Latin America use traditional medicine (TM) to help meet some of their primary health care needs. In Africa, up to 80% of the population uses traditional medicine for primary health care and many of these medicines involve the use of plant extracts or their active principles (WHO, 2008). In view of the fact that up to 80% of the population of Africa uses various forms of traditional medicine, further research is needed to confirm the claims of efficacy and establish the safety of the practices and medicinal plants used by traditional medicine systems.

Ghana is one of the few African countries which have made remarkable progress in the promotion, development and practice of traditional medicine as part of their healthcare delivery

systems. To give legal backing and show commitment to incorporate traditional medicine into the orthodox medicine practice, the Parliament of Ghana passed an act which was signed into law by government (The Traditional Medicine Practice Act 595, 2000). The Act defines traditional medicine as "practice based on beliefs and ideas recognized by the community to provide health care by using herbs and other naturally occurring substances" and herbal medicines as "any finished labelled medicinal products that contain as active ingredients aerial or underground parts of plants or other plant materials or the combination of them whether in crude state or plant preparation".

1.2 JUSTIFICATION

In various countries, especially the developing ones, Ghana inclusive, the population depends largely on traditional forms of medicine namely; Ayurveda in India, herbal medicine especially in Africa and traditional Chinese medicine (TCM) in China. Plants or plant parts constitute the main ingredients in all these practices. In Ghana there are a number of drawbacks in our healthcare delivery system, particularly the orthodox or western medicine. These include difficulty in accessing the services, high cost of treatment even in the advent of the National Health Insurance Scheme and the unacceptability of orthodox medicine by some social set-ups and religious sects.

An appreciable number of people among the population of Ghana still depends on medicinal plants or other forms of herbal remedies for healthcare needs hence the need for investigation to establish the scientific basis for the uses of these plants. A greater percentage of the patrons

of these herbal remedies are mainly those who want a panacea to mitigate or control acute or chronic pain.

Pain is a normal manifestation of everyday life and serves as a vital defensive function. However, uncontrolled pain can dramatically diminish quality of life (Curtis *et al.*, 2002) and for this reason compounds that can control or eliminate pain have to be looked for through research. Pain is a nagging issue and affects everybody. Man is constantly making effort to mitigate pain which is a problem for all mankind especially as man advances in age.

The leaves and roots plant *P.hirsuta* have been used by folklore to control pain and inflammation and such use has also been confirmed scientifically by researchers at the Department of Pharmacology, Faculty of Pharmacy and Pharmaceutical Sciences. Further research into the plant is therefore needed to establish the constituents responsible for the analgesic and anti-inflammatory activities of the plant. This research and others into plant medicine could help so that herbal preparations could be well-formulated and standardized to be acceptable by all. Again, investigations into the active principles of plants may provide useful analgesic and anti-inflammatory agents or "lead" compounds/constituents which could further be developed into useful agents for analgesia and anti-inflammation.

1.3 AIMS AND OBJECTIVES OF PRESENT STUDY

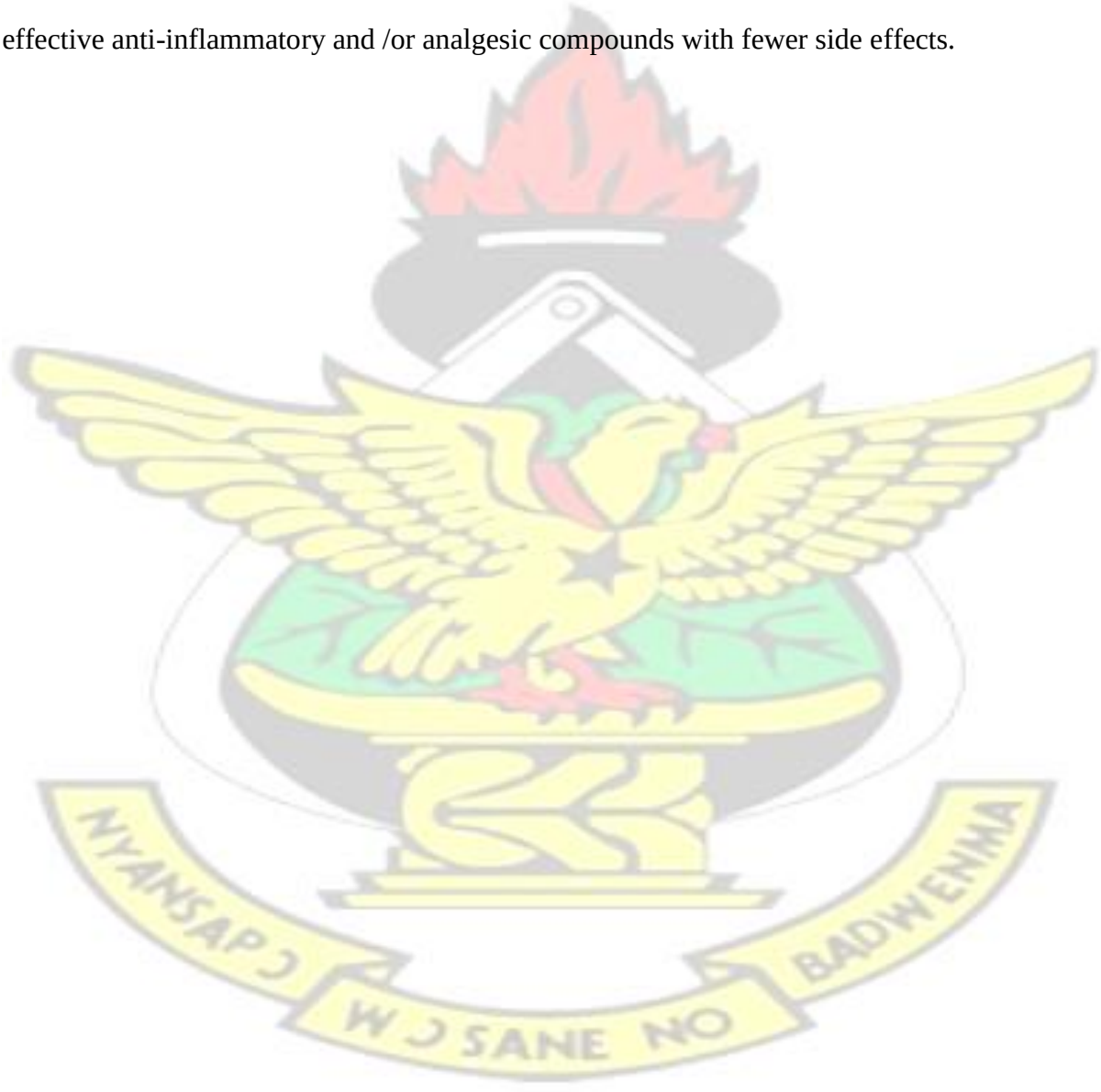
The research aims at;

1. Screening the roots of *P.hirsuta* for anti-inflammatory, anti-oxidative and analgesic activities.
2. Fractionating the extract by bioassay guided method.

3.Isolate and characterize the constituent(s) that may be responsible for anti-inflammatory and analgesic activities.

4. Possibly present the isolates that have anti-inflammatory and analgesic properties as alternatives for pain management.

6. Present the isolates as possible 'lead' compounds for the syntheses of stronger or more effective anti-inflammatory and /or analgesic compounds with fewer side effects.



Chapter 2 LITERATURE REVIEW

2.1 THE FAMILY COMMELINACEAE

Commelinaceae is a family of flowering plants, also known as the spiderwort family. The family is divided into two subfamilies, Commelinoideae and Cartonematoideae; comprising forty genera and six hundred and fifty two species. The subfamily Commelinoideae consists of thirty-eight genera and six hundred and forty species whereas the Cartonematoideae has two genera and twelve species. The genus *Palisota* belongs to the subfamily Commelinoideae (Watson and Dallawitz, 1992). They are typically found at an altitude of 0 to 902 meters.

2.1.1 Morphology

Plants of the family may be annual or perennial and sometimes woody at base. They have stems with prominent nodes and internodes. The leaves alternate, distichous or spirally arranged. The lamina is simple and the margin entire. The flowers are bisexual and rarely unisexual. The fruits, loculicidal, 2 or 3 valved capsule, rarely baccate and indehiscent. The seeds are few and large with a copious endosperm (www. ZipcodeZoo.com)

2.1.2 The genus Palisota

This genus and especially the species *P.hirsuta* is the most commonly used of the Commelinaceae family (Akobundu and Aggakwa, 1987). The genus consists of approximately 41 species of evergreen perennial plants native to tropical Africa. They spread by rhizomes, forming a clump of lush foliage and producing erect spikes of small

flowers that are followed by berries that are often red when ripe. Outside the tropics *Palisota* species and cultivars are usually seen as greenhouse plants (www.litertaure/photos-house-plants-palisota.html).

2.1.3 Palisota hirsuta

This is a robust herb with whitish to pinkish lateral branches, white to purplish flowers found mostly in lowland rain forest. It is synonymously known as *Dracena hirsuta* (Thumberg) and *Palisota maclaudii* (Cornu, 1896).

Local names

In West Africa, *Palisota hirsuta* is known by the local names leone ndomn (Mendes tribe of Sierra Leone), Kordrubo (Massa tribe of Liberia) lebo (Nekedie tribe of Ivory Coast) and Ikpereafunu (Igbo tribe of Nigeria).

In Ghana, it is known by the local names 'Some-nini' (Ashanti), 'Sombenyin' (Fante), 'nzahuara' (Nzema) and 'Adutsyrohoti' (Ewe).

2.1.4 Botanical description

P.hirsuta is a robust plant in forest re-growths about 1.2-4m high with distinct main stem, large leaves in terminal rosettes and white to purplish flowers in lax inflorescence which open from late afternoon (around 4p.m) until dusk. The internodes reach up to 30cm in length with thickly swollen nodes. This character is noted in the Liberian Massa name meaning 'swollen knee', and Nigerian Igbo 'sheep's knee'.

The leaves, mostly in terminal rosettes, measures 15-40 cm by 4-11.5 cm and are obovate to oblanceolate in shape. It is acute at the apex, narrow at the base and terminating in flat densely hairy petioles of about 3cm in length. The flowers occur in lax, elongated inflorescence of 10-30cm length with many slender, whitish-pink lateral branches 1-2cm long. The fruits occur as black berries, of about 0.6cm in diameter with glossy disc-shaped seeds (Akobundu and Aggakwa, 1987).

Aerial parts of *Palisota hirsuta*

Figure 2.2.1Aerial parts of *Palisota hirsuta*

2.1.5 Ecological and geographical distribution

It is commonly distributed from Senegal to Zaire and found in abundance in southern Ivory Coast. It occurs as a weed on farmlands and in forest zones. The plant is grown as an ornamental and is sometimes a component of hedges (Burkill, 1985).

2.1.6 Traditional medicinal uses

Palisota hirsuta is used traditionally for its pain-relieving and antiseptic effects. In Gabon, twig scrapings are used for quick calcitrisation of wounds. Warmed leaves help to ease back pains. A decoction of the twigs is used for gonorrhoea. In the Ivory Coast, root scrapings are used as a suppository for vomiting, diarrhoea, female sterility and as an aphrodisiac. The plant juice and crushed pieces of twigs are used as a poultice for fractures, bruises, joint pains, nail ulcers and lymph gland inflammation. The whole plant decoction is used for urethral problems. Leaf preparations are used against lice(Bellomaria and Kacou, 1995). In Ghana and Nigeria the stem is chewed as a sedative and for cough (Ainslie, 1937).

In Sierra Leone, the boiled leaves are considered to be very effective for gonorrhoea. Dried leaves are smoked like tobacco in southern Nigeria for headaches. The Igbos use the extract for steam bath to treat malaria. Decoction of the root is gargled for toothache and sore throats. The extracts of the leaves and stems are medication for arthritis and inflammation. The young leaves are eaten in Nigeria (Dokosi, 1998). The roots and leaves are traditionally used to treat infertility and the roots alone used to treat anaemia, dysentery and rheumatism (Mshana *et al.*, 2000).

2.1.7 Biological activity

Benson *et al.* (2008) investigated the use of *Palisota hirsuta* leaves as an aphrodisiac in traditional herbal medicine in Côte d'Ivoire. Assessment of sexual stimulant potential of total flavonoids extracted from the leaves was done. Total flavonoids extracted from *P.hirsuta* leaves modified the sexual parameters such as the latent time of observation (LTO) and the number of tentative intromissions (NTI) (vaginal penetration) among the male rats. Indeed, administration of unique dose (27.59 mg kg^{-1} of body weight) of flavonoids to male rats led to a reduction of LTO and an increase of NTI. This revealed the sexual stimulation activity of the leaves of *Palisota hirsuta* justifying its use as an aphrodisiac in traditional medicine.

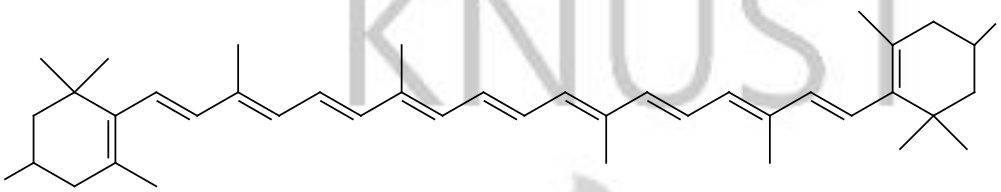
In another study, (Boakye-Gyasi *et al.,* 2009a), investigated the anti-arthritic and antipyretic activities of ethanolic extract of the leaves of *P. hirsuta* using the Freund's adjuvant induced arthritis in rats' model. The extract at 30-300mg/kg significantly reduced the arthritic oedema in the ipsilateral paw with the highest dose used giving a maximum inhibition of 13.02 ±8.77%. It also prevented the spread of the oedema from the ipsilateral paw indicating inhibition of systemic spread (Boakye-Gyasi *et al.,* 2008). The methanol extract of the leaves of the plant *Palisota hirsuta* showed antiviral activity against herpes simplex, sindbis virus and poliovirus (Anani *et al*., 2000; Hudson *et al.,* 2000)

2.1.8 Phytochemistry

Kusamba *et al.,* (1995) isolated two rare phytoecdysones (ecdysteroids), ecdysterone and 20, 26-dihydroxyecdysone along with stigmasterol and eicosanoic acid from the rhizomes.

In a previous study, Egger and Kleinig-Voigt (1968) had reported carotenoids from the fruits with β-citrannine as the main pigment. It is esterified with the saturated fatty acid

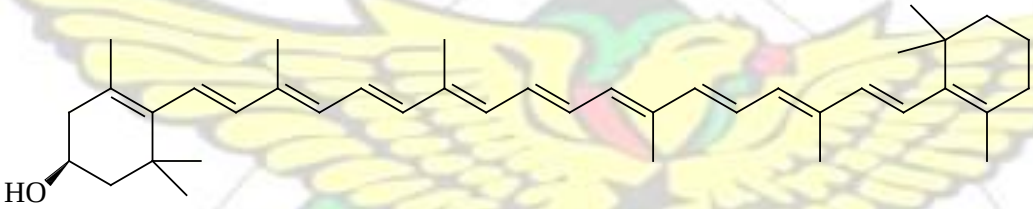
from C_4, C_6 up to C_{18}. The major fatty acid is lauric acid. Minor pigments are zeaxanthine, cryptoxanthine, *β*-carotene and *β*-apo-8' carotenol (Egger and Kleinig-Voigt, 1968).



OH

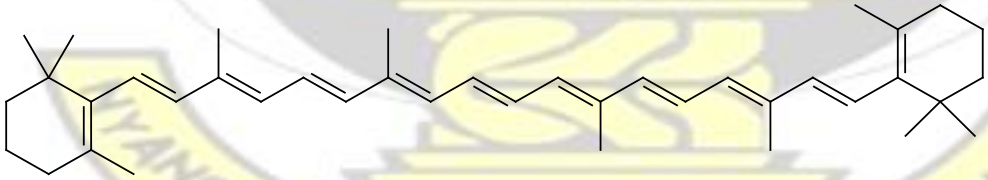
HO

Zeaxanthine

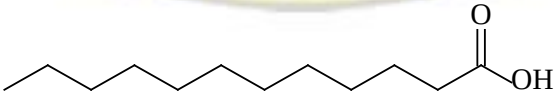


HO

Cryptoxanthine



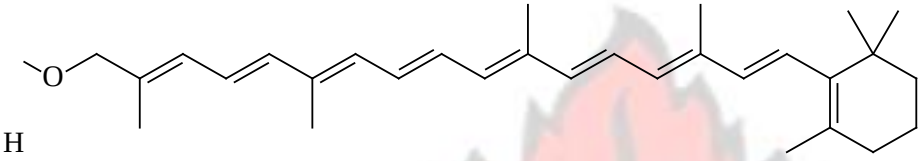
***β*-carotene**



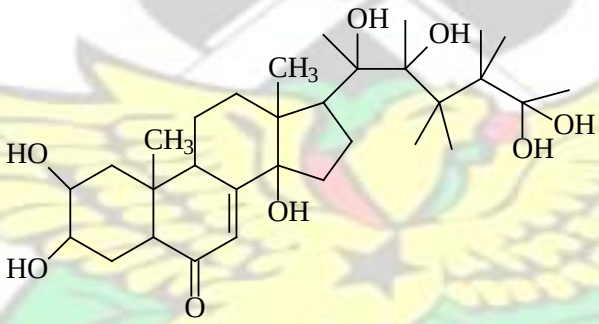
O

OH

Lauric acid



***β*-apo-8′-carotenol**



20, 26- dihydroxyecdysone

There is a report on the chemical composition and some anti-nutritive components of the fresh leaves from the apical portion of the branches of *Palisota hirsuta*. The leaves contain 93.60% dry matter, 15.34% crude protein, 10.90%crude fibre, 2.10% ether extract, 10.80% ash, 54.46% nitrogen free extract, 48.75% acid detergent fibre, 49.4% neutral detergent fibre and a percentage hemi-cellulose content of 0.66%. Also the leaves contain 1.91%

tannins, 17.40 mg g^{-1}phytin/phytic acid and 1.72 mg g^{-1} of hydrogen cyanide acid (Okoli *et al.*, 2003).

2.2 PAIN

In medicine, pain is considered as highly subjective. A definition that is widely used in nursing was first given as early as 1968: "Pain is whatever the experiencing person says it is, existing whenever he says it does" (McCarffery, 1968). It is a major symptom in many medical conditions, significantly interfering with a person's quality of life and general functioning. Diagnosis is based on characterizing pain in various ways, according to duration, intensity, type (dull, burning, throbbing or stabbing), source, or location in body. Usually pain stops without treatment or responds to simple measures such as resting or taking an analgesic, and it is then called 'acute' pain. But it may also become intractable and develop into a condition called chronic pain, in which pain is no longer considered a symptom but an illness by itself. The study of pain has in recent years attracted many different fields such as pharmacology, neurobiology, nursing, dentistry, physiotherapy, and psychology. Pain medicine is a separate subspecialty figuring under some medical specialties like anesthesiology, physiatry, neurology, and psychiatry.

Pain typically consists of unpleasantness, motivation to withdraw or protect, and an awareness of the quality, location, intensity and duration of the pain, though it is possible to experience pain in the absence of one or more of these elements. Pain is often accompanied by negative emotions (e.g., fear, anxiety, and rage) and cognitive impairment (e.g., attention and working memory deficits) (Merskey, 1994)

For scientific and clinical purposes, pain is defined by the International Association for the Study of Pain (IASP) as an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage (Merskey, 1994). Pain is unquestionably a sensation in a part or parts of the body, and is also always unpleasant and therefore also an emotional experience.

Experiences which resemble pain but are not unpleasant, e.g., pricking, should not be called pain. Unpleasant abnormal experiences (dysesthesias) may also be pain but are not necessarily so because, subjectively, they may not have the usual sensory qualities of pain. Many people report pain in the absence of tissue damage or any likely pathophysiological cause; usually this happens for psychological reasons. There is usually no way to distinguish such experience from that due to tissue damage if it is taken as the subjective report. Inflammation and nociception both produce pain.

2.2.1 Classification of pain

Pain has constantly been described as a symptom. However, current advances in the understanding of neural mechanisms have confirmed that unrelieved pain may lead to changes in the nervous system and as such pain, particularly chronic pain, may be considered a disease in itself (Smith *et al.*, 2000). Pain is categorized according to its duration, location and etiology. Pain classified by location is helpful in communicating and treating pain; chest pain will suggest angina or myocardial infarction which will need treatment according to cardiac care standards. Burns pain and post herpetic neuralgia are however examples of pain described by aetiology. Pain can be divided into two broad categories generally: acute and chronic pain. Acute pain is also referred to as adaptive pain

since it serves to protect the individual from further injury or promote healing. However, chronic pain has been called maladaptive, a pathologic function of the nervous system or pain as a disease (Calvino *et al*, 1968).

2.2.1.1 Acute pain

Acute pain is a warning that something is not right in the body. It is pain that occurs as a result of injury or surgery and is usually self-limiting, subsiding when the injury heals. It is of short duration and lasts less than 3 to 6 months. Acute pain is of recent onset and (probably) limited duration, usually having an identified temporal and causal relationship to injury or disease. Intensity of acute pain is from mild to severe and it is use to describe conditions, such as post-operative pain, pain following trauma or procedural pain. Somatic acute pain arises from injury to skin, bone, joint, muscle, and connective tissue, and it is generally localized to the site of injury (Abdel-Salem and El-Batran, 2005; Calvino *et al*., 1988; Schim and Stang, 2004).

2.2.1.2 Chronic pain

Chronic pain is pain that persists beyond the point at which healing would be expected to be complete, or that occurring in disease processes in which healing does not take place, may be accompanied by severe psychological and social disturbance and usually lasts longer than six months and ranges in intensity from mild to severe (DeLeo, 2006). Chronic pain associated with malignancy includes the pain of cancer, acquired immunodeficiency syndrome (AIDS), multiple sclerosis, sickle cell disease, and end-stage organ system failure. The exact cause of chronic pain of a non-malignant nature may or may not be known (DeLeo, 2006). This type of pain includes the pain associated with various

neuropathic and musculoskeletal disorders such as headaches, fibromyalgia, rheumatoid arthritis, and osteoarthritis. It is now known that in chronic pain, presynaptic receptors on sensory nerve terminals in the periphery contribute to increased excitability of sensory nerve endings (peripheral sensitization) (Agarwal *et al.*, 2007). The hyperexcitable sensory neuron bombards the spinal cord, leading to increased excitability and synaptic alterations in the dorsal horn. Such changes appear to be important in chronic inflammatory and neuropathic pain states (Agarwal *et al.*, 2007; Trevisani *et al.*, 2007).

2.2.2 Types of pain

Several distinct types of pain have been described based on their pathophysiology: nociceptive, inflammatory, neuropathic, and functional. The description of the pain may be indicative of the type of pain, for example the pain may be described as sharp, dull, pin and needle, burning, knawing, etc.

2.2.2.1 Nociceptive pain

Nociceptive pain is a transient pain in response to a noxious stimulus at nociceptors that are located in cutaneous tissue, bone, muscle, connective tissue, vessels, and viscera. Nociception may be thermal, chemical, or mechanical. The nociceptive system extends from the receptors in the periphery to the spinal cord, brain stem, and to the cerebral cortex where pain sensation is perceived. This system is a key physiological function that prevents further tissue damage due to the body's autonomic withdrawal reflex (Baron and Treede, 2007; Messeguer *et al.*, 2006; Suardiaz *et al.*, 2007).

When tissue damage occurs despite the nociceptive defense system, inflammatory pain ensues. The body now changes focus from protecting against painful stimuli to protecting the injured tissue. The inflammatory response contributes to pain hypersensitivity that serves to prevent contact or movement of the injured part until healing is complete, thus reducing further damage (Anseloni and Gold, 2008; Harvey and Dickenson, 2008).

2.2.2.2 Neuropathic pain

Neuropathic pain is defined as spontaneous pain and hypersensitivity to pain associated with damage to or pathologic changes in the peripheral nervous system as in painful diabetic peripheral neuropathy (DPN), acquired immunodeficiency syndrome (AIDS), polyneuropathy, post-herpetic neuralgia (PHN); or pain originating in the central nervous system (CNS), that which occurs with spinal cord injury, multiple sclerosis, and stroke (Amado *et al.*, 2007; Baron and Treede, 2007; Garcia-Larrea and Magnin, 2008). Functional pain, a relatively newer concept, is pain sensitivity due to an abnormal processing or function of the central nervous system in response to normal stimuli.

2.2.3 Mechanism

Stimulation of a nociceptor, due to a chemical, thermal, or mechanical event that has the potential to damage body tissue, may cause nociceptive pain.

Damage to the nervous system itself, due to disease or trauma, may cause neuropathic (or neurogenic) pain. Neuropathic pain may refer to peripheral neuropathic pain, which is caused by damage to nerves, or to central neuropathic pain, which is caused by damage to the brain, brainstem, or spinal cord.

Nociceptive pain and neuropathic pain are the two main kinds of pain when the primary mechanism of production is considered.

Nociceptive pain may be classified further in three types that have distinct organic origins and felt qualities.

1. Superficial somatic pain (or cutaneous pain) is caused by injury to the skin or superficial tissues. Cutaneous nociceptors terminate just below the skin, and due to the high concentration of nerve endings, produce a sharp, well-defined, localized pain of short duration. Examples of injuries that produce cutaneous pain include minor wounds, and minor (first degree) burns.

2. Deep somatic pain originates from ligaments, tendons, bones, blood vessels, fasciae, and muscles. It is detected with somatic nociceptors. The scarcity of pain receptors in these areas produces a dull, aching, poorly-localized pain of longer duration than cutaneous pain; examples include sprains, broken bones, and myofascial pain.

3. Visceral pain originates from body's viscera, or organs. Visceral nociceptors are located within body organs and internal cavities. The even greater scarcity of nociceptors in these areas produces pain that is usually more aching or cramping and of a longer duration than somatic pain. Visceral pain may be well-localized, but often it is extremely difficult to localize, and several injuries to visceral tissue exhibit "referred" pain, where the sensation is localized to an area completely unrelated to the site of injury.

Nociception is the unconscious afferent activity produced in the peripheral and central nervous system by stimuli that have the potential to damage tissue. It is initiated by nociceptors that can detect mechanical, thermal or chemical changes above a certain threshold. All nociceptors are free nerve endings of fast-conducting myelinatedA delta fibers or slow-conducting unmyelinatedC fibers, respectively responsible for fast, localized, sharp pain and slow, poorly-localized, dull pain. Once stimulated, they transmit signals that travel along the spinal cord and within the brain. Nociception, even in the absence of pain, may trigger withdrawal reflexes and a variety of autonomic responses such as pallor, diaphoresis, bradycardia, hypotension, lightheadedness, nausea and fainting(Feinstein *et al.,* 1954).

2.2.4 The dimensions of pain

Pain has been described in terms of its three "dimensions" (Melzack *et al*., 1968);

- Sensory-discriminative (location, intensity, quality, duration)
- Motivational-affective (unpleasantness and urge to escape the unpleasantness)
- Cognitive-evaluative

The effect of thinking on the intensity and unpleasantness of pain has been closely studied and an enormous effort has been put into devising cognitive and behavioural techniques for the amelioration of pain (Vlaeyen and Morley, 2005).

Rainville *et al* (1997) tested Melzack and Casey's 1968 model using PET to monitor brain activity while their subjects' left hands were immersed in painfully hot water. Using hypnotic suggestion, these researchers were able to vary the unpleasantness experienced

by their subjects, while keeping both the stimulus intensity and their subjects' perception of the pain intensity constant. It was found that this variation in perceived unpleasantness was mirrored by variation in activity in the anterior cingulate cortex (ACC) and concluded that, though many parts of the brain exhibit heightened activity during pain, and these regions are highly interactive, nevertheless "there appears to be at least a partial segregation of function between pain affect and sensation, with ACC activity possibly reflecting the emotional experience that provokes our reactions to pain" (Melzack *et al*., 1968; Rainville *et al*., 1997).

Neuroimaging support for the involvement of the anterior cingulate cortex in pain's unpleasantness was not entirely unexpected. It had been known for decades that surgical removal of the anterior cingulate relieves the distress of pain without affecting its intensity, and this surgical intervention is used even today in cases of extreme and otherwise incurable chronic pain (Foltz and White, 1962).

2.2.5 Diagnosis and assessment

To establish an understanding of an individual's pain, health-care practitioners will typically try to establish certain characteristics of the pain: site (localization), onset and offset, character, radiation, associated symptoms, time pattern, exacerbating and ameliorating factors, and severity. According to its duration, pain may be categorized as acute (short term), sub- acute (medium term), or chronic (long term).

By using the gestalt of these characteristics, the source or cause of the pain can often be established. A complete diagnosis of pain will require also looking at the patient's general condition, symptoms, and history of illness or surgery. The physician may order blood

tests, X-rays, scans, EMG, etc. Pain clinics may investigate the person's psychosocial history and situation.

2.2.5.1 Verbal characterization

A key characteristic of pain is its quality. Typical descriptions of pain quality include sharp, stabbing, tearing, squeezing, cramping, burning, lancinating (electric-shock like), or heaviness. It may be experienced as throbbing, dull, nauseating and shooting or a combination of these. Indeed, individuals who are clearly in extreme distress such as from a myocardial infarction may not describe the sensation as pain, but instead as an extreme heaviness on the chest. Another individual with pain in the same region and with the same intensity may describe the pain as tearing which would lead the practitioner to consider aortic dissection. Inflammatory pain is commonly associated with some degree of itch sensation, leading to a chronic urge to rub or otherwise stimulate the affected area. The difference between these diagnoses and many others rests on the quality of the pain. The McGill Pain Questionnaire is an instrument often used for verbal assessment of pain.

2.2.5.2 Intensity

Pain may range in intensity from slight through severe to agonizing and can appear as constant or intermittent. The threshold of pain varies widely between individuals. Many attempts have been made to create a pain scale that can be used to quantify pain, for instance on a numeric scale that ranges from 0 to 10 points. In this scale, zero would be no pain at all and ten would be the worst pain imaginable. The purpose of these scales is to monitor an individual's pain over time, allowing care-givers to see how a patient responds to therapy

for example. Accurate quantification can also allow researchers to compare results between groups of patients.

2.2.5.3 Localization

Pains are usually called according to their subjective localization in a specific area or region of the body: headache, toothache, shoulder pain, abdominal pain, back pain, joint pain, myalgia, etc. Localization is not always accurate in defining the problematic area, although it will often help narrow the diagnostic possibilities. Some pain sensations may be diffuse (radiating) or referred. Radiation of pain occurs in neuralgia when stimulus of a nerve at one site is perceived as pain in the sensory distribution of that nerve. Sciatica, for instance, involves pain running down the back of the buttock, leg and bottom of foot that results from compression of a nerve root in the lumbar spine. Referred pain usually happens when sensory fibers from the viscera enter the same segment of the spinal cord as somatic nerves, i.e., those from superficial tissues. The sensory nerve from the viscera stimulates the nearby somatic nerve so that the pain localization in the brain is confused. A well-known example is when the pain of a heart attack is felt in the left arm rather than in the chest.

2.2.6 Management

In general, physicians are more comfortable treating acute pain, which usually is caused by soft tissue damage, infection and/or inflammation among other causes. It is usually treated simultaneously with pharmaceuticals, commonly analgesics, or appropriate techniques for removing the cause and for controlling the pain sensation. The failure to treat acute pain properly may lead to chronic pain in some cases (Dahl and Moiniche, 1998; Greco *et al.*, 2000).

2.2.6.1 Anesthesia

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

2.2.6.2 Analgesia

Analgesia is an alteration of the sense of pain without loss of consciousness. The body possesses an endogenous analgesia system, which can be supplemented with painkillers or analgesic drugs to regulate nociception and pain. Analgesia may occur in the central nervous system or in peripheral nerves and nociceptors. The perception of pain can also be modified by the body according to the gate control theory of pain.

The endogenous central analgesia system is mediated by three major components : the periaqueductal grey matter, the nucleus raphe magnus, and the nociception-inhibitory neurons within the dorsal horns of the spinal cord, which act to inhibit nociceptiontransmitting neurons also located in the spinal dorsal horn. The peripheral regulation consists of several different types of opioid receptors that are activated in response to the binding of the body's endorphins. These receptors, which exist in a variety of areas in the body, inhibit firing of neurons that would otherwise be stimulated to do so by nociceptors (Straube *et al*, 2009).

2.2.7 Alternative and complementary medicine

A survey of American adults found pain was the most common reason that people use complementary and alternative medicine. Traditional Chinese medicine views pain as a 'blocked' qi, akin to electrical resistance, with treatments such as acupuncture claimed as more effective for non-traumatic pain than traumatic pain. Although the mechanism is not fully understood, acupuncture may stimulate the release of large quantities of endogenous opioids (Sapolsky, 1968).

Pain treatment may be sought through the use of nutritional supplements such as curcumin, glucosamine, chondroitin, bromelain and omega-3 fatty acids.

Hypnosis as well as diverse perceptional techniques provoking altered states of consciousness have proven to be of important help in the management of all types of pain (Ornstein and Sobel, 1988). Some kinds of physical manipulation or exercise are showing interesting results as well (Degood *et al*., 1997).

2.2.8 Animal models of nociception

Animal models have been used extensively in basic pain research based on the premise that these models can serve as surrogate assays that can reliably predict the potency and efficacy of the pharmacologic action of, and, in some cases, the molecular response to, agents that work in human pain states (Yaksh, 1999). In contrast to the polymorphic nature of pain that is described as a sensation in humans, pain in animals can best be estimated only by examining their reactions to various chemical, thermal, and mechanical stimuli, with the latency or nature of response altered in the “pain” state. There has been a progressive increase in publications on the use of the formalin model and the various tests that involve

withdrawal of the paws from mechanical stimuli, or tactile allodynia (LeBars *et al.*, 2001). This certainly reflects the heightened interest in understanding the mechanisms of pain and the need to have reliable methods to test interventions preclinically in rat models. One easy way to describe and delineate the great variety of animal models for pain would be to separate them by differences in the stimulus required, the time course of development of the injury or response, and identification of the afferent nociceptive fibers or spinal or supra spinal systems involved (if known) (LeBars *et al.*, 2001; Yaksh, 1999).

2.2.8.1 Short-Duration Stimuli Tests (Acute Phasic Pain)

Acute tests, such as hot-plate, tail-flick, and paw pressure tests, require a high-intensity stimulus (such as thermal, mechanical, or chemical) and do not test a pre-injured animal.

The response measured(Argoff, 2002) is immediate (or within seconds), (Goldstein, 2001) uses the Aδ- and C-fiber input, and (MacPherson, 2002) is known to activate the spinal dorsal horn, the cells of which are nociceptive-specific and/or wide dynamic range (WDR) neurons. In addition, the response is proportional to the frequency of stimulus and the fiber class of afferent input. Some of these acute tests are based on the use of thermal stimuli, such as the tail-flick test, which uses a radiant heat source and an automated timer to determine the withdrawal time of the tail (Bass and Vanderbrook, 1952). A variation of this test increases the area of stimulation and, rather than aiming a hot stimulus at the base of the tail, requires a complete immersion of the animal's tail in hot water (Ben-Bassat *et al.*, 1959). Such thermal tail-flick tests are most widely and reliably used for revealing the potency of opioid analgesics, useful for predicting analgesic effects in humans (Grumbach, 1964). Another acute pain test that uses a thermal stimulus is the hot-plate test, in which a rat or mouse is placed in an open-ended cylindrical space with a floor capable of being

precisely heated (O'Callaghan and Holzman, 1975). The plate floor, heated to a constant temperature, produces two responses, measured in terms of their reaction times: paw licking and jumping/ lifting. Both are considered supraspinally integrated responses. Such "chaotic defensive movements" are complex in the rat (compared with the mouse), making observation and identification difficult. Thus, this can be a very inconsistent test to use. The paw-pressure, or mechanical hyperalgesia, test uses a pressure of increasing intensity applied to a punctiform area on the hind paw or, far less commonly, on the tail. In practice, the paw or tail is placed between a plane surface and a blunt, plastic-coated point mounted on top of a system of cogwheels, with a cursor that can be displaced along the length of a graduated beam (Green *et al*., 1951) for an automated readout. The application of increasing pressure is interrupted when the animal removes its tail, an action that is read out as force in grams for the threshold of response. However, the intensity is difficult to measure reproducibly, and is more often used when the paw is injured beforehand, by inflammation or nerve injury; then the threshold is compared to the non-injured paw (Randall and Selitto, 1957).

2.2.8.2 Long-Duration Stimuli Tests (Tonic Pain)

These tests use an irritant, foreign chemical agent as the nociceptive stimulus. They differ from most other pain tests (Argoff, 2002) in that they do not measure a threshold response; (Goldstein, 2001) they quantitatively measure the resulting behavior after the stimulus, which varies in potency with time; (MacPherson, 2002) they are not models of chronic pain, since the duration of the behaviors is short, usually minutes or tens of minutes. Hence, long-duration stimuli tests are considered models of tonic pain. They are usually based on intradermal or intraperitoneal injections of the agent.

Closely related are the weeks-long, chronic inflammatory pain models that use the intracapsular administration of urate crystals, Freund's adjuvant, capsaicin, or carrageenan (Coderre and Wall, 1987; Otsuki *et al*, 1986; Tonussi and Ferreira, 1992). Such long-term tonic pain in rats has been used to model human arthritis and to examine the safety and efficacy of various nonsteroidal anti-inflammatory drugs (NSAIDs) (Fiorucci *et al.,* 2001), including the COX-1 and COX-2 inhibitors commonly used by patients for inflammatory pain (Guilianao and Warner, 2002).

2.2.9 Intradermal injections

Formalin, a 37% solution of formaldehyde, is the most commonly used agent for intradermal paw injection to induce nociception (the formalin test) (Heapy *et al*., 1987). Other agents less commonly used are hypertonic saline (Hwang and Wilsox, 1986), Freund's adjuvant (Teiger, 1976), ethylene diamine tetra-acetic acid (Iadarola *et al*., 1988), capsaicin (Sakurada *et al.,* 1992), or bee sting (Lariviere and Melzack, 1996). A 0.5 - 15 percent solution of formalin (usually about 3.5%) injected into the dorsal or plantar surface of the rat fore or hind paw produces a biphasic painful 60 minutes after the injection. Typical responses include the paw being lifted, licked, nibbled, or shaken (Lariviere and Melzack, 1996); these responses are considered nociceptive, since formalin predominantly evokes activity in C- fibres, and not in Aδ- afferents (Humnskaar *et al.,* 1985). The initial phase of the response, which lasts 3 to 5 min, is probably due to direct chemical stimulation of nociceptors(Watson *et al*., 1997); this is followed by 10-15 min during which animals display little behavior suggestive of nociception. The second phase of this response starts about 15 - 20 min after the formalin injection and lasts 20-40 min, initially rising with both number and frequency of nociceptive behaviors, reaching a peak, and then falling off. The

intensities of these nociceptive behaviours are dependent on the concentration of formalin used, and the second phase involves a period of sensitization during which inflammatory phenomena occur. These inflammatory phenomena are possibly a result of central processes triggered by the neuronal activation during the first phase, since glutamate NMDA receptor antagonists significantly and dose-dependently reduce nociceptive activity during the second phase of the formalin test when they are given before the formalin (Berrino *et al*., 2003). A few approaches have been used to compute a composite pain score that is weighted according to the time spent in each behavioural category—for example, per 5 min interval over 60 min after injection (Watson *et al.*, 1997). This method and its modifications are all based on the concept that the different behaviors express degrees of a single nociceptive experience, and can be expressed as a single number, or pain score. In rats, other behaviors, such as flinching or jerking of the injected paws, have also been quantified; but scoring these common behavioural responses becomes more difficult in mice, due to rapid movements in these animals. Again, scoring of the time spent licking, or licking and biting, the injected paw is the most common method of behavioral assessment in mice (Calvino *et al.*, 1988). Opioid analgesics provide analgesia for both phases of the behavioral response (but the second, delayed phase is more sensitive), while agents such as NSAIDs only suppress the second phase (McCormack *et al.*, 1998). Thus, the formalin test is best used to examine opioid mimetics.

2.2.9.1 Intraperitoneal Injections of Irritants ("Writhing Test")

Intraperitoneal injection of agents (originally phenylbenzoquinone) that are irritating to serous membranes provokes a stereotypical behaviour in rodents that is characterized by abdominal contractions, whole body movements, contortions of the abdominal muscles,

and reduced motor activity and incoordination. In this test, commonly called the "writhing test," the behaviors are considered reflexive, and are evidence of peritoneovisceral or visceral pain associated with visceral chemoreceptors (Hammond, 1989). Unfortunately, the frequency of cramps decreases spontaneously with time to such an extent, and with such variability, that is difficult to evaluate the effect of an analgesic on the behaviors of any single animal (Loux *et al.*, 1978). Even with multiple modifications in the nature of the chemical irritant used, the concentration, temperature, and volume of the injectant, and other modifications to simplify the test and measurements of behaviors, the test lacks specificity, because these tests work so well for all major and minor analgesics, as well as non-analgesic substances such as muscle relaxants. Even with poor specificity of action, the writhing test can predict effective analgesic doses for agents that can be used in humans (Dubinsky *et al*., 1987).

2.3 INFLAMMATION

Inflammation is considered to be a morbid process affecting some part of the body, characterized by excessive heat, swelling, pain, and redness. It is a common factor in arthritic diseases or osteoarthritis (Andrejus, 1988) and a cardinal host defense response to injury, tissue ischaemia, autoimmune responses or infectious agents. Many diseases are now recognized to have an inflammatory component as part of the pathophysiology (e.g. rheumatoid arthritis). Inflammation often elicits a generalized sequence of events known as the acute phase response which is characterized by classical features of swelling, redness, heat and often pain. The essence of inflammation is to contain and eradicate local injury and then initiate repair of the damage. It is a major component of the damage caused

by auto-immune diseases, and is also a fundamental contributor to diseases such as cancer, diabetes, and cardiovascular disease. In health, acute inflammation is often self-limiting, but when the acute inflammatory response is "deranged" aberrant and exacerbated neutrophil responses can lead to tissue damage and chronic inflammatory sequelae. Thus, chronic inflammation occurs when acute inflammation remains unresolved. Chronic inflammation is a slow smoldering reaction that continues for months or even years and involves destruction of tissue as well as local proliferation of cells and connective tissue. In terms of what is happening locally within the tissues, the changes can be divided into vascular and cellular events.

2.3.1 The vascular events

These result in fluid exudation from vessels to the site of injury. The fluid exudates contain a variety of mediators which influence the cells in the vicinity and the blood vessels themselves. These include the components of four proteolytic enzymes cascades: the complement system, the coagulation system, the fibrinolytic system, and the kinin system. These components are proteases that are inactive in their native form; they are activated by proteolytic cleavage, each activated component then activating the next. The activation of these components gives rise to more inflammatory mediators.

2.3.2 The cellular events

The cells that are involved in the mediation of inflammation include neutrophils, mast cells, tissue macrophages, platelets and leucocytes. From the blood, platelets and leucocytes move into the inflammatory area but the neutrophils, mast cells and tissue macrophages are normally present in tissues (Rang *et al.*, 2003).

Neutrophils: These are characteristic of inflammation in the early stages - they are the first cells to appear in an infected area, and any section of recently inflamed (within a couple of days or so) tissue viewed under a microscope will appear packed with them. They perform many important functions, including phagocytosis and the release of extracellular chemical messengers. Neutrophils only live for a couple of days in these interstitial areas, so if the inflammation persists for a longer duration then they are gradually replaced by longer lived monocytes.

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

Mast cells: They are a type of innate immune cell that resides in the connective tissue and in the mucous membranes, and are intimately associated with pathogen defense and wound healing, and are often also associated with allergy and anaphylaxis. When activated by stretch receptors, mast cells rapidly release characteristic granules, rich in histamine and heparin, along with various hormonal mediators, and chemokines, or chemotactic

cytokines into the environment. Histamine dilates blood vessels, causing the characteristic signs of inflammation, and also recruits neutrophils and macrophages. This is especially important in cases of trauma. Generally, mast cells make molecules that help repair traumatic injuries, including those involved with blood clotting (Stvrtinova *et al,* 1995; Johnson, 1994; www.cc.columbia.edu/cu/cup/Morefrom encyclopedia).

Leucocytes: Various leucocytes are involved in the initiation and maintenance of inflammation. These cells can further be stimulated to maintain inflammation through the action of adaptive cascade through lymphocytes: T Cells, B Cells, and antibodies.

2.3.3 Characteristics of inflammation

The redness/heat associated with an inflamed tissue is caused by increased blood supply to the affected area. Blood vessels are vasodilated upstream of an infected area while capillary permeability to the affected tissue is increased, resulting in a loss of blood plasma. Vasoconstriction downstream of the infected area further increases oedema/swelling. The swelling distends the tissues, compresses nerve-endings and thus causes pain. The WBCs/leucocytes take on an important role in inflammation; they extravasate from the capillaries into tissues, and carry on as phagocytes picking up bacteria and cellular debris. They may also aid by walling off an infection and preventing its spread. If inflammation persists, released cytokines IL-1 and Tumor Necrosis Factor (TNF) will activate endothelial cells to upregulate receptors VCAM, ICAM-1, E-selection and L-selection for various immune cells. Receptor upregulation increases extravasation of neutrophils, monocytes, activated T-helper and T-cytotoxic and memory T and B cells to infected site.

2.3.4 Importance of inflammation

It is one of the first responses of the immune system to infection or irritation. Inflammation serves to establish a physical barrier against the spread of infection and to promote healing of any damaged tissue following the clearance of pathogens. Mechanistically, inflammation results in the recruitment of cells, called neutrophils, to the site of injury. Neutrophils then trigger the immune system by releasing factors that summon other innate immune cells and lymphocytes. The inflammatory response is characterized by the following quintet: redness, heat, swelling, pain and possible dysfunction of the organs or tissues involved. Inflammation is stimulated by chemical factors released by injured cells. These factors (histamine, bradicine, serotonin, leucotrienes) sensitize pain receptors, cause vasodilation of the blood vessels at the scene, and attract phagocytes, especially neutrophils (Stvrtinova *et al.*, 1995)(www.cc.columbia.edu/cu/cup/Morefrom encyclopedia).

2.3.5 Resolution of inflammation

When inflammation subsides, the damaged tissue is repaired. Depending on the severity of the inflammation and the type of tissue involved repairs may or may not be complete; in minor inflammations of the skin, for example, the tissue is capable of complete regeneration whereas in nervous tissue regeneration may be limited and the damaged cells may be replaced with scar tissue (www.cc.columbia.edu/cu/cup/Morefrom encyclopedia).

2.3.6 Experimental models of inflammation

Paw oedema, sponge implantation, pleurisy and air pouch granulomas are among the models that are used for inflammation. These models employ a variety of agents like formalin, Freunds adjuvant, carrageenan, monosodium urate crystals and zymosan (Higgs,

1989; (Singh *et al.*, 2000). Other agents include vasoactive agents e.g. platelet activating factor and histamine, weakened bacteria such as *E. coli*, chemotactic factors [e.g. N-formyl-norleucyl-phenylalanine], injection of polymorphonuclear leucocyte (PMNL) leucotriene B4, and arachidonic acid in acetone (Issekutz, 1989).

2.3.7 Models of acute inflammation

Injecting inflammatory agents such as carrageenan, yeast, dextran, latex, PMNL, chemotactic factors, leucotriene B4,zymosan, vasoactive agents [e.g. platelet activating factor and histamine], and arachidonic acid into various parts of the body may induce acute inflammatory responses (Issekutz, 1989). The inflammatory effect can then be assessed by monitoring the responses/reactions such as foot volume increase produced by oedema (e.g. in the chick's paw), presence of plasma markers in the skin, measurement of inflammatory mediators in plasma exudates, local rise in the temperature of the skin, hyperaemia (an increase in vascular permeability), monocyte infiltration, PMNL and lymphocyte accumulation, quantization of haemorrhage, platelet deposition and thrombosis using diverse techniques. Hyperaemia and the emigration of leucocytes are the basic manifestations of the acute inflammatory reaction (Issekutz, 1989). Among the lot, the most acceptable preliminary screening test for antirheumatic activity is the carrageenan induced acute footpad oedema in laboratory animals. This model has been widely used to screen new anti-inflammatory drugs (Singh *et al.*, 2000) and will be used in this current investigation because of its simplicity and lower cost.

2.4 OXIDANTS AND ANTI-OXIDANTS

Oxidants also known as oxidizing agents are chemical compounds that easily transfer oxygen or substances that gain electrons in a redox chemical reaction. Anti-oxidants help organisms' deal with oxidative stress, caused by free radical damage. Free radicals are chemical species, which contain one or more unpaired electrons which make them highly unstable and cause damage to other molecules by extracting electrons from them in order to attain stability (Shahin *et al.*, 2008). Excess free radicals can result from tissue damage and hypoxia, overexposure to environmental factors (smoking, ultraviolet radiation, and pollutants), a lack of antioxidants, or destruction of free radical scavengers. When the production of damaging free radicals exceeds the capacity of the body's antioxidant defenses to detoxify them, a condition known as oxidative stress occurs. The cellular injury caused by oxidative stress has been linked to over 200 clinical disorders (Kohen *et al*., 1999).

Any free radical involving oxygen is referred to as reactive oxygen species (ROS) (McDermott, 2000). The most commonly formed ROS are superoxide anion radical ($O^{2\bullet-}$) and hydroxyl radical (•OH) (Wilson and Mogil, 2001; Kendler, 1995). $O^{2\bullet-}$ is formed when one electron is added to an oxygen molecule, and is considered the least reactive type of ROS (Kohen and Nyska, 2002). Once $O^{2\bullet-}$ is produced, it triggers a rapid cascade of events that creates other free radicals, eventually terminating in the formation of H_2O. In humans, $O^{2\bullet-}$ is the most commonly produced free radical. Phagocytic cells such as macrophages and neutrophils are prominent sources of $O^{2\bullet-}$. In an inflammatory response, these cells generate free radicals that attack invading pathogens such as bacteria.

Production of $O^{2\bullet-}$ by activated phagocytic cells in response to inflammation is one of the most studied free radical-producing systems (Gutteridge and Mitchell, 1999).

If oxygen attracts two hydrogen molecules, hydrogen peroxide (H_2O_2) is formed. H_2O_2, though not technically considered an oxygen free radical, is a member of the ROS family and may selectively participate in free radical generation (Kerr *et al*., 1996). The majority of the H_2O_2 is broken down to oxygen and water by the cellular enzyme catalase. In addition to catalase, the enzyme glutathione peroxidase is responsible for the breakdown of H_2O_2 and any peroxides that form on lipids within the body (Gutteridge and Mitchell, 1999).

The hydroxyl radical (•OH) is the most reactive of the free radical molecules (Droge, 2002) and damages cell membranes and lipoproteins by a process called lipid peroxidation. Lipid peroxidative damage to lipids in low density lipoprotein (LDL) plays an important role in atherosclerosis (Kerr *et al.*, 1996).

2.4.1 Antioxidants

Antioxidants help organisms deal with oxidative stress, caused by free radical damage. Reactive oxygen species (ROS) formed *in vivo*, such as superoxide anion, hydroxyl radical and hydrogen peroxide, are highly reactive and potentially damaging transient chemical species. These are continuously produced in the human body, as they are essential for energy supply, detoxification, chemical signaling and immune function. ROS are regulated by endogenous superoxide dismutase, glutathioneperoxidase and catalase but due to over-production of reactive species, induced by exposure to external oxidant substances or a failure in the defense mechanisms, damage to cell structures, DNA, lipids and proteins

(Valko *et al.*, 2006) occur which increases risk of more than 30 different disease processes (Arouma, 1998). The most notorious among them being neurodegenerative conditions like Alzheimer's disease (AD) (Smith *et al*, 1996; Smith *et al*, 2000) mild cognitive impairment (MCI) (Guidi et al., 2006) and Parkinson's disease (PD) (Bolton *et al.*, 2000). Other neurodegenerative diseases significantly associated with oxidative stress include multiple sclerosis, Creutzfeldt–Jacob disease and meningoencephalitis. Other diseases include highly disabling vascular pathologies like cardiovascular disease (CVD) and cardiac failure (Jha *et al.*, 1995), alcohol-induced liver disease (ALD) (Arteel, 2003) and Ulcerative colitis (Ramakrishna *et al*., 1977) and cancer caused by a complex of different causes, of which RNS/ROS is a component. Valko *et al*.,(2007) have done an extensive review on the effect of free radicals and antioxidants in normal physiological functions and human disease. All these diseases are associated with significant increase in the specific and persistent lipid peroxidation marker F2-isoprostane (Greco *et al.*, 2000). The hydroxyl radical is known to react with all components of the DNA molecule, damaging both the purine and pyrimidine bases and also the deoxyribose backbone (Halliwell and Gutteridge, 1999). Besides DNA, ROS also attack other cellular components involving polyunsaturated fatty acid residues of phospholipids (Siems *et al*., 1995), side chains of all amino acid residues of proteins, in particular cysteine and methionine residues (Stadtman, 2004). The body interestingly possesses defence mechanisms against free radical-induced oxidative stress, which involve preventative mechanisms, repair mechanisms, physical defenses and antioxidant defenses. Enzymatic antioxidant defenses include superoxide dismutase (SOD), glutathioneperoxidase (GPx) and catalase (CAT). The non-enzymatic antioxidants include ascorbic acid (vitamin C), a-tocopherol (vitamin E), glutathione (GSH),

carotenoids and flavonoids. All these act by one or more of the mechanisms like reducing activity, free radical-scavenging, potential complexing of pro-oxidant metals and quenching of singlets oxygen. A brief description of mechanism of actions of some antioxidants in different diseases is described in Table 2.1 (Ali *et al*, 2008). It is possible to reduce the risks of chronic diseases and prevent disease progression by either enhancing the body's natural anti-oxidant defenses or by supplementing with proven dietary antioxidants (Stanner *et al.*, 2004). This is one of the reasons why discovery and synthesis of novel antioxidants is a major active area. Synthetic antioxidants like butylatedhydroxytoluene (BHT) and butylatedhydroxyanisole (BHA) commonly used in processed foods have side effects and are carcinogenic (Branen, 1995; Ito *et al.*, 1983). In recent years, the use of natural antioxidants present in food and other biological materials has attracted considerable interest due to their presumed safety, nutritional and therapeutic value (Ajila *et al*., 2007). Nutraceuticals are supposed to hold the key to a healthy society in the coming future. Anti-oxidants derived from fruits, vegetables, spices and cereals are very effective and have reduced interference with the body's ability to use free radicals constructively (Kahkonen *et al*, 1999; Wolfe *et al*, 2003). Natural antioxidants mainly come from plants in the form of phenolic compounds, flavonoids, phenolic acids and alcohols, stilbenes, tocopherols, tocotrienols, ascorbic acid and carotenoids. The quest for natural antioxidants for dietary, cosmetic and pharmaceutical uses has become a major industrial and scientific research challenge over the last two decades. Efforts to gain extensive knowledge regarding the power of antioxidants from plants and to tap their potential are therefore on the increase.

Table.2.1 Mechanism of action of various anti-oxidants against different disease

Compound	Pathology	Mechanism of action	References
Catalase (CAT)	Cancer diabetic retinopathy	Destroys hydrogen peroxide in high concentration by catalyzing its Two-electron dismutation into oxygen and water	(Shaunbaum and Chance, 1976)
Glutathione Peroxide (GPx)	Neurodegenerative disease	Catalyse the reduction of hydroperoxide at the expense of GSH. In this process, hydrogen is reduced to water whereas organix Hydroperoxides are reduced to alcohol	(Ursini *et al*, 1995)
Superoxide Dismuthase	Neurodegenerative	Catalyse the one-electron dismutation of superoxide into hydrogen peroxide oxygen	(Fridovich, 1995)
Alkaloids	Cancer, Neurodegenerative Disease, Chronic Inflammation	Shown a variety of biologicl activities such as inhibition of topoisomerase I and and II; cytotoxicity against different tumor cell lines	(Radisky *et al*, 1995) (Gunasekera *et al*, 2003)
Catechins	Neurodegenerative disease	Enhance activity of SOD and catalse	(Levites *et al*, 2001)
Carotenoids	Cancer, diabetic retinopathy, Chronic inflammation	Mainly act as physical quenches of reactive oxygen	(Sundquist *et al*, 1994)
A-tocopherol	Cancer, Neurodegenerative Disease, Chronic Inflammation	Scavenges lipid peroxyl radicals (LOO) through hydrogen atom transfer	(Burton and Ingold, 1994)

Compound	Pathology	Mechanism of action	References
(-)-EGCG	Neurodegenerative conditions	Decreases the expression of proapototic genes (bax, bad, caspase-1 And -6, cyclin dependent kinase inhibitor) thus maintaining the Integrity of the mitochondrial membrane	(Levites *et al*, 2003)
(-)-EGCG	Cancer, diabetic retinopathy Chronic inflammation	Suppression of angiogenesis by inhibiting growth factor triggered activation of receptors and Pkc. Down regulation of VEGF production in tumour cells. Repression of AP-1,NF-B, and STAT 1 Transcription factor pathways	(Wollin and Jones, 2003)
Ferulic acide	Diabetes	Decrease lipid peroxidation and enhances the level of glutathione and Antioxidant enzymes	(Balasubashini *et al*, 2003)
Glutathione	Cancer	Glutathione in the nucleus maintains the redox state of critical protein Sulphydryls that are necessary for DNA repair and expression.	(Monnier and Chaudiere, 1996)
Proanthocyanidin	Cardiovascular	Inhibitory effects on proapoptic and cardioregulatory genes. Modulating apoptic regulatory bcl-XL,p53 and c-myc genes	(Bagchi *et al*, 2003)
Phenolics	Cancer, diabetic Retinopathy, Chronic	Inhibit the oxidation of lipids, fats and proteins (RH) by donation of a phenolic hydrogen atom to free radical	(Aruoma *et al*, 1993)

In developing countries like Ghana where poverty and malnutrition is rampant, knowledge of plant derived antioxidants could reduce the cost of health care by their consumption since they remove oxidants which might cause oxidative stress and inflammatory diseases. Ghana has a rich history of using various herbs and herbal components for treating various

diseases. Many Ghanaian plants have been investigated for their beneficial use as antioxidants or source of antioxidants using presently available experimental techniques.

2.4.2 *In vitro* anti-oxidant models

Several methods are used to measure the antioxidant activity of a biological material. The most commonly used ones are those involving chromogen compounds of radical nature that stimulate the reductive oxygen species. These methods are popular due to their ease, speed and sensitivity. The presence of antioxidants leads to the disappearance of these radical chromogens; the most widely used ones being the ABTS and DPPH methods. Some other commonly used assays like FRAP assay, ORAC assay, PCL assay, etc are mentioned below.

2.4.2.1 ABTS or TEAC assay

The Trolox equivalent antioxidant capacity (TEAC) assay was reported first by (Miller *et al.,* 1993)and (Rice-Evans and Miller, 1994) and then modified by (Re *et al.,* 1999). The TEAC assay is based on the inhibition by antioxidants of the absorbance of the radical cation of 2, 20-azinobis (3-ethylbenzothiazoline-6-sulfonate) (ABTS), which has a characteristic long-wavelength absorption spectrum showing maxima at 660, 734 and 820 nm. Generation of the ABTS radical cation forms the basis of one of the spectrophotometric methods that have been applied to the measurement of the total antioxidant activity of various substances. The experiments are carried out using a decolorization assay, which involves the generation of the ABTS chromophore by the oxidation of ABTS with potassium persulphate. It is applicable to both hydrophilic and lipophilic compounds. The ABTS solution is diluted and absorbance measured in about 10 min after the initial mixing

of different concentrations of the extracts with 1ml of the solution. Trolox, a water-soluble analogue of vitamin E is used as the reference standard. The assay has been widely used in many recent studies related to detection of antioxidant property of plant (Dalmalchi *et al*, 2007; Srinivasan *et al.*, 2007).

2.4.2.2 DPPH method

This method was designed by (Brand-Williams *et al.*, 1995) and later modified by (Sanchez-Moreno *et al*., 1998). It is one of the most extensively used antioxidant assay for plant samples. Recently the assay has been used to determine antioxidant activity in Tanacetum (Tepe and Soknen, 2007), Moldavianbalm (Dastmalchi *et al*., 2007) and *Phyllanthus amarus* (Lim and Murtijaya, 2007). This method is based on scavenging of the 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH) from the antioxidants, which produces a decrease in absorbance at 515 nm. When a solution of DPPH is mixed with a substance that can donate a hydrogen atom, the reduced form of the radical is generated accompanied by loss of colour. This delocalization is also responsible for the deep violet colour, characterized by an absorption band in ethanol solution at about 520 nm. Representing the DPPH radical by Z* and the donor molecule by AH, the primary reaction is: Z*+ AH ----→ ZH + A

2.4.2.3 FRAP assay

Ferric reducing ability of plasma (FRAP) assay is a technique to determine the total antioxidant power interpreted as the reducing capability. The FRAP assay was first given by (Benzie and Strain, 1999). This assay was very recently used by (Lim and Murtijaya,

2007)and (Netzel *et al.*, 2007) for establishing the antioxidant activity of different plant species. In this assay reductants (‘‘antioxidants’’) in the sample reduce Fe (III)/tripyridyltriazinecomplex, present in stoichiometric excess, to the blue ferrous form, with an increase in absorbance at 593 nm. ¢A is proportional to the combined (total) ferric reducing/antioxidant power (FRAP value) of the antioxidants in the sample. The final results were expressed as micromole Trolox equivalents (TE) per gram on dried basis (µmol TE/g, db).

$$\mathbf{FE\ (TPTZ)_2(III) + ArOH \longrightarrow Fe\ (TPTZ)_2(II) + ArOH^+}$$

2.4.2.4 ORAC assay

The oxygen radical absorbance capacity (ORAC) assay is based largely on the work reported by (Glazer, 1990). It uses beta-phycoerythrin (PE) as an oxidizable protein substrate and 2, 20-azobis (2-amidinopropane) dihydrochloride (AAPH) as a peroxyl radical generator or Cu_2+–H_2O_2 as a hydroxyl radical generator. To date, it is the only method that takes free radical action to completion and uses an area-under-curve (AUC) technique for quantitation and thus, combines both inhibition percentage and the length of inhibition time of the free radical action by antioxidants into a single quantity. The assay has been widely used in many recent studies related to plant (Almeida *et al.*, 2008).

2.4.2.5 TRAP assay

The total radical trapping parameter (TRAP) assay of (Wayner *et al.*, 1985) was the most widely used method for measuring total antioxidant capacity of plasma or serum during the last decade. (Schlesier *et al.*, 2002) employed TRAP assay to evaluate the antioxidant property of tea and various fruit juice while (Actis-goretta et al., 2002) did antioxidant

assay in red wine. The TRAP assay uses peroxyl radicals generated from 2, 20-azobis (2-amidinopropane) dihydrochloride (AAPH) and peroxidizable materials contained in plasma or other biological fluids. After adding AAPH to the plasma, the oxidation of the oxidizable materials is monitored by measuring the oxygen consumed during the reaction. During an induction period, this oxidation is inhibited by the antioxidants in the plasma. The length of the induction period (lag phase) is compared to that of an internal standard, Trolox (Aldrich, Milwaukee, WI, USA) (6-hydroxyl-2, 5, 7, 8,-tetramethylchroman-2-carboxylic acid), and then quantitatively related to the antioxidant capacity of the plasma.

2.4.2.6 Dichlorofluorescin-diacetate (DCFH-DA) based assay

Another method that also measures TRAP is the spectrophotometric assay reported recently by (Valkonen and Kuusi, 1997). (Amado *et al.*, 2007) have reported a rapid and easy assay method involving dichlorofluorescin-diacetate. This assay uses AAPH to generate peroxyl radicals and DCFH-DA as the oxidizable substrate for the peroxyl radicals. The oxidation of DCFH-DA by peroxyl radicals converts DCFH-DA to dichlorofluorescein (DCF). DCF is highly fluorescent (Ex 480 nm, Em 526 nm) and also has absorbance at 504 nm. Therefore, the produced DCF can be monitored either fluorometrically or spectrophotometrically. DCFH-DA assay has been used to determine the antioxidant property of *Gongronema latifolium* leaves (Ugochukwu and Cobourne, 2003), Labrador tea (*Ledumgroen landicum*, Retzius) leaves (Dufour *et al*., 2007), and grape juice (Rozenberg *et al*., 2006).

2.4.2.7 Cyclic voltammetry method

The cyclic voltammetry procedure reported by (Kohen *et al.*, 1999) evaluates the overall reducing power of low molecular weight antioxidants in a biological fluid or tissue homogenate. Following preparation, the sample is introduced into a well in which three electrodes are placed: the working electrode (e.g., glassy carbon), the reference electrode (Ag/AgCl), and the auxiliary electrode (platinum wire). The potential is applied to the working electrode at a constant rate (100 mV/s) either toward the positive potential (evaluation of reducing equivalent) or toward the negative potential (evaluation of oxidizing species). During operation of the cyclic voltammetry, a potential current curve is recorded (cyclic voltammogram). Recently quantitative determination of the phenolic antioxidants using voltammetric techniques was described by (Raymundo *et al.*, 2007). In case of plant samples (Chatterjee *et al.*, 2007) determined the antioxidant property of green pepper (*Piper nigrum* L.) and lignans from fresh mace (*Myristica fragrans*), while (Kilmartin and Hsu, 2003)determined antioxidant property of green, oolong and black teas, and in coffee using cyclic voltammetry method.

2.4.2.8 TOSC assay

(Winston *et al.*, 1998)recently reported an assay called total oxyradical scavenging capacity (TOSC) assay. (MacLean *et al.*, 2003) used this assay to determine the antioxidant activity of plant tissues. It is based on the oxidation of alpha-ketocmethiolbutyric acid (KMBA) to ethylene by peroxyl radicals produced from AAPH. The ethylene formation, which is partially inhibited in the presence of antioxidants, is monitored by gas chromatographic

analysis of head space from the reaction vessel. The TOSC is calculated according to the equation:

TOSC = 100-($SA/\int CA \times 100$); where $\int SA$ and $\int CA$ are the integrated areas from the curve defining the sample and control reactions, respectively.

2.4.2.9 Photochemiluminescence (PCL) assay

PCL assay was initially used by (Popov *et al*., 1987). This technique was extensively studied to determine water-soluble and lipid-soluble antioxidants (Popov and Lewin, 1994; Popov and Lewin, 1996). The photochemiluminescence measures the antioxidant capacity, towards the superoxide radical, in lipidic (ACL) and water (ACW) phase. This method allows the quantification of both the antioxidant capacity of hydrophilic and/or lipophilic substances, either as pure compounds of complex matrix from different origin: synthetic, vegetable, animal, human, etc. The PCL method is based on an acceleration of the oxidative reactions in vitro about 1000 faster than the normal conditions, because the presence of an appropriate photosensitizer.

The PCL is a very quick and sensitive measurement method. Using the PCL assay (Wang *et al.*, 2006)determined antioxidant property in marigold flowers.

2.4.2.10 Other antioxidant models in use

There are a number of anti-oxidant models in use which determine the reducing power or capacity of plant extracts and compounds isolated from plants as described by (Oyaizu, 1986) and later modified by (Amarowicz *et al.*, 2005), the extent of lipid peroxidation in rat brain homogenate using thiobarbituric acid (TBA) as described by(Auddy *et al*, 2003;

Ohkawa *et al.*, 1979) or lipid peroxidation using linoleic acid autoxidation as described by Mitsuda (Mitsuda *et al.*, 1967).

CHAPTER 3 MATERIALS AND METHODS

3.1 PLANT COLLECTION AND PROCESSING

Roots of the plant *P.hirsuta* were collected from Esaase-Bontefufuom in the Amansie West district of Ashanti Region of Ghana, in May 2006. The plant was authenticated by Mr. E Amissah, the curator of the Kwame Nkrumah University of Science and Technology Botanic Garden; a voucher specimen has been kept in the Faculty of Pharmacy and Pharmaceutical Sciences' Herbarium (No. FP 1008). The roots were thoroughly washed and sun-dried for seven days before milling.

3.2 EXTRACTION

A total of 1.3kg of powdered root material was extracted continuously in batches of 400g, 450g and 450g by Soxhlet's apparatus using a total of 3litres of 70% ethanol as the solvent for extraction. The extraction was done till exhaustion (determined after a sample was taken from the percolate in the thimble and taken through thin layer chromatography (TLC) and sprayed with anisaldehyde, to detect the presence of secondary metabolites). The extract (coloured yellowish brown) was then concentrated to dryness with a rotary evaporator(R-114, Buchi, Switzerland) at a temperature of 60° C under vacuum and the yield was154.6g (11.69%.).This was subsequently labelled as PHC and kept in a desiccator awaiting further work.

A weight of 50g of the crude extract (PHC) was dissolved in 80% methanol and serially extracted with 50ml aliquots of Petroleum ether (Fisons) 40-60° until exhaustion (by testing for the presence of phytoconstituents that were extractable in petroleum ether). Both the

methanol and the Pet. ether fractions were evaporated to dryness on a water bath, labelled PHM and PHE respectively and kept in a desiccator.

3.3 PRELIMINARY PHYTOCHEMICAL SCREENING

Preliminary phytochemical screening was performed to identify the main phytochemical groups or secondary metabolites present in the roots of the plant. The tests for alkaloids, anthraquinones, phenolic compounds, glycosides, flavonoids, triterpenes and steroids, saponins, fixed oils and essential oils were performed.

3.3.1 PHYTOCHEMICAL METHODS

3.3.1.1 Tannins

The powdered roots of *Palisota hirsuta*, 0.5 g, were boiled with 25ml of water for 5 minutes. It was then cooled, filtered and the volume adjusted to 25ml. To 1ml aliquot of the aqueous extract was added 10ml of water and 5 drops of 1% lead acetate. The colour and amount of precipitate, if any, was noted and recorded. The procedure was repeated using 5 drops of 1% ferric chloride (Sofowora, 1993).

3.3.1.2 Alkaloids

The root powder of *Palisota hirsuta* (0.5g) was extracted with 30ml of ammoniacal alcohol (ammonia: alcohol, 1:9) and filtered. The filtrate was then evaporated to dryness and the residue extracted with 1% H_2SO_4. This was then filtered and the filtrate rendered alkaline with dilute ammonia solution. The alkaline solution of the extract was then put in a separating funnel and partitioned with chloroform. The chloroformic layer was then separated and evaporated to dryness. The residue was again dissolved in 1% H_2SO_4 and

few drops of Dragendorff's reagent added. An orange precipitate indicates the presence of alkaloid (Sofowora, 1993).

3.3.1.3 Phytosterols (Lieberman's test)

The powdered roots (0.5g) of *Palisota hirsuta* was extracted with chloroform. Two ml of acetic anhydride was then added to the chloroformic extract and few drops of conc. H_2SO_4 were added along the sides of the test tube. A violet to blue coloration indicates the presence of steroid nucleus (sterol) (Sofowora, 1993).

3.3.1.4 Terpenoids (Salkowski test)

The root powder (0.5g) of *Palisota hirsuta* was extracted with 70% ethanol and mixed with 5 ml chloroform. It was then warmed for 30 minutes. The chloroform solution was then treated with a small volume of concentrated sulphuric acid and mixed properly. A reddish brown coloration of the interface shows a positive result for the presence of terpenoids (Sofowora, 1993).

3.3.1.5 Flavonoids

The method outlined by Sofowora (1993) was followed with some modification. The powdered roots (0.5g) of *P. hirsuta* was extracted with 15 ml of ethanol (98%).To the ethanolic extract was added a small piece of zinc metal, this was followed by drop wise addition of concentrated hydrochloric acid. Colours ranging from orange to red indicated flavones, red to crimson indicated flavonols, crimson to magenta indicated flavonones (Sofowora, 1993).

3.3.1.6 General test for Glycosides (Reducing sugars)

About 200 mg of the powdered root sample was warmed with 5 ml dilute H_2SO_4 on a water bath for 2 minutes. It was then filtered and the filtrate rendered distinctly alkaline with 2 to 5 drops of 20% NaOH. 1 ml each of Fehling's solution A and B was then added to the filtrate and heated on the water bath for 2 minutes. A brick red precipitate indicates the presence of glycosides (Sofowora, 1993). A test for free reducing sugars was performed to eliminate the possibility of such masking that for glycosides.

3.3.1.7 Anthracene glycosides

To test for anthracene glycosides, 0.5g of *P. hirsuta* root powder was boiled with dilute H_2SO_4 for five minutes and filtered whilst still hot and the filtrate allowed to cool. The filtrate was then mixed with an equal volume of chloroform in a separation funnel. The chloroformic layer was separated and dilute NH_3 added and observed (Sofowora, 1993).

3.3.1.8 Saponins

An amount (0.2g) of the powdered root material was shaken with 5 ml of water in a test tube and the mixture observed for the presence of a froth which does not break readily upon standing (Trease and Evans, 1983)

3.4 ANTI-INFLAMMATORY ASSAYS

3.4.1 Animals

Cockerels (*Gallus gallus*; strain: Shaver 579, one-day post-hatched were obtained fromAkropong Farms, Kumasi, Ghana) and housed in stainless steel cages (34 × 57 × 40

cm) at a population density of 12- 13 chicks per cage. Food (Chick Mash, from GAFCO, Tema, Ghana) and water were available *ad libitum* through one-quart gravity-fed feeders and waterers. The temperature in the room was maintained at 29°C, and overhead incandescent illumination was maintained on 12-hr. light-dark cycle. Daily maintenance was conducted during the first quarter of the light cycle. Chicks were checked for weight and good health at seven days of age. Group sample sizes of 5-6 were used throughout the study.

3.4.2 Carrageenan-induced oedema in chicks

The carrageenan foot oedema model of inflammation in the chick with some modifications (Roach and Sufka, 2003; Boakye-Gyasi *et al*, 2008) was used to evaluate the antiinflammatory properties of the extract and compared to dexamethasone and diclofenac as reference drugs. Carrageenan (10 µl of a 2% suspension in saline) was injected subplantar into the right footpads of the chicks. The foot volume was measured by water displacement plethysmography as described by (Fereidoni *et al.*, 2000) before injection and at hourly intervals for 5 hours after injection. The oedema component of inflammation was quantified by measuring the difference in foot volume before carrageenan injection and at the various time points.

3.4.3 Preliminary Bioassay of PHC

The experiment was aimed at investigating the effect of the extract and reference drugs (diclofenac and dexamethasone) on oedema in the paws of the chicks. The extract was administered via the oral route one hour and the reference drugs via the intraperitoneal route thirty minutes before carrageenan challenge. The chicks were randomly selected and

grouped into fives to perform each of the study: Positive control; Diclofenac (10, 30 and 100 mg kg^{-1}, *i.p.*); Dexamethasone (0.1, 1.0 and 3.0 mg kg^{-1}, *i.p.*).

Extracts: (30, 100, and 300mg kg^{-1}, *p.o.*).

The doses for the extract (PHC) were prepared by triturating a known weight of the extract with Tween 60 polysorbate to get it into solution. The reference drugs and extract solutions were prepared such that not more than 1ml of extract was given orally and not more than 0.5ml of the standard drugs was injected intraperitoneally. All drugs and the extracts as well were freshly prepared. The control group received only intraplantar injection of carrageenan suspension. All changes in ipsilateral paw volume were represented as percentage increase from the pretreatment value obtained at time 0, which was calculated according to the formula;

Calculation of percentage increase in foot volume

Percentage increase in foot volume = $[P_x\text{-}P_o/P_o] \times 100\%$

Where P_x as a parameter measurement is the time after injection and P_o is the time before the carrageenan injection. Data was presented as the effect of drugs on time course and the total oedema response in carrageenan induced paw oedema for 5-6 hours using Graph Pad Prism for Windows version 5(Graph Pad Software, San Diego, CA, USA).

3.4.4 Anti-inflammatory assays of Petroleum ether and Methanol Fractions

The fractions designated PHE (Pet. ether portion, 7.28g) and PHM (Methanol portion, 41.54g) were tested for anti-inflammatory activity using the method described in section

3.4.3.

3.4.5 Anti-inflammatory assays of isolates

The isolates PH 1-VI were tested for anti-inflammatory activity using the method described in section **3.4.3.** The doses used for the isolates were 3, 10 and 30 $mgkg^{-1}$body.

3.5 ANTI-NOCICEPTIVE ASSAY OF PHC, PHE AND PHM

FORMALIN-INDUCED NOCICEPTION

Drugs: Formalin was obtained from BDH, Poole, England and Morphine hydrochloride was purchased from Phyto-Riker Pharmaceuticals, Accra, Ghana.

Animals: Mice [Imprinting Control Region (ICR)] (30-40g) were purchased from Centre for Scientific Research into Plant Medicine Akwapim Mampong, Ghana and housed in the animal facility of the Department of Pharmacology, Kwame Nkrumah University of Science and Technology (KNUST). The animals were housed in groups of six in stainless steel cages (34×47×18 cm) with soft wood shavings as bedding, fed with normal commercial pellet diet (GAFCO, Tema), given water *ad libitum* and maintained under laboratory conditions (temperature 24-28 °C, relative humidity 60-70%, and 12 hour lightdark cycle). All procedures and techniques used in these studies were in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals (NIH, Department of Health and Human Services publication no. 85 - 23, revised 1985).

All protocols used were approved by the Department of Pharmacology Ethics Committee, KNUST, Kumasi.

Formalin-induced nociception. The formalin test first described by (Dubuisson and Dennis, 1977) was carried out as described by (Malmberg and Yaksh, 1995) with some modifications. Each animal was assigned and acclimatized to one of 20 formalin test chambers (a Perspex chamber 15 × 15 × 15 cm) for thirty minutes before formalin injection (Wilson *et al.,* 2002). The mice were then pre-treated with the test drugs (30 min for i. p. route and 1 hr for oral route) before intraplantar injection of 10 μl of 5 % formalin. The animals were immediately returned individually into the testing chamber. A mirror angled at 45˚ below the floor of the chamber allowed a complete view of the paws. The behaviour of the animal was then captured over a 60 minute period for analysis by a camcorder (Everio™ model GZ-MG1300, JVC, Tokyo, Japan) placed in front of the mirror. Pain response was scored for 60 min, starting immediately after formalin injection.

The first phase of the formalin test was time 0-10 minutes and the second phase as`15-60 minutes.

Nociceptive behaviour was resolved quantitatively by counting the incidents of spontaneous biting/licking of the injected paw (Hayashida *et al.,* 2003) using the public domain software JWatcher™ Version 1.0 (University of California, Los Angeles, USA and Macquarie University, Sydney, Australia available at http://www.jwatcher.ucla.edu/). Nociceptive score was determined for each 5-minutes time block in each phase by measuring the amount of time spent biting/licking the injected paw. The product of the

frequency and duration of biting/licking was used as nociceptive score. The mice were selected in random pattern for each of the following study groups:

Group I Morphine (1, 3, and 10 mg kg^{-1})

Group II *Palisota* extract (30, 100 and 300 mg kg^{-1}).

Group III Vehicle treated control

Group IV……Methanol Fraction/extract (30,100 and 300 mg kg^{-1})

Group V…….Petroleum Ether Fraction /Extract (30,100 and 300mg kg^{-1})

Group VI……...PH I (1, 10 and 30mg kg^{-1})

Group VI……...PH II (1, 10 and 30mg kg^{-1})

Group VII……..PH III (1, 10 and 30mg kg^{-1})

Group IX……PH IV (1,10and 30 mg kg^{-1})

Group X ……PH V (1, 10 and 30mg kg^{-1})

Group XI ……..PH VI (1, 10 and 30mg kg^{-1})

Extracts were prepared with Tween 60 surface active agent. Emulsions of the extracts were prepared such that not more than 1ml of extracts was given orally and for the standard drugs, solutions not more than 0.5 ml of the drugs was injected intraperitoneally. All drugs were freshly prepared.

Dose-response curves were presented as the sum of total nociceptive score for the first phase (0-10 min) and second phase (10-60min) post formalin injection using GraphPad Prism for Windows version 5(GraphPad Software, San Diego, USA).

3.6 ANTI-OXIDANT ASSAY OF SOME ISOLATES OF *PALISOTA HIRSUTA*

In the present study the antioxidant activities of the isolates PH I, II, III, IV, V and VI were evaluated using three different assays: reducing power test, 1, 1-Diphenyl-2picrylhydrazyl (DPPH) scavenging activity and lipid peroxidation assays.

3.6.1 *In vitro* qualitative DPPH test

The qualitative test for antioxidant activity was performed using the rapid DPPH radical scavenging assay (Cuendet *et al.,* 1997). 10µl of the extracts PHC, PHE and PHM were applied on silica gel plates and allowed to dry completely. The plates were then sprayed with a solution of 2% DPPH in methanol. A pale yellow to white spot over a purple background indicated a radical scavenging activity of the particular extract.

3.6.1.1 Quantitative antioxidant assays of some isolates of *P. hirsuta*

3.6.1.2 Reducing power assay

Reducing activities of the isolates PH I, II, III, IV, V, and VI was carried out by using the method of (Oyaizu, 1986). Different concentrations (0.5 mg, 0.75 mg, 1.0 mg and 1.5 mg/ml) of the isolates and; as well as the standard anti-oxidant N-propyl gallate(3 µg/ml, 7.5 µg/ml, 15 µg/ml and 30µg/ml) were prepared in aqueous methanol (50% $^{v}/_{v}$) and 1 ml each taken into test tubes in triplicates. To the test tubes, 2.5 ml of sodium phosphate buffer and 2.5 ml of 1% Potassium ferric cyanide solution were added and contents were mixed

well and were incubated at 50 ˚C for 20 minutes. After incubation, 2.5 ml of 10% trichloroactetic acid (TCA) was added and the mixture centrifuged at 3000 revolutions per minute (rpm) for 10 minutes. After centrifugation 2.5 ml of supernatant was added to 2.5 ml of distilled water. To this about 1 ml of 0.1% ferric chloride was added to it. About 200 µl of this mixture was taken into a micro plate with 96 wells and the absorbance at 700 nm was read with a spectrometer (Cecil CE 7200).

3.6.1.3 Scavenging of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical assay

The free radical scavenging activity was determined as described by (Govindarajan *et al*., 2003) with a few modifications. 1 ml each of the isolates PH I, II, III, IV, V and VI of various concentrations (0.5, 1.0, 2.0 and 3 mg ml^{-1} in methanol) was added to 3 ml methanolic solution of DPPH solution (20 mg l^{-1}) in a test tube. The reaction mixture was kept in a water bath at 25°C for 30mins. The absorbance of the residual DPPH was determined at 517 nm in a spectrophotometer (Cecil CE 7200 spectrophotometer, Cecil Instrument Limited, Milton Technical Centre, England). One millilitre (1 ml) methanol (99.8%) was added to 3 ml DPPH solution incubated at 25°C for 30minutes and used as control. N-propyl gallate (1-30 µg ml^{-1}) was used as a standard free radical scavenger.). The concentration required to cause a 50% decrease in the absorbance was calculated (EC_{50}). Each test was carried out using three replicates.

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

% DPPH scavenging effects = $(Ac - At)/Ac \times 100$

Where

Ac = Absorbance of the control

At = Absorbance of the test drug/ extracts

3.6.1.4 Total antioxidant (Phosphomolybdenum) assay

The assay is based on the reduction of molybdenum, (Mo^{+6} to Mo^{+5}), by the extracts and subsequent formation of a green phosphate-molybdate (Mo^{+5}) complex at acidic pH.(Prieto *et al*., 1999). Test tubes containing l ml each of the isolates (0.50-3 mg/ml) and 3ml of the reagent solution (0.6 M sulphuric acid, 28 mM disodium phosphate and 4mM ammonium molybdate) were incubated at 95ºC for 90minutes. After the mixture had cooled to room temperature, the absorbance of the solution was measured at 695 nm. Four concentrations of ascorbic acid were used to construct a calibration curve. A blank solution was prepared by adding every other solution but without extract or standard drug. The antioxidant capacity was expressed as an ascorbic acid equivalent (AAE).

3.6.1.5 Linoleic acid auto-oxidation assay

The method of Mitsuda *et al*., (1967) was used. The isolates (0.5-3 mg/ml) in absolute alcohol were compared with n-propyl gallate (0.001-0.03 mg/ml) in absolute alcohol as a reference antioxidant. 2 ml of the extract, 2 ml of 2.5% linoleic acid in absolute ethanol, 4 ml of 0.05M phosphate buffer (pH=7) and 1.9 ml of distilled water were put into test tubes with a screw cap and placed in an oven at 40ºC in the dark for 7 days. After the seven day period, 2 ml each of the isolate and standard drug was added to 20% aqueous trichloroactetic acid (TCA) solution and 1ml of 0.6% aqueous thiobarbituric acid solution (TBA). This mixture was placed in boiling water bath for 10 minutes and after cooling, was centrifuged at 3000 rpm for 10 minutes. The absorbance of the supernatant was

measured. Each test was carried out using three replicates. Percentage inhibition of lipid peroxidation by the test drugs was determined by comparing the absorbance of the drug test with that of the control (linoleic acid mixture without any drug). Data was presented as percentage inhibition of lipid peroxidation against concentration. The % inhibition of linoleic acid autoxidation was calculated as follows:

$$\%\, inhibition = \left[1 - \frac{D - Do}{Co - C}\right] \times 100$$

Where

Co= the degree of lipid peroxidation in the absence of antioxidant (Full reaction mixture)

C= the underlying lipid peroxidation before the initiation of lipid peroxidation

D= any absorbance produced by the isolate/linoleic acid mixture
Do= the absorbance produced by the isolate alone.

3.7 CHROMATOGRAPHY

3.7.1 Column chromatography

Silica gel (Merck, 60-120 mesh ASTM) was used as the stationary phase for the column to separate the sample into fractions which were appropriately labelled. The fractions so obtained from the initial separation were further separated into smaller fractions using silica gel (Merck, 230-400 mesh ASTM).The packing of the column was done by the wet method while gradient elution was employed in eluting the column starting with hexane and followed by petroleum ether and along the ascending order of the eluotropic series.

3.7.2 Analytical thin layer chromatography

Precoated silica gel plates 60 F_{254} (Merck, 0.25 mm thick, Catalog No.OB 315394) were used with the solvent the solvent systems in Table 3.1.

3.7.3 Preparative thin layer chromatography

Silica gel PF_{254} (Merck, 1 mm thick) prepared and activated at 105°C for 30 minutes before use with the solvent systems overleaf.

3.7.4 Detection of spots in analytical thin layer chromatography

The spots on TLC plates corresponding to separate compounds were detected by spraying with anisaldehyde 0.5%w/v in acetic acid-H_2SO_4-methanol (10:5:85) followed by heating at 105°C for 5-10 minutes.

3.7.5 Solvents and chemicals

The solvents and chemicals used for the work were obtained from BDH, Poole, England and Fisons, some of which were of analytical grade while the others were of general purpose grade.

3.7.6 Solvent systems

The solvents listed below were used to run the column chromatography to obtain fractions of similar composition, using the silica gel with mesh size 60-120 ASTM as stationary phase; petroleum ether (40°-60°), ethyl acetate and methanol in order of increasing polarity. The bulk fractions were further fractionated using the above-mentioned solvents in various proportions.

In the preparative and analytical thin layer chromatography, the under-mentioned solvent systems were employed, and the R_f values calculated based on the respective solvent systems.

Table 3.1 List of solvent systems for analytical TLC.

Code	Solvent System	Proportions
SS1	Pet. Ether (40°-60°) –EtOAc-n-Butanol	40:30:30
SS2	Pet.Ether (40°-60°) – EtOAc-n-Butanol	60:30:10
SS3	Pet. Ether (40°-60°)-EtOAc-Methanol	70:20:10
SS4	$CHCl_3$-MeOH-Pet.Ether (40°-60°)	25:70:5

SS5	Pet. Ether (40°-60°)-EtOAc	70:30
SS6	$CHCl_3$-MeOH	40:60
SS7	EtOAc –MeOH	40:60

3.8 ISOLATION OF COMPOUNDS

3.8.1 Column Chromatography of Methanol Fraction

An amount of 500g of silica gel (60-120 ASTM) was packed into a large size column (80cm x 5cm) by the wet method. A weight of 54.7g of the methanol fraction of *Palisota hirsuta* was re-dissolved in a minimum amount of methanol and mixed with about 100g of silica gel and allowed to dry to attain the same consistency as the silica gel and gently placed on top of the packed column. A wad of cotton wool was placed on top of the packed column to avoid disturbing the surface of the packing. The column was eluted in a gradient manner starting with petroleum ether-ethyl acetate (80-20°C), petroleum ether-ethyl acetate (50:50), ethyl acetate (100%), ethyl acetate-methanol (80:20), ethyl acetate-methanol (50:50), ethyl acetate-methanol (20:80), methanol (100%). Fractions designated as PH 1 to PH 9 were collected based on the increasing polarity of the eluants as formulated above. The fractions collected were concentrated under reduced pressure using the rotary evaporator (Buchi Rotavapor R-210), weighed and subjected to thin layer chromatography.

3.8.2 Isolation of Compounds PH I-III **Chromatography**

of Fraction PH 1

A quantity of 100g of silica gel [(0.040-0.60mm) 230-400 mesh ASTM] was packed into a column (50cmx 3.5cm) using the wet method. About one (1)gram of the PH 1 was dissolved in enough of petroleum ether (40°-60°) and mixed with adequate silica gel to a consistency of the silica gel and carefully placed on top of the packed column. A wad of cotton wool was placed on top to prevent disturbing the surface. The column was eluted in a gradient manner starting with petroleum ether (40°-60°) and ending with 20% ethyl acetate in petroleum ether (40-60°). **PH I** was obtained by elution with only petroleum ether and analysed by TLC which gave overlapping tailings and was hence subjected to gas chromatography coupled with mass spectrometry (GC/MS), to separate and identify the constituents. **PH II** was eluted and was also analysed by TLC and subjected to GC-MS to separate and identify the constituents because there were overlapping tailings.

3.8.3 Isolation of Compounds PH IV, V and VI

Chromatography of Fraction PH 2

About 50g of Sephadex LH-20 (Bead size 25-100µ, Sigma-Aldrich) which has been previously soaked overnight in methanol was packed wet into a column (50cm x 3.5cm).An amount of 1.5g of PH 2 was dissolved in methanol and in a gentle manner applied to the top of the packed column. The column was eluted with only methanol and the fractions collected in 5 ml portions checked for the presence of compounds using analytical TLC and anisaldehyde. The portions (three of them) that had like compounds were pooled

together and labeled **2A, 2B** and **2C** respectively. **PH IV** was isolated from **2A**. The eluate was evaporated and the residual crude crystals continually washed with petroleum ether followed by ethyl acetate. The next to be isolated in the same mode was **PH V** from portion **2B**, which was also thoroughly cleaned by washing with chloroform after it crystallized out from evaporation. **PH VI** was obtained from **2C** after evaporation and purified by washing with chloroform.

3.8.4 Isolation of Compounds PH VII, VIII and IX

Chromatography of Fraction PH 3

A quantity of 50g of Sephadex LH-20 (Bead size 25-100μ, Sigma-Aldrich) which has been previously soaked overnight in methanol was packed wet into a column (50cm x 3.5cm).The column was eluted with methanol after 2g of PH 3 had been dissolved in methanol and gently placed on top of the packed column. The column was eluted with only methanol and the fractions collected. **PH VII** was eluted first, with both **PH VIII** and **PH IX** following in that order. Each was washed with petroleum ether and recrystallised with acetone. These isolates were identified during the column chromatography by monitoring with TLC; however these did not show desired biological activities.

3.8.5 Column Chromatography of Petroleum Ether Fraction

A weighed amount of 150g of silica gel [(0.040-0.060mm) 230-400 ASTM] was packed into a column (500mm x 35mm), using the wet method. About 8.8g of the petroleum ether

fraction was dissolved in chloroform and mixed with enough silica gel, allowed to dry and to achieve a consistency of the silica gel. This was placed on top of the packed column and a wad of cotton wool was put on top of the packing to prevent disturbing the surface. The column was eluted in a gradient manner, starting with hexane and ending with ethyl acetate. Five fractions were collected after pooling together the fractions that had similar analytical TLC properties. All the compounds or mixtures of compounds obtained were found to be without the desired biological activities with the exception of **PH III** which was identified as a fatty acid with the assistance of GC/MS.

3.9 IDENTIFICATION OF COMPOUNDS

The methods usually involved in the identification of compounds are a combination of different techniques. These might include Nuclear Magnetic Resonance (NMR), Mass Spectrometry (MS), Ultra Violet (UV) and Infra Red (IR) spectroscopy. NMR and MS were employed as the techniques to identify the structure and identity of compounds in this project/study. GC-MS was also utilized to resolve the compounds which were mixtures of fatty acids and for that matter difficult to separate with silica gel.

3.9.1 Gas chromatography-mass spectrometry (GC/MS)

The separation and detection of components from a mixture of organic compounds is achievable by gas chromatography. Mass spectrometry, because of its high sensitivity and fast scan speeds, is used to provide definite structural information from small quantities of compounds eluted from a gas chromatograph (Dudley and Fleming, 1995). The association of the two techniques therefore provides a means of structural identification of the

components of natural organic mixtures including fatty acids (Fabianska, 2004). Mass spectra of acceptable quality could be obtained for every component that may be separated by the gas chromatograph even though the component may be present in minute quantities (Dudley and Fleming, 1995).

3.9.2 Identification of PH I, PH II and PH III (fatty acids), using GC/MS

GC/MS analysis was used to identify the components of KI because simple column and preparative TLC could not fully resolve all components of the fatty acid mixture. Samples PH I, PH II and PH III were derivatised into the methyl ester by refluxing with tetramethylsilane (TMS) at 100°C for thirty minutes. Methyl esters of fatty acids were analysed with a Fisons MD 800 mass (quadrupole) –GC 8000 series instrument equipped with silica column (30m x 0.25mm I.D.) coated with OV1. Samples were injected by hot split method, with the injector and detector maintained at 270°C and 250°C respectively. The temperature program was 40°C for four minutes after injection, then increased to 280°C (8°C/min) with a hold at 280°C for twenty minutes. Helium (20psi) was as carrier gas with flow rate of 0.5ml/min. The gas chromatograph was directly coupled to a mass spectrometer working in electron impact ionization mode at 70eV, and scanned masses in the range of 40-650 Da. Data were acquired in full scan mode and processed. Compounds were identified by their mass spectra, interpretation of MS fragmentation patterns and comparing retention times of their peaks to those of standard compounds analysed previously. Quantification of methyl esters of fatty acids was performed by integration of appropriate peak areas in Total Ion Chromatogram (TIC).

Chapter 4

RESULTS AND DISCUSSION

4.1 ANTI-INFLAMMATORY ACTIVITY OF *P. HIRSUTA* EXTRACT

Total oedema produced by the treatment is expressed in arbitrary units as AUC of the timecourse curve. PHC (30-300 mg kg^{-1}) dose dependently and significantly reduced the total foot oedema with maximal effect of 64.01±10.90 for PHC administered prophylactically (Figure 4.1a and b). PHE (10-100 mg kg^{-1}) also dose dependently and significantly reduced the total foot oedema with maximal effect of 57.86±11.89% when administered prophylactically (Figure 4.2a and b). PHM (10-100mgkg^{-1}) dose dependently and significantly reduced the total foot oedema with a maximal effect of 72.91±4.06% when prophylactically administered (Figure 4.3a and b). Diclofenac (3-10 mg kg^{-1}), an NSAID dose-dependently reduced the total foot oedema with a maximal effect of71.71± 10.46% (Figure 4.4a and b) when administered by the pre-emptive paradigm. In a similar manner, dexamethasone (0.1-1 mg kg^{-1}), a steroidal antiinflammatory drug reduced total foot oedema with a maximal effect of 56.76± 10.21% (Figure
4.5a and b).

a.

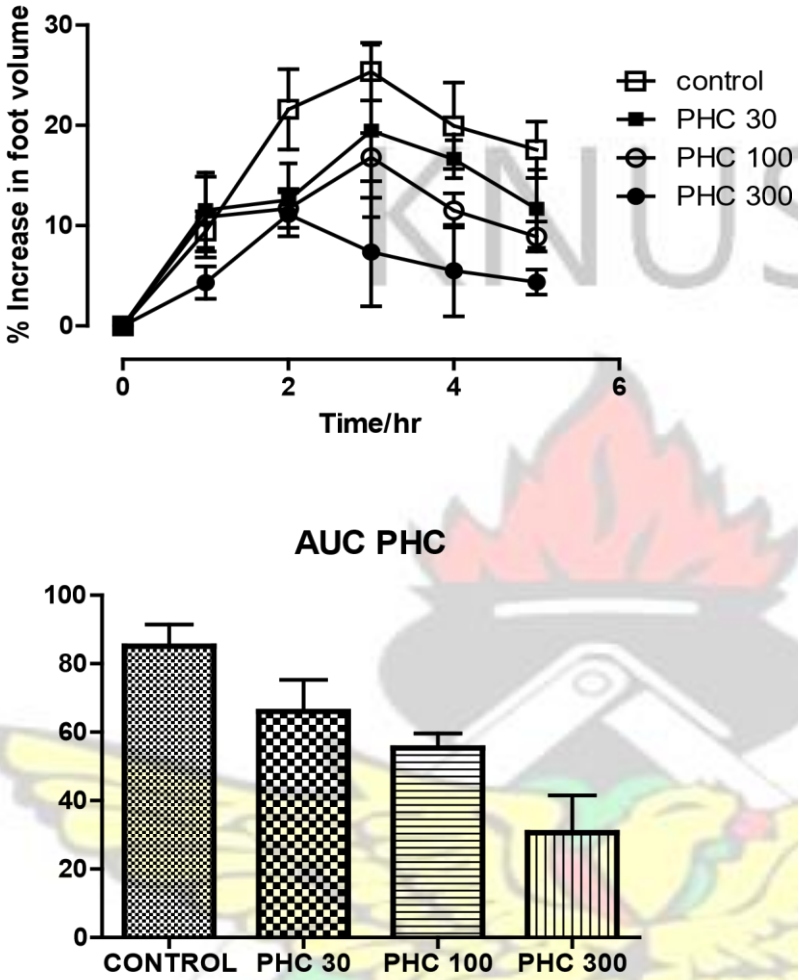


Figure 4.1 Effect of PHC (30-300 mg/kg p.o) on the time course curve (a) and total oedema response expressed as AUC (b) in carrageenan induced paw oedema in Chicks. Values are mean± S.E.M (N=6) *** P<0.001, ** P<0.01, * P<0.05 compared to the vehicle treated group (ONE-WAY ANOVA followed by Newman Keul's *post hoc* test).

a.

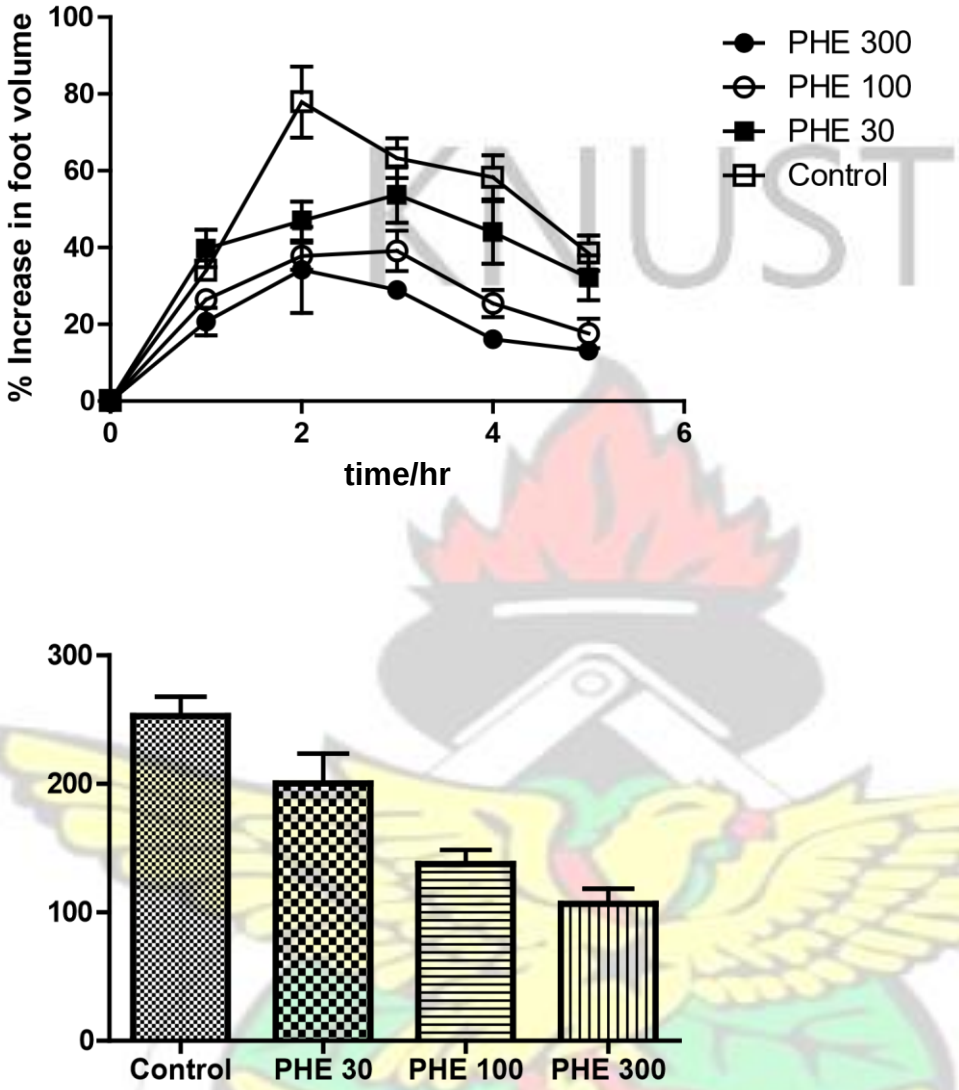


Figure 4.2 Effect of PHE(10-100 mg/kg *p.o*) on the time course curve (a) and total oedema response expressed as AUC (b) in carrageenan induced paw oedema in Chicks. Values are mean± S.E.M (N=6) *** P<0.001, ** P<0.01, * P<0.05 compared to the vehicle treated group (ONE-WAY ANOVA followed by Newman Keul's *post hoc* test).

a.

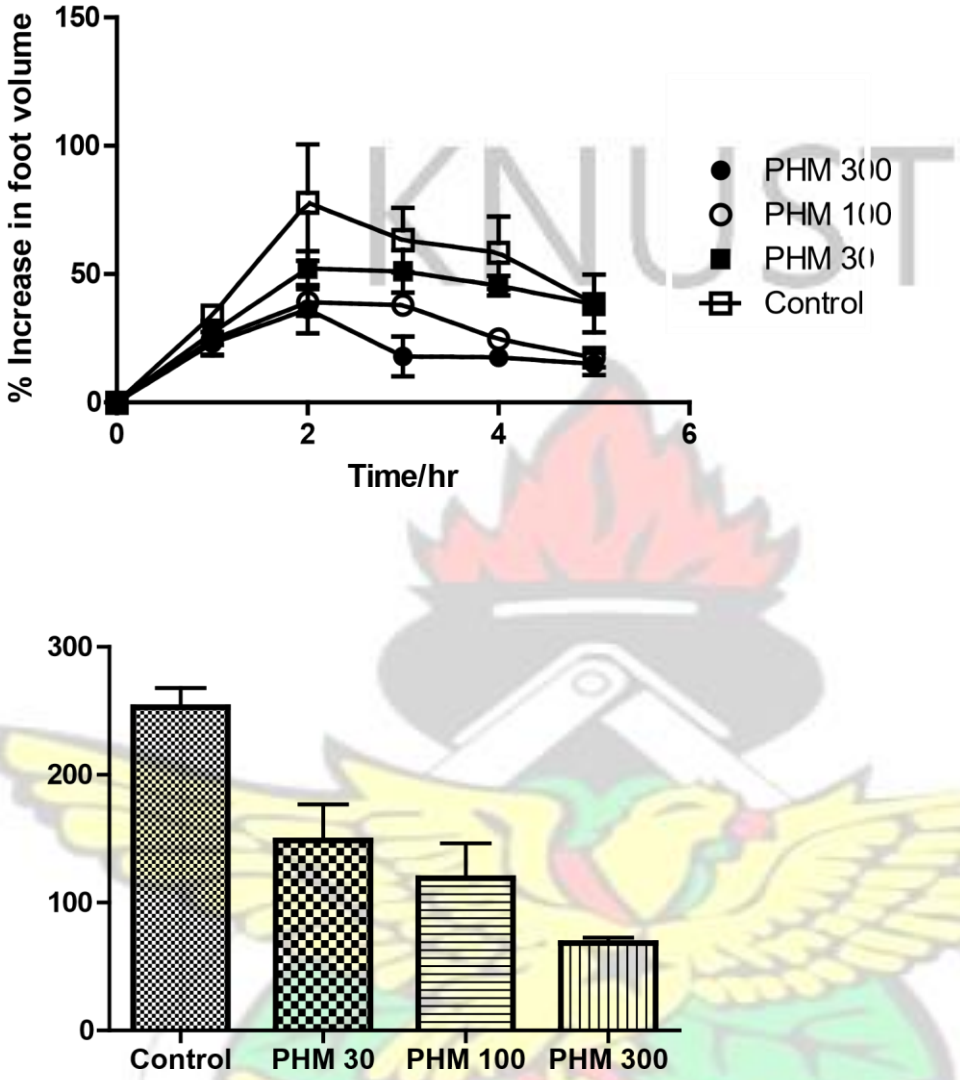


Figure 4.3 Effect of PHM(10-100 mg/kg *p.o*) on the time course curve (a) and total oedema response expressed as AUC (b) in carrageenan induced paw oedema in Chicks. Values are mean± S.E.M (N=6) *** P<0.001, ** P<0.01, * P<0.05 compared to the vehicle treated group (ONE-WAY ANOVA followed by Newman Keul's *post hoc* test).

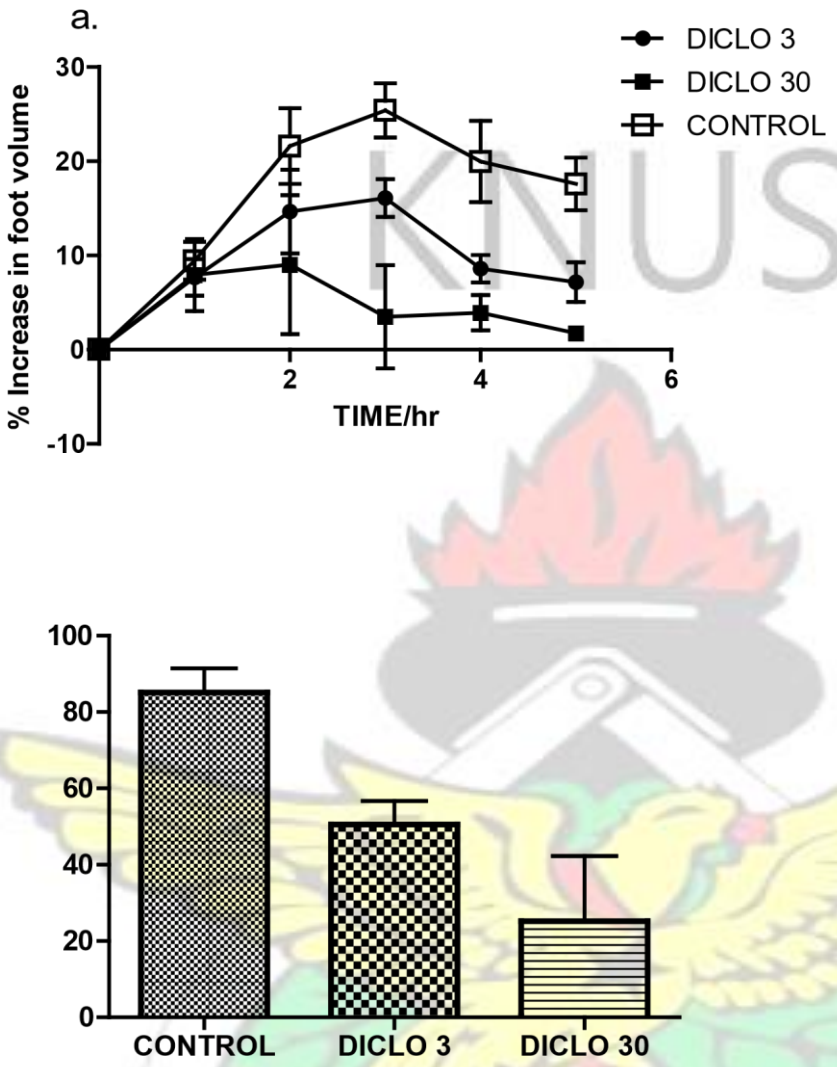


Figure 4.4 Effect of Diclofenac(3-30 mg/kg i.p) on the time course curve (a) and total oedema response expressed as AUC (b) in carrageenan induced paw oedema in Chicks. Values are mean± S.E.M (N=6) *** P<0.001, ** P<0.01, * P<0.05 compared to the vehicle treated group (ONE-WAY ANOVA followed by Newman Keul's *post hoc* test).

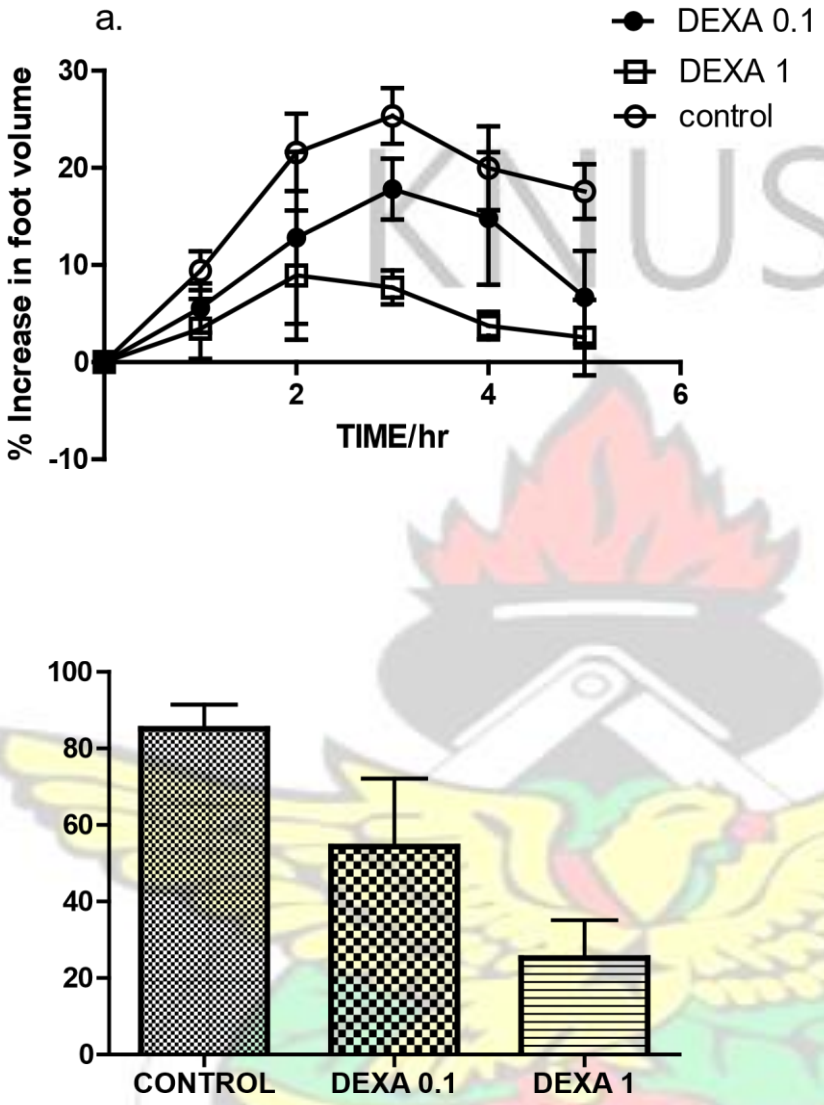


Figure 4.5 Effect of dexamethasone (0.1-1 mg/kg i.p) on the time course curve (a) and total oedema response expressed as AUC (b) in carrageenan induced paw oedema in Chicks. Values are mean± S.E.M (N=6) *** P<0.001, ** P<0.01, * P<0.05 compared to the vehicle treated group (ONE-WAY ANOVA followed by Newman Keul's *post hoc* test).

4.2 ANTI-INFLAMMATORY ACTIVITY OF ISOLATES

The carrageenan-induced acute footpad oedema in laboratory animals as designed by Winter et al, 1962 has been widely used as a tool to screen new anti-inflammatory drugs and perhaps remains an acceptable preliminary screening test for anti-inflammatory activity (Niemegeers *et al*., 1975; Singh *et al*., 2000). The method has commonly been used to evaluate non-steroidal anti-inflammatory drugs [NSAIDs] as proposed by Rosa and Willoughby, 1971. In this current study, chicks were used instead of rodents that are commonly known to be used for such experiments. The reasons for using the chicks in this study are twofold;

1. Carrageenan-induced oedema has been validated in the chicks by (Roach and Sufka, 2003), and is much more economical than rodent models.

2. Chicks are easier to handle and studies have demonstrated that intraplantar injection of carrageenan in the 7-day-old chick elicits a measurable, reliable and relatively short-lasting state of oedema. This is differentially attenuated by the systemic administration of typical antiinflammatory compounds (Roach and Sufka, 2003) and compares favorably with the more commonly used rodent models (rat and mice) in the screening of drugs with anti-inflammatory activities.

The isolated compounds PHI, IV, V and VI, from the roots of *Palisota hirsuta* dose dependently and significantly reduced the total foot oedema with maximal effects of 55.39±5.95%,66.51±5.43%,69.04±22.61% and 64.52±5.49% respectively. The compounds PH II and III even though significantly reduced the total oedema with maximal effects of 75.25±28.84% and 82.52±7.20% respectively, however their effects were not dose dependent.

The dose dependency of the isolates in mitigating carrageenan-induce oedema in foot pads is evidence to the potential of the isolates PH I-VI for reducing acute oedema.

As adduced by Vinegar *et al*., 1987, the development of the carrageenan-induced paw oedema derives from the release of cytoplasmic enzymes and serotonin from mast cells and the increase of prostaglandin in the inflammatory area. The initial phase of inflammation (0-2 hr) has been attributed to the release of histamine and kinins, this is followed by a late phase (2.5-6 hr) which is sustained principally by the release of prostaglandins (Rosa, 1972). Evidence more recently has attributed late phase to the induction of cyclooxygenase-2(COX-2) in the tissue(Muniappar and Sundararaj, 2003).

Even though the actual mechanisms of action of the extract of *Palisota hirsuta* and for that matter the compounds isolated from the roots *P. hirsuta* have not been determined , it is possible that, the anti-inflammatory activity exhibited by these could be attributed to the inhibition of the synthesis, release or action of inflammatory mediators that are known to be involved in carrageenan-induced inflammation which include cytoplasmic enzymes and serotonin from mast cells and also bradykinin, prostaglandins and other cyclooxygenase products (Boakye-Gyasi,2010).

The extract, fractions and isolates were compared to the standard drugs diclofenac and dexamethasone which both showed a dose-dependent inhibition of carrageenan-induced edema. Diclofenac, an NSAID exhibits anti-inflammatory effect by mediation mainly through inhibition of the cyclooxygenase pathway (COX 1 and COX 2) and thus inhibit the release of arachidonic acid metabolites particularly prostaglandins which are well known mediators of

inflammation (Al-Majed *et al*, 2003; Seibert *et al*, 1994; Wise *et al*, 2008).The antiinflammatory property of dexamethasone, a steroidal anti-inflammatory drug, is elicited through the suppression of the inflammatory cytokines and on other lipid and glucolipid mediators of inflammation (Enomoto *et al*, 2007; Kaur *et al*, 2004; Li *et al*, 2007; Masferrer *et al*, 1994). The effect before the induction of inflammatory (pre-emptive protocol) paradigm was used for the assessment of inflammation. The crude extract PHC, methanolic and petroleum ether fractions, PHM and PHE of *P. hirsuta* (30-300 mg/kg, p.o.) dose dependently reduced oedema with maximal effects of 64.01 ± 10.90 %, 72.91±4.06% and 57.86±11.89% (prophylactic) respectively. The isolates PHI, IV, V and VI also dose-dependently reduced oedema with maximal effects of 55.39±5.95%, 66.51±5.435%, 69.04±22.61% and 64.52±5.95% (prophylactic) respectively. The NSAID, diclofenac (3-30 mg kg^{-1}, i.p) used as reference drug dose-dependently reduced the oedema with a maximal effect of 71.71±10.46% (prophylactic). Dexamethasone a steroidal anti-inflammatory drug, (0.1-1mg kg^{-1}, *i.p*) another reference drug inhibited the oedema with a maximal effect of 56.76±10.21% (prophylactic). The potency exhibited by the methanolic fraction was similar to the reference drug diclofenac; however, the potency exhibited by the methanolic fraction was higher compared to dexamethasone used when administered one hour before carrageenan injection. Similarly, the potency exhibited by the petroleum ether fraction was similar to that of dexamethasone but less than that of diclofenac administered one hour before carrageenan injection.

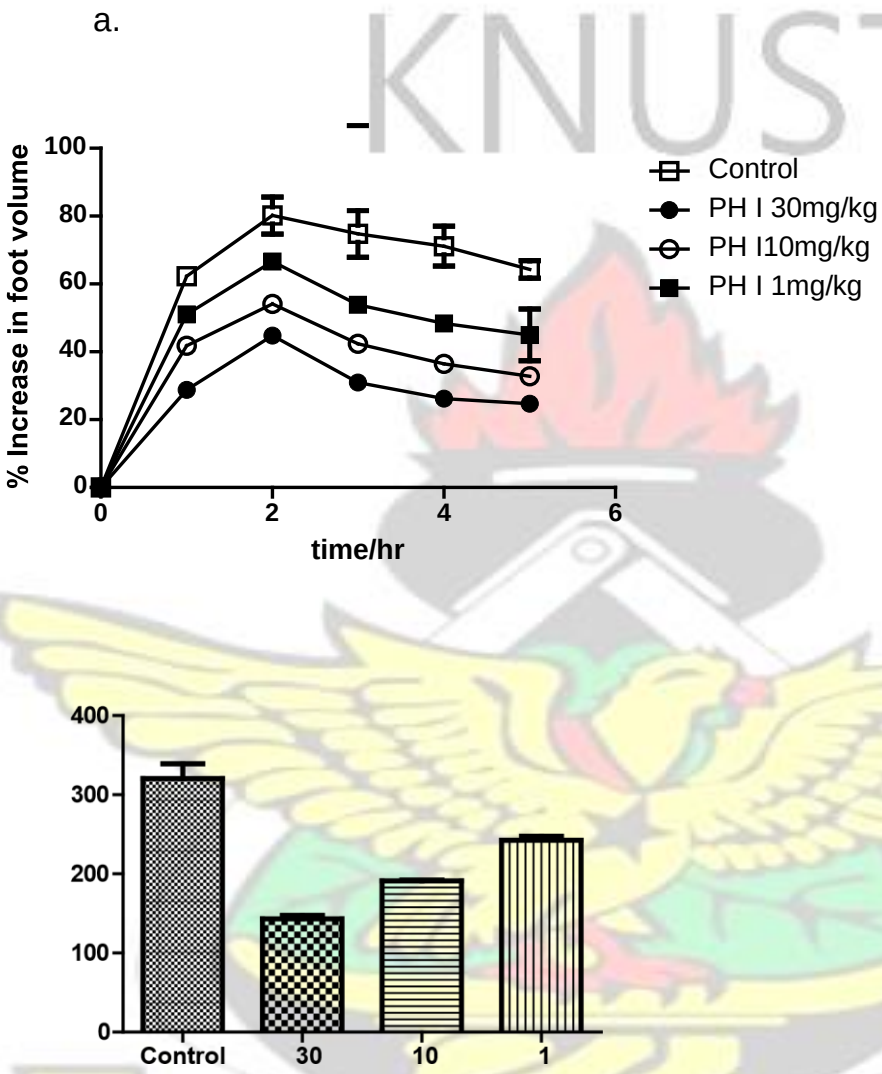


Figure 4.6 Effect of PH 1 (1-30 mg/kg p.o) on the time course curve (a) and total oedema response expressed as AUC (b) in carrageenan induced paw oedema in Chicks. Values are mean± S.E.M (N=6) *** P<0.001, ** P<0.01, * P<0.05 compared to the vehicle treated group (ONE-WAY ANOVA followed by Newman Keul's *post hoc* test).

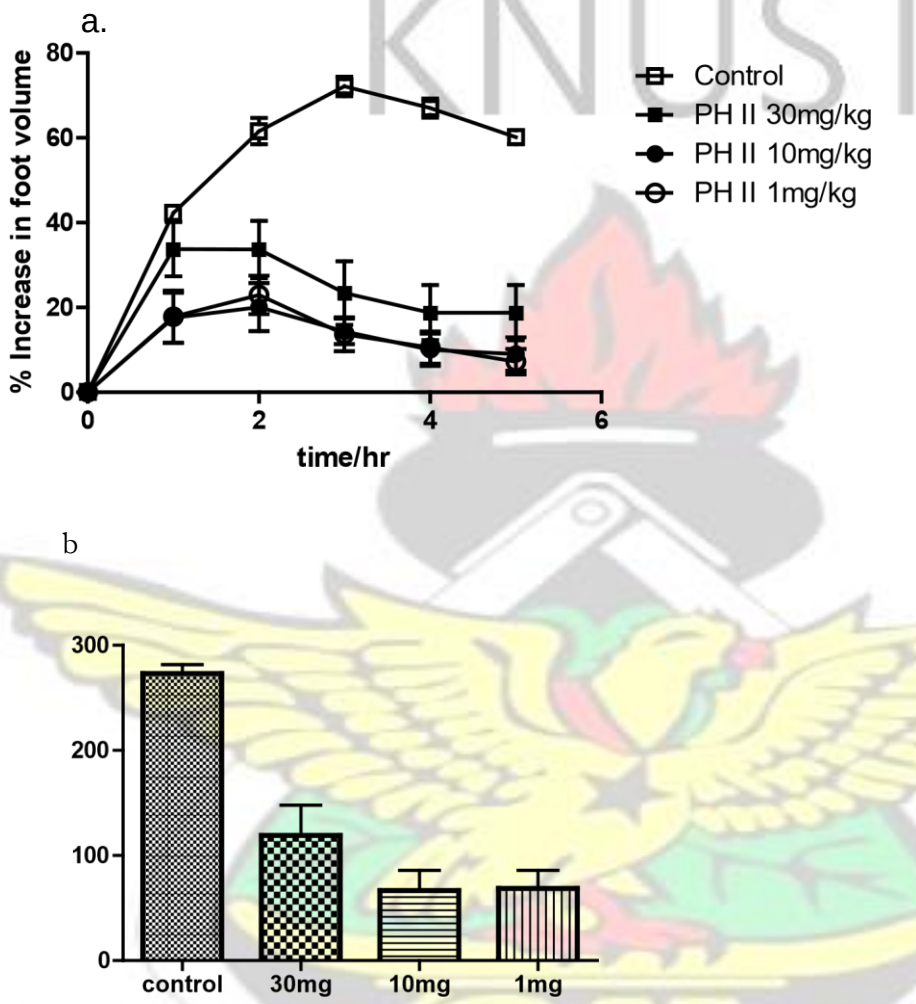


Figure 4.7 Effect of PH II (1-30 mg/kg p.o) on the time course curve (a) and total oedema response expressed as AUC (b) in carrageenan induced paw oedema in Chicks. Values are mean± S.E.M (N=6) *** P<0.001, ** P<0.01, * P<0.05 compared to the vehicle treated group (ONE-WAY ANOVA followed by Newman Keul's *post hoc* test).

a.

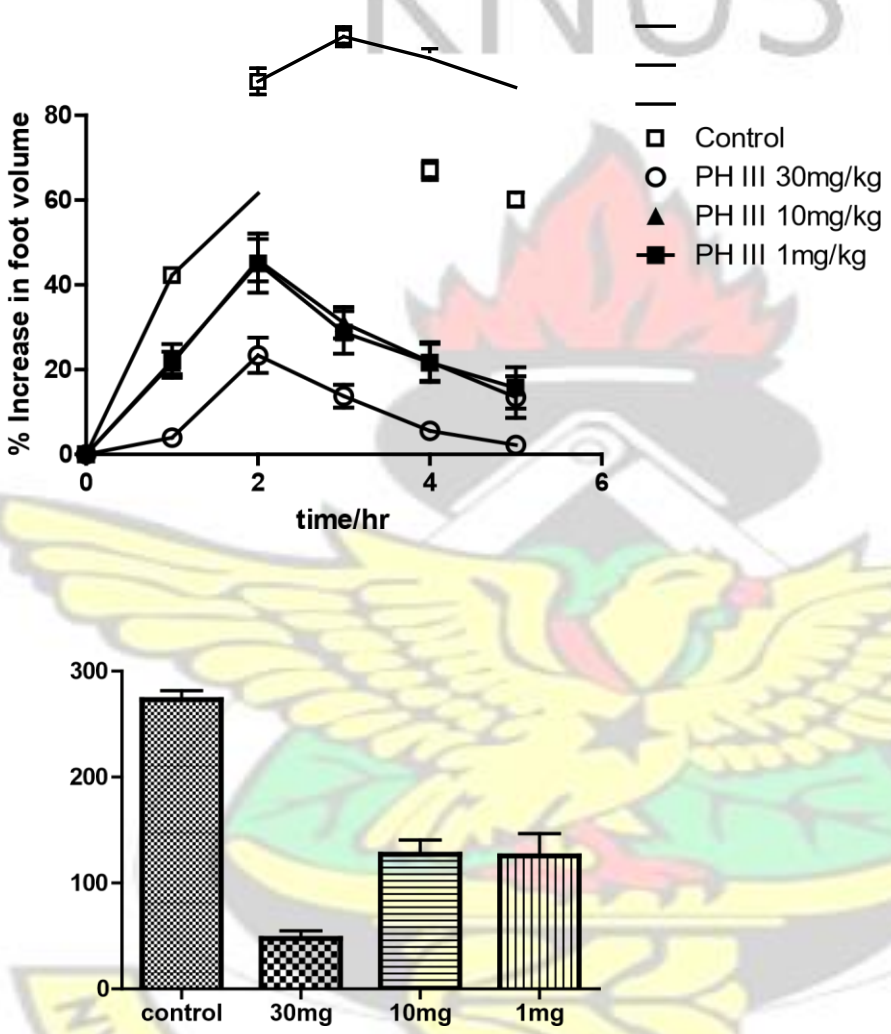


Figure 4.8 Effect of PH III (1-30 mg/kg *p.o*) on the time course curve (a) and total oedema response expressed as AUC (b) in carrageenan induced paw oedema in Chicks. Values are

mean± S.E.M (N=6) *** P<0.001, ** P<0.01, * P<0.05 compared to the vehicle treated group (ONE-WAY ANOVA followed by Newman Keul's *post hoc* test).

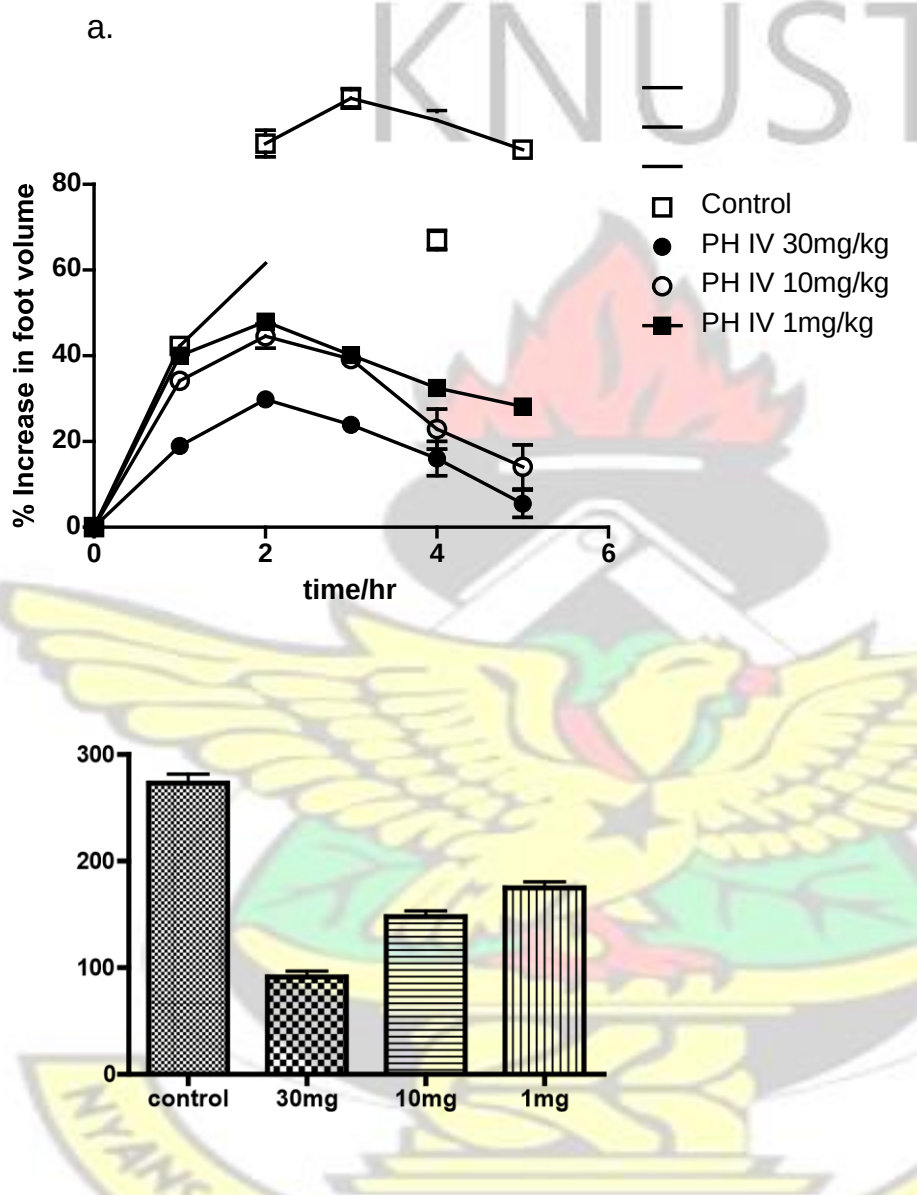


Figure 4.9 Effect of PH IV (1-30 mg/kg *p.o*) on the time course curve (a) and total oedema response expressed as AUC (b) in carrageenan induced paw oedema in Chicks. Values are

mean± S.E.M (N=6) *** P<0.001, ** P<0.01, * P<0.05 compared to the vehicle treated group (ONE-WAY ANOVA followed by Newman Keul's *post hoc* test).

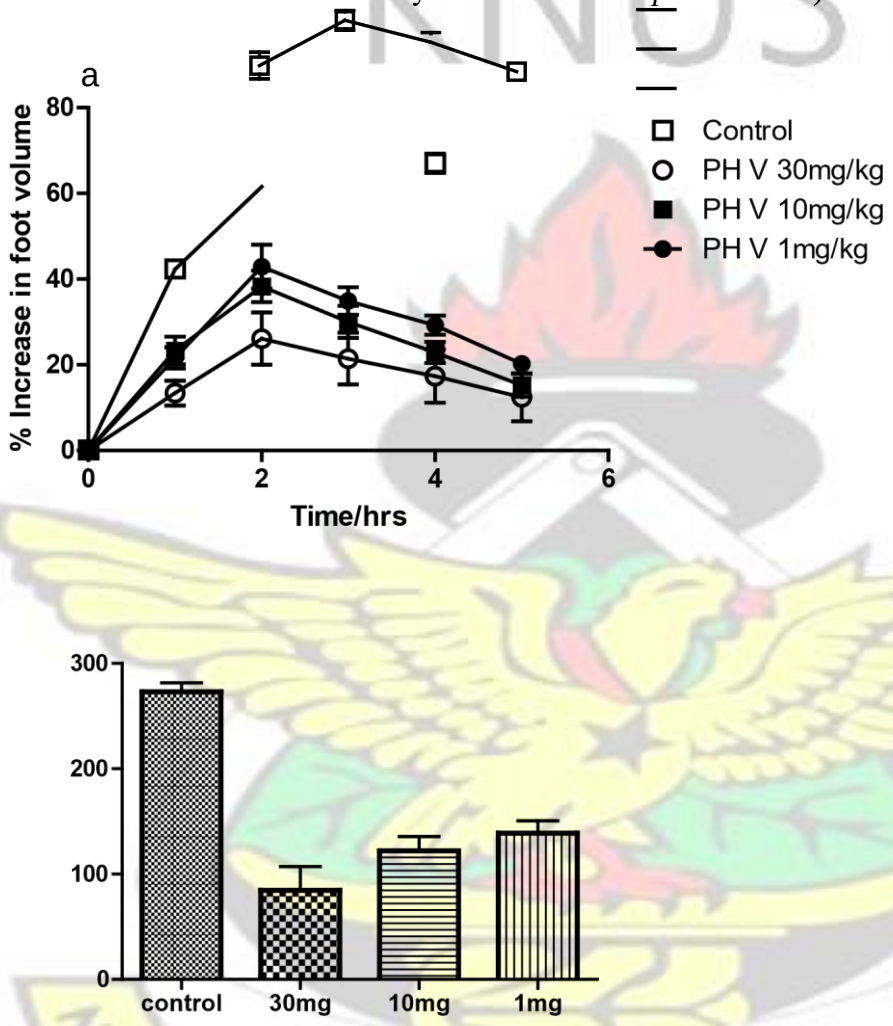


Figure 4.10 Effect of PH V (1-30 mg/kg p.o) on the time course curve (a) and total oedema response expressed as AUC (b) in carrageenan induced paw oedema in Chicks. Values are

mean± S.E.M (N=6) *** P<0.001, ** P<0.01, * P<0.05 compared to the vehicle treated group (ONE-WAY ANOVA followed by Newman Keul's *post hoc* test).

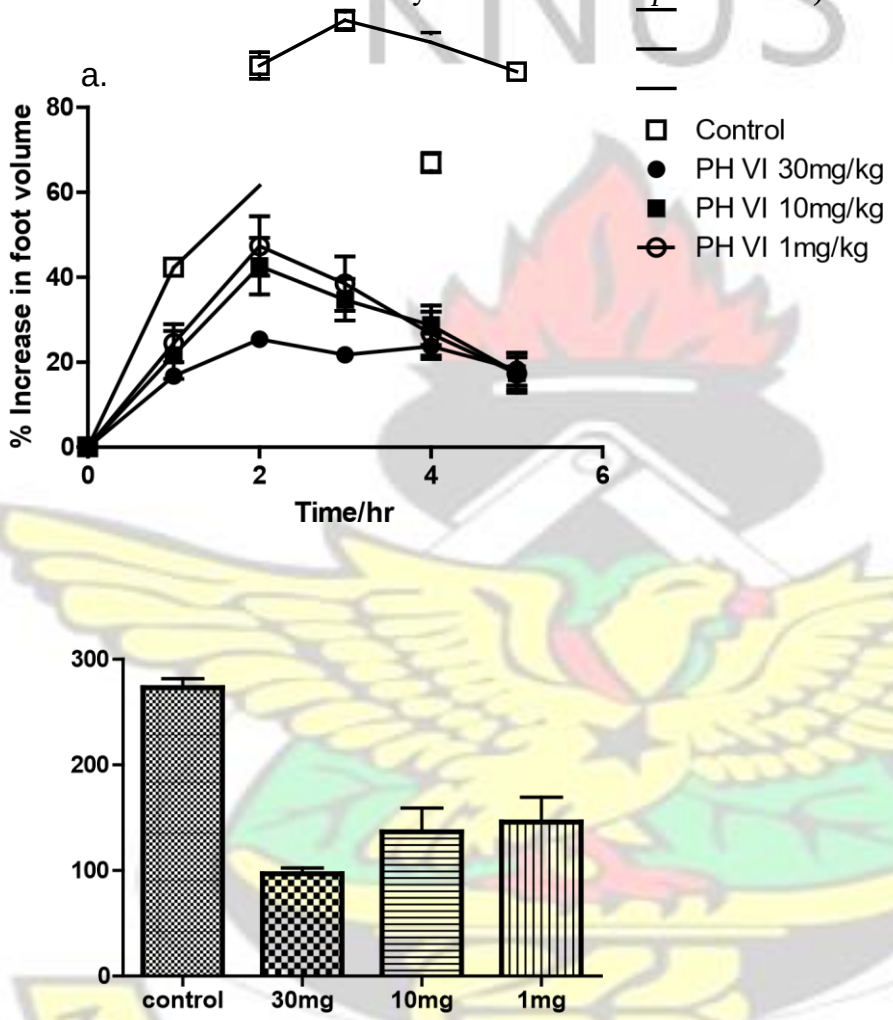
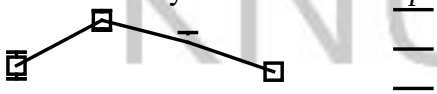
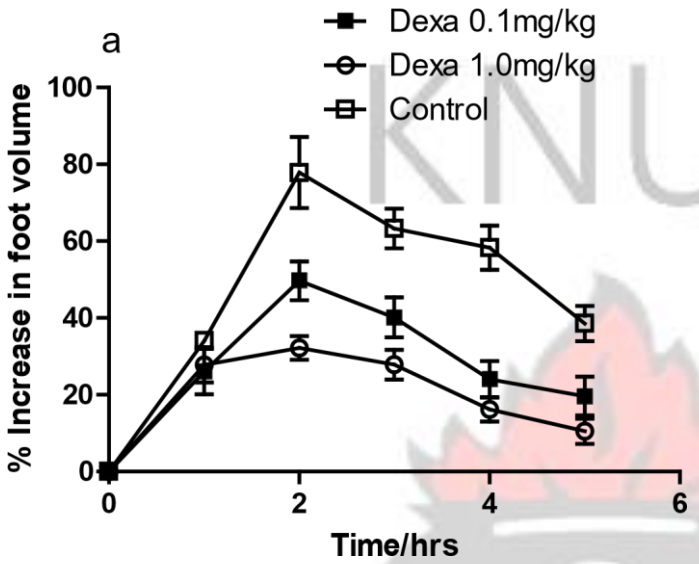


Figure 4.11 Effect of PH VI (1-30 mg/kg *p.o*) on the time course curve (a) and total oedema response expressed as AUC (b) in carrageenan induced paw oedema in Chicks. Values are

mean± S.E.M (N=6) *** P<0.001, ** P<0.01, * P<0.05 compared to the vehicle treated group (ONE-WAY ANOVA followed by Newman Keul's *post hoc* test).





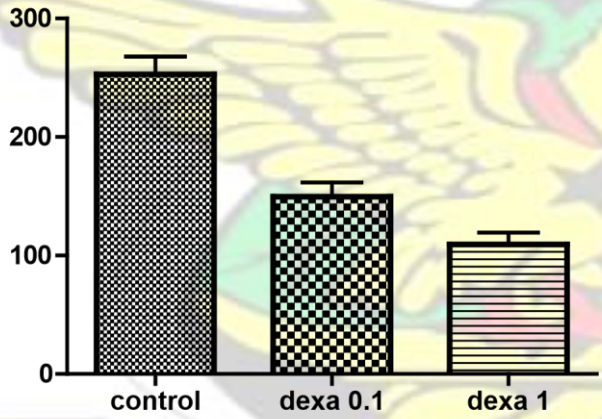
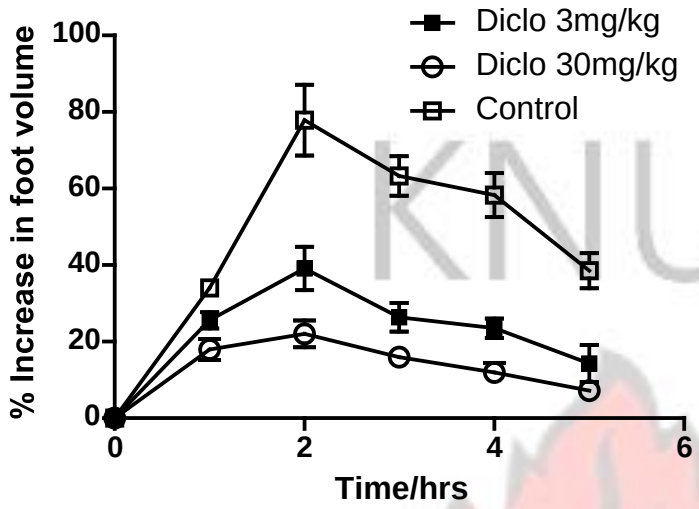


Figure 4.13 Effect of dexamethasone (0.1-1 mg/kg *i.p*) on the time course curve (a) and total oedema response expressed as AUC (b) in carrageenan induced paw oedema in Chicks. Values are mean± S.E.M (N=6) *** P<0.001, ** P<0.01, * P<0.05 compared to the vehicle treated group (ONE-WAY ANOVA followed by Newman Keul's *post hoc* test).

a



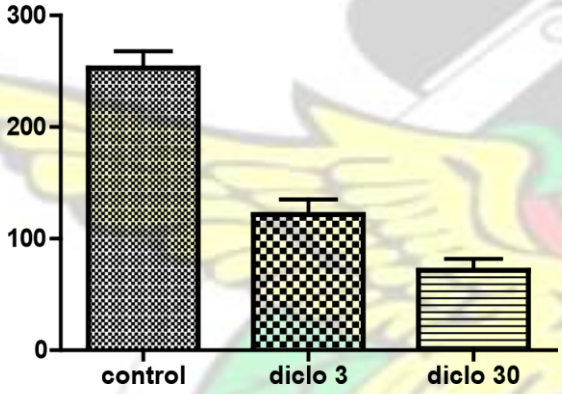


Figure 4.14Effect of diclofenac (3-30 mg/kg *i.p*) on the time course curve (a) and total oedema response expressed as AUC (b) in carrageenan induced paw oedema in Chicks. Values are mean± S.E.M (N=6) *** P<0.001, ** P<0.01, * P<0.05 compared to the vehicle treated group (ONE-WAY ANOVA followed by Newman Keul's *post hoc* test).

4.3 ANTI-OXIDANT ASSAY OF ISOLATES

The isolates (PH 1-VI) were assessed for antioxidant activity using selected antioxidant assays.

4.3.1 DPPH scavenging activity

The isolates caused a concentration dependent DPPH scavenging effect as shown by the concentration response curve (Figure 4.15). This is an indication of the isolates' ability to directly interact with free radicals to produce less harmful products. This property is exhibited by chain breaking and scavenging antioxidants like tocopherol (vitamin E) and ascorbic acid (vitamin C) by donating an electron to stabilize an existing free radical (Cui *et al*, 2004; Sakai *et al*, 1999; Scheibmeir *et al*, 2005).The IC_{50} for the isolates PH I-VI and n-propyl gallate are listed in Table 4.1

Table 4.1 DPPH Scavenging activity of isolates and *n*-propyl gallate

Drug	**IC_{50}**
PH 1	3.304
PH 11	3.636
PH III	0.241
PH IV	0.880
PH V	4.414
PH VI	0.163
n-propyl gallate	0.058

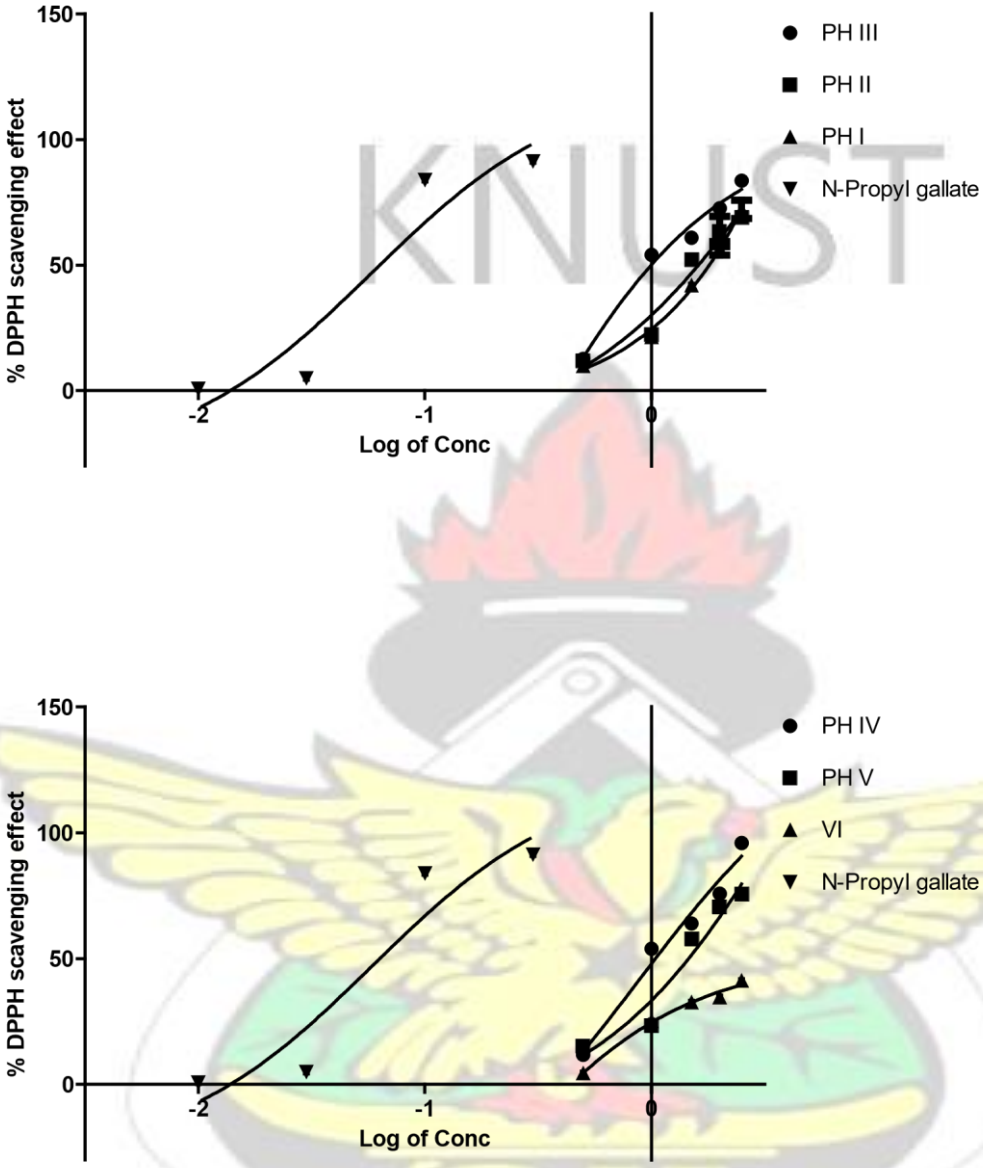


Figure 4.15 Free radical scavenging ability of PH I-VI compared to *n*-propyl gallate (0.01-0.3 mg/ml) in the DPPH radical assay. Each point represents mean ± S.E.M (n=3)

4.3.2 Reducing power

The isolates PH I-VI (0.5- 1.5mg ml^{-1}) and the standard antioxidant *n*-propyl gallate (0.3-3 mg ml^{-1}) concentration dependently reduced Fe^{3+} to Fe^{2+} resulting in concentration dependent increase in absorbance (Figure 4.2). From the EC_{50} (in $mgml^{-1}$; Table 4.2) obtained for PH I-VI and *n*-propyl gallate, PH I-III were found to be about three fold less potent than *n*-propyl gallate. PH V was equipotent to *n*-propyl gallate. However PH IV was found to be about three times more potent than the standard antioxidant*n*-propyl gallate.

Table 4.2 Reducing power activity of isolates and *n*-propyl gallate

Extract/Drug	**EC_{50}**
PH I	5.874
PH II	5.122
PH III	5.238
PH IV	0.524
PH V	1.742
PH VI	4.501
***n*-propyl gallate**	1.723

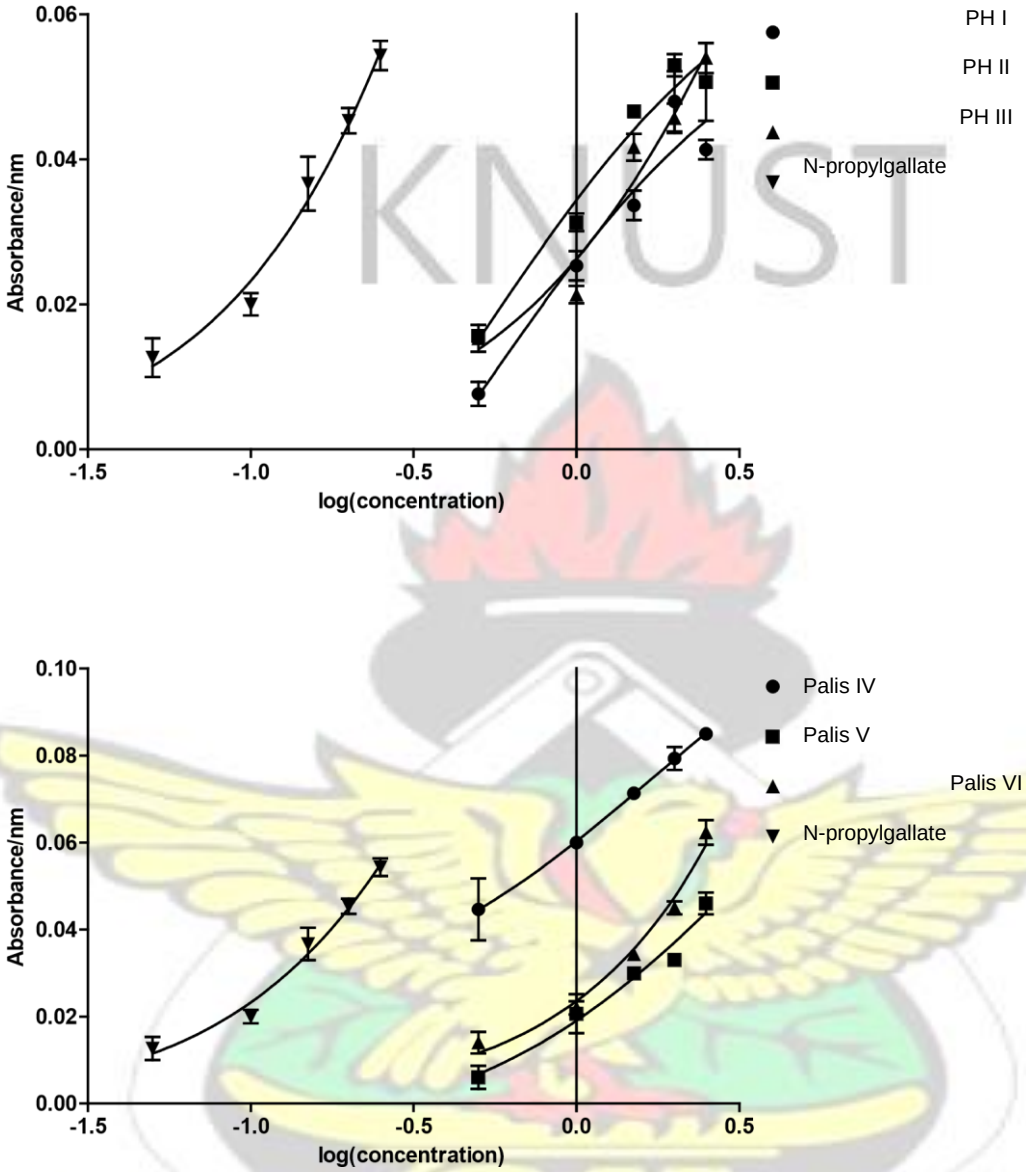


Figure 4.16 Reducing power of PH I-VI (0.5-1.5 mg/ml) compared to *n*-propyl gallate (0.3-3 mg/ml). Each point represents the mean± S.E.M (n=3).

4.3.3 Lipid peroxidation using linoleic acid

In biological systems, lipid peroxidation generates a number of degradation products, such as malondialdehyde (MDA), and it is found to be an important cause of cell membrane destruction

and cell damage (Yoshikawa *et al*., 1997). Lipid peroxidation is an oxidative alteration of polyunsaturated fatty acids in the cell membranes that generates the aforementioned degradation. Lipid peroxidation is considered a marker of oxidative stress (Janero, 1990).

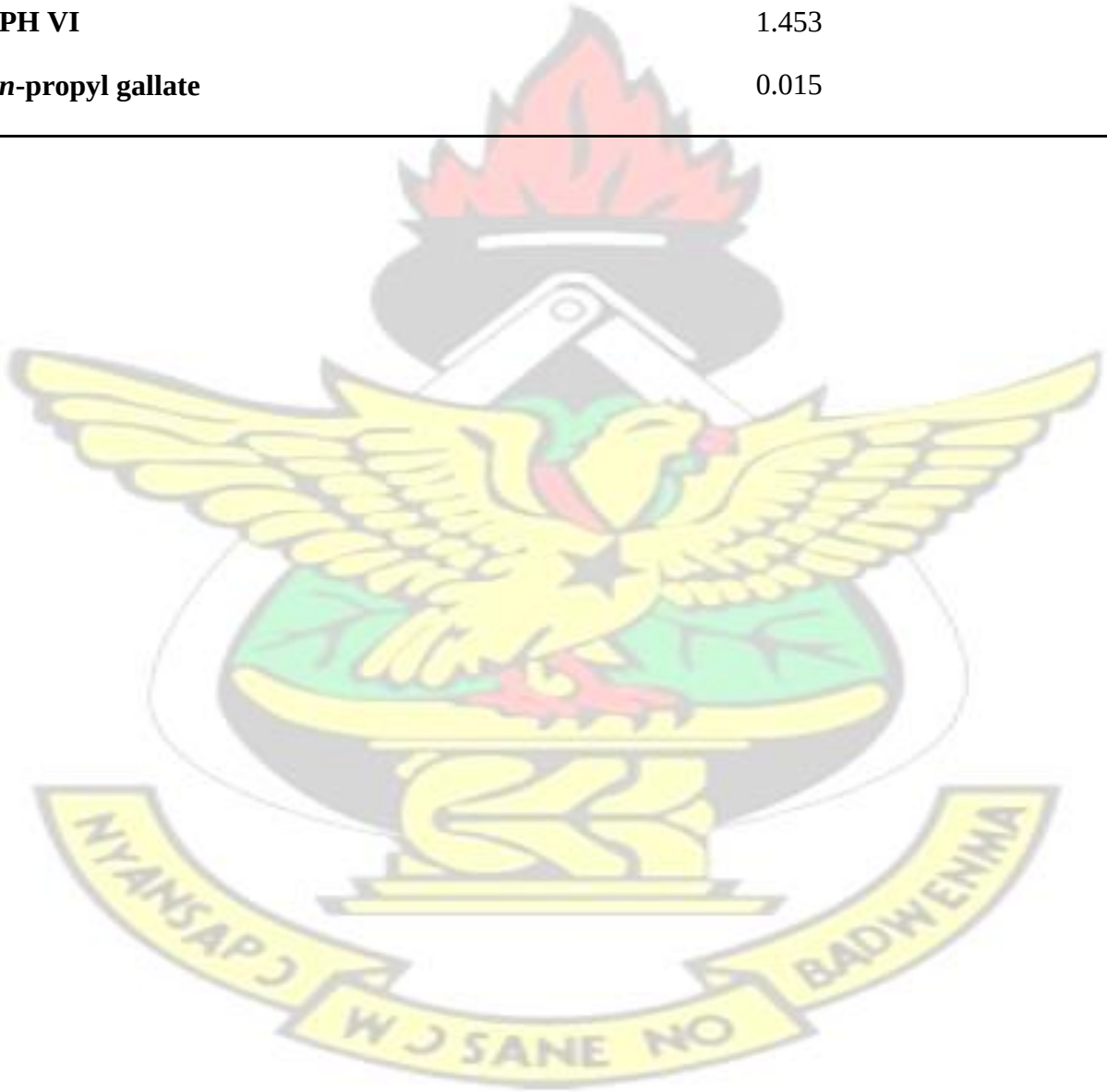
Most researchers use the homogenized brain as their source of polysaturated fatty acid (PUFA) base. Linoleic acid autoxidation assay was chosen due to its relatively simple methodology, cheap in terms of cost (compared to cost of rats), and less intensive.

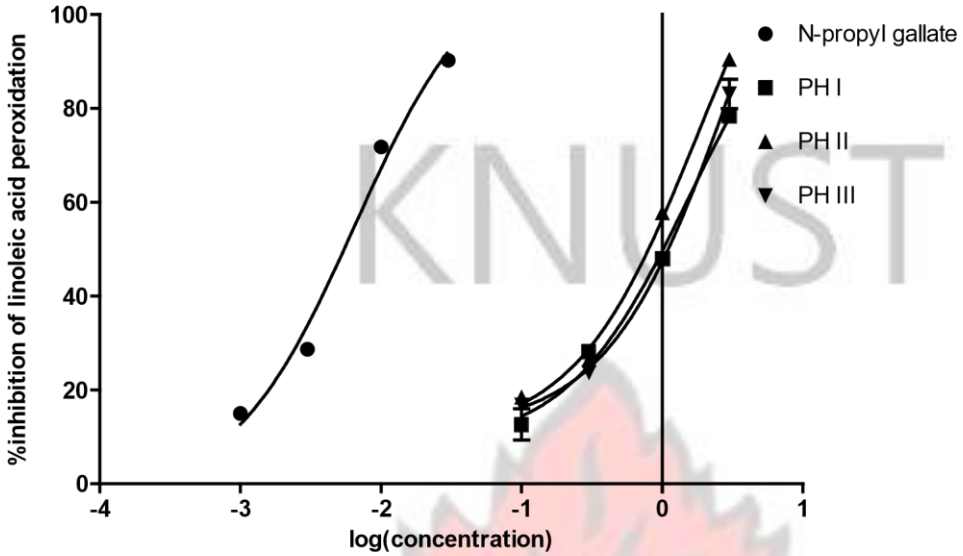
The isolates PH I, II, III, IV, V and VI as well as the standard drug effectively inhibited lipid peroxidation, a proof of their potent antioxidant properties. The IC_{50} values for the various isolates and the standard drug *n*-propyl gallate are listed in Table 4.3.

Table 4.3Lipid peroxidation activity of isolates and *n*-propyl gallate

Extract/Drug	**IC_{50}**

PH I	0.260
PH II	0.295
PH III	1.545
PH IV	0.007
PH V	1.769
PH VI	1.453
***n*-propyl gallate**	0.015





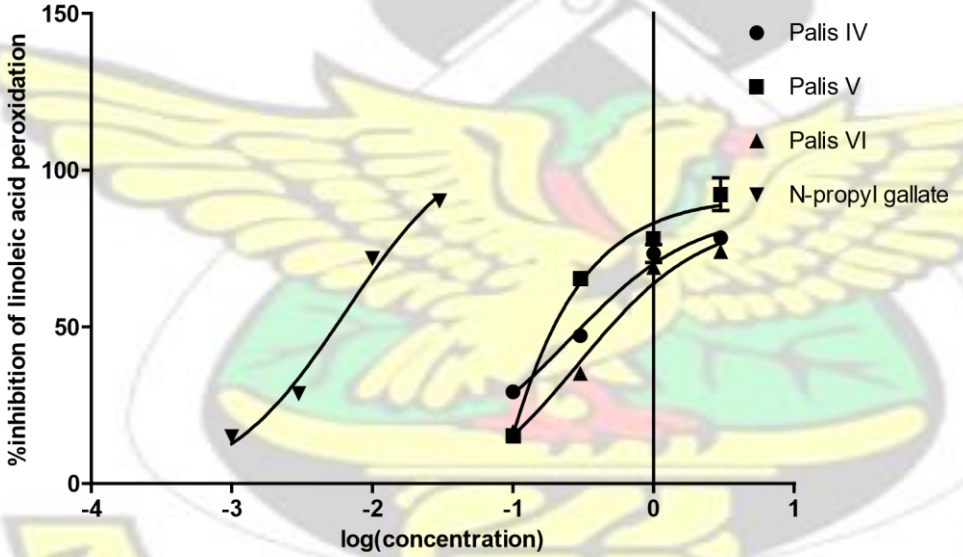


Figure 4.17 Percentage inhibition of linoleic acid autoxidation by PH I-VI (0.5-3mg/ml) compared to *n*-propyl gallate (0.001-0.3mg/ml). Each point represents the mean S.E.M (n=3)

4.4 ANTI-NOCICEPTIVE ASSAY

Formalin-induced nociception

Formalin administration into the right hind paw produced a typical pattern of flinching and licking/biting behaviour. The first phase (early phase) of nociception which is mainly due to direct irritation (direct stimulation of nociceptors) by the formalin started immediately after administration of formalin diminished gradually in about 10 minutes. The second phase (late phase, during which inflammatory phenomena occur) started at about 15 minutes after the formalin injection and lasted until one hour. Treatment of mice with the various crude fractions and isolates which have been designated PHC,PHE,PHM, PH I,II,III,IV,V & VI (10–300 mg kg^{-1}, *p.o.*, 60 minute earlier for the extract and fractions and 10-30 mg kg^{-1},*p.o.*, 60 minutes earlier for the isolates) respectively (Figures 4.18a & b- 4.26 a & b), produced a marked and dose-related inhibition of both phases of formalin-induced nociception first phase ($P<0.0001$) second phase ($P<0.0001$).The highest dose causing maximal inhibitions of 40.89±24.10% and 66.25±32.08% for PHC. For the fraction, PHE the highest dose causing maximal nociceptive inhibition of45.02±29.81% and 80.50±4.628%. The maximal inhibitions caused by the highest dose of the fraction PHM was 31.72±15.29% and 61.0±41.97%.

Considering the isolates, the pattern of inhibition was not regular for the various isolated compounds. For instance, with PH I, the maximal inhibitions caused by the doses (phase 1: $10mgkg^{-1}$ and phase $30mgkg^{-1}$) were 55.08±14.90% and 63.57±25.91%. About PH II, the maximal inhibitions as anti-nociceptive activity were 90.86±6.40% and 67.47±20.84%, and these were produced by the doses $1mgkg^{-1}$ for phase 1 and $30mgkg^{-1}$ for phase 2 respectively.

PH III caused maximal inhibitions of 57.87±8.43 and 79.90±7.05% and these were obtained from the doses 1mgkg^{-1} for phase 1 and 10mgkg^{-1} for phase 2. The highest dose was that caused maximal inhibitions of nociception of 71.39±9.19% and 89.19±3.81% for PH IV and 61.34±11.07% and 82.89±3.97% for PH V was 30mgkg-1. A dose of 10mgkg-1 caused maximal inhibitions of 70.14±8.60% and 86.18±7.42% of the licking time in the early and late phase respectively for PH VI (As demonstrated by Fig. 4.18a&b -4.26 a&b). Similarly, morphine (1-10 mg kg^{-1}, i.p.) produced marked inhibition of both the neurogenic ($P<0.0001$) and inflammatory ($P<0.0001$) pain phases (Fig. 4.18a and b). Morphine, reduced the duration of formalin evoked nociceptive behavior by a maximum percentage of 92.49±4.67% in the early phase and 95.16±5.49% in the late phase of the formalin test (Fig. 4.18a and b). Examining the results obtained in the formalin test, it was deduced that; PHC, PHE, PHM, PH I-VI and morphine significantly reduced the time spent in licking the injured paw, an indication that these compounds possess anti-nociceptive or analgesic properties.

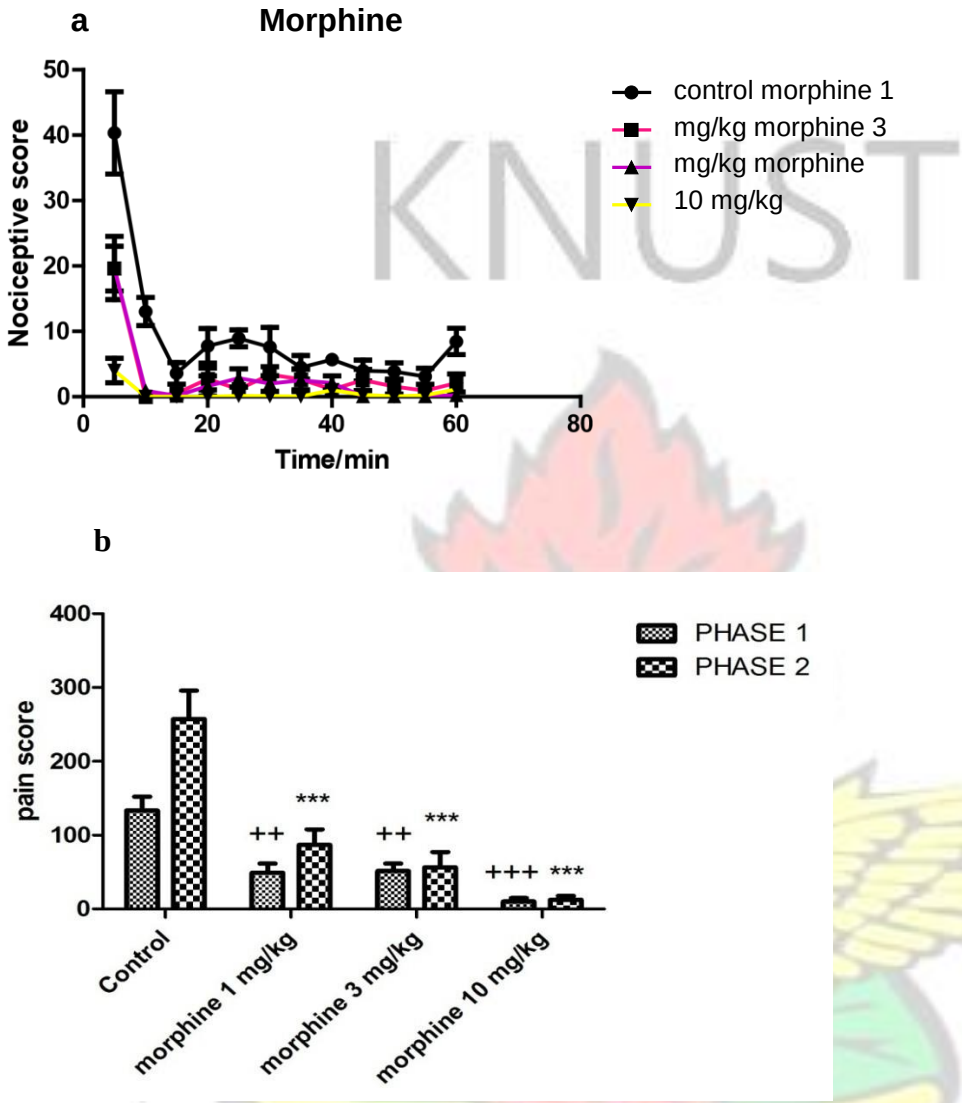
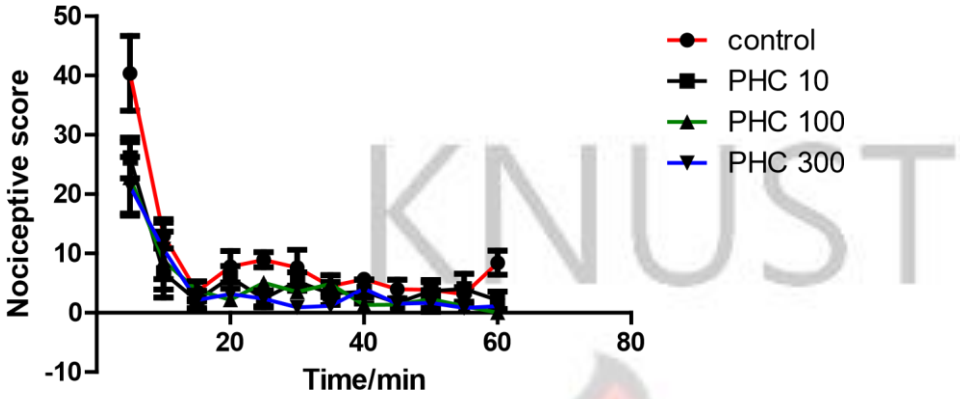


Figure 4.18 Effect of Morphine (1-10 mg kg^{-1} *i.p.)* on the time course of formalin induced pain in mice (a). Nociceptive/pain scores are shown in 5 min blocks up to 60 min post formalin injection. Each point represents Mean ± S.E.M (n = 5). *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$ compared to respective controls (two-way repeated measures ANOVA; the AUC (total response) for phase 1 and phase 2. Each column in (b) represents the mean ± S.E.M. $^{+}P \leq 0.05$, $^{++}P \leq 0.01$, $^{+++}P \leq 0.001$ (one-way ANOVA followed by Newman-Keuls *post hoc* test)

a **PHC**



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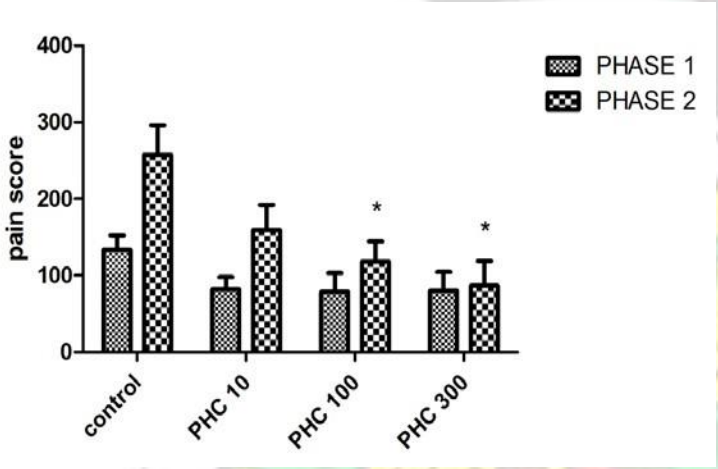


Figure 4.19 Effect of PHC (10-300 mg kg^{-1} *i.p.)* on the time course of formalin induced pain in mice (a). Nociceptive/pain scores are shown in 5 min blocks up to 60 min post formalin injection. Each point represents Mean ± S.E.M (n = 5). $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$ compared to respective controls (two-way repeated measures ANOVA; the AUC (total response) for phase 1 and phase 2. Each column in (b) represents the mean ± S.E.M. $^{+}P \leq 0.05$, $^{++}P \leq 0.01$, $^{+++}P \leq 0.001$ (one-way ANOVA followed by Newman-Keuls *post hoc* test)

a **PHM**

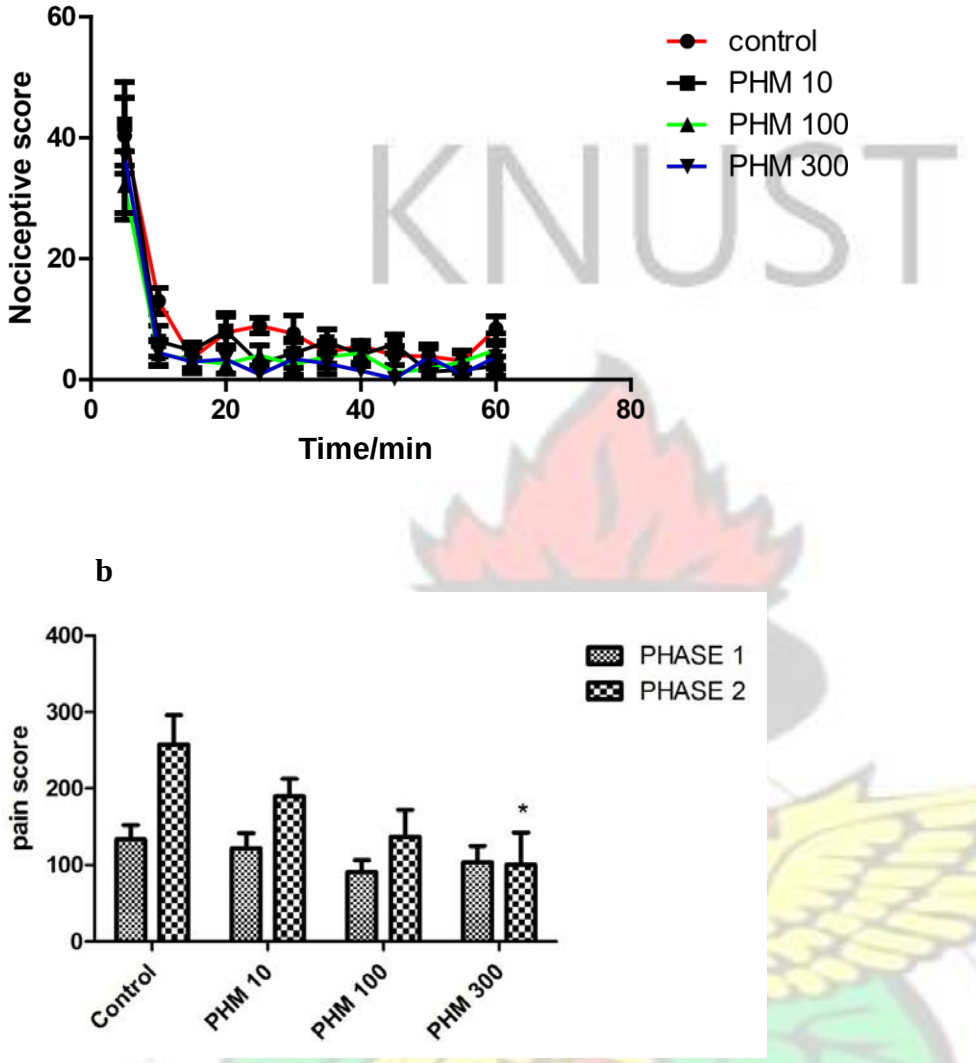
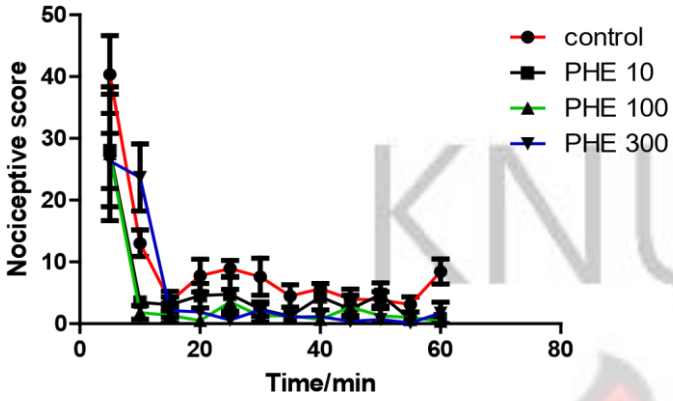


Figure 4.20 Effect of PHM (1-10 mg kg^{-1} *i.p.)* and on the time course of formalin induced pain in mice (a). Nociceptive/pain scores are shown in 5 min blocks up to 60 min post formalin injection. Each point represents Mean ± S.E.M (n = 5). $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$ compared to respective controls (two-way repeated measures ANOVA; the AUC (total response) for phase 1 and phase 2. Each column in (b) represents the mean ± S.E.M. $^{+}P \leq 0.05$, $^{++}P \leq 0.01$, $^{+++}P \leq 0.001$ (one-way ANOVA followed by Newman-Keuls *post hoc* test)

a **PHE**



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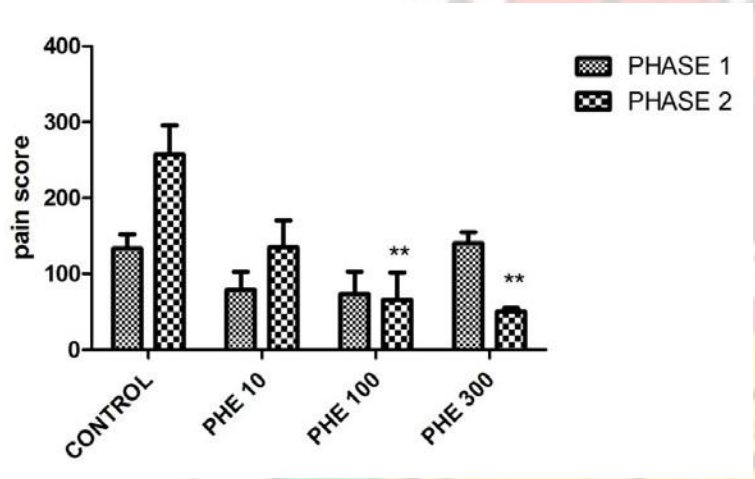


Figure 4.21 Effect of PHE (10-300 mg kg^{-1} *i.p.)*on the time course of formalin induced pain in mice (a). Nociceptive/pain scores are shown in 5 min blocks up to 60 min post formalin injection. Each point represents Mean ± S.E.M (n = 5). $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$ compared to respective controls (two-way repeated measures ANOVA; the AUC (total response) for phase 1 and phase 2. Each column in (b) represents the mean ± S.E.M. $^{+}P \leq 0.05$, $^{++}P \leq 0.01$, $^{+++}P \leq 0.001$ (one-way ANOVA followed by Newman-Keuls *post hoc* test)

a **PH I**

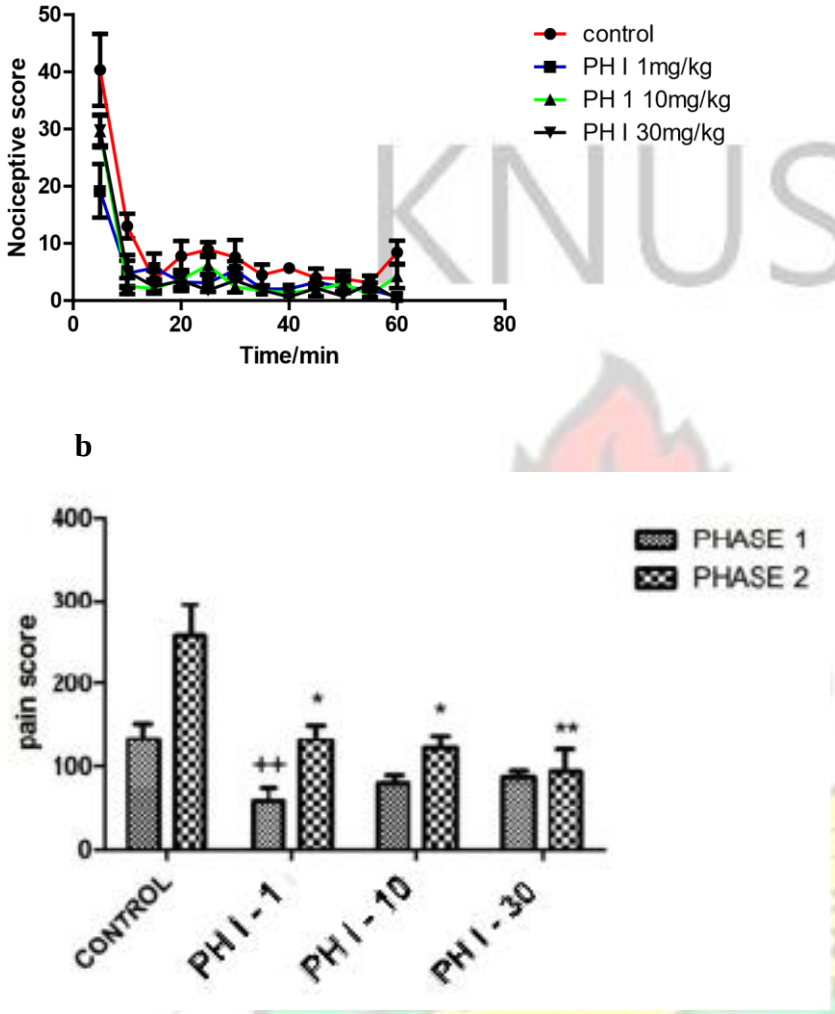


Figure 4.22 Effect of PH I (1-30 mg kg^{-1} *i.p.)*on the time course of formalin induced pain in mice (a). Nociceptive/pain scores are shown in 5 min blocks up to 60 min post formalin injection. Each point represents Mean ± S.E.M (n = 5).*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$ compared to respective controls (two-way repeated measures ANOVA; the AUC (total response) for phase 1 and phase 2. Each column in (b) represents the mean ± S.E.M. $^{+}P \leq 0.05$, $^{++}P \leq 0.01$, $^{+++}P \leq 0.001$ (one-way ANOVA followed by Newman-Keuls *post hoc* test)

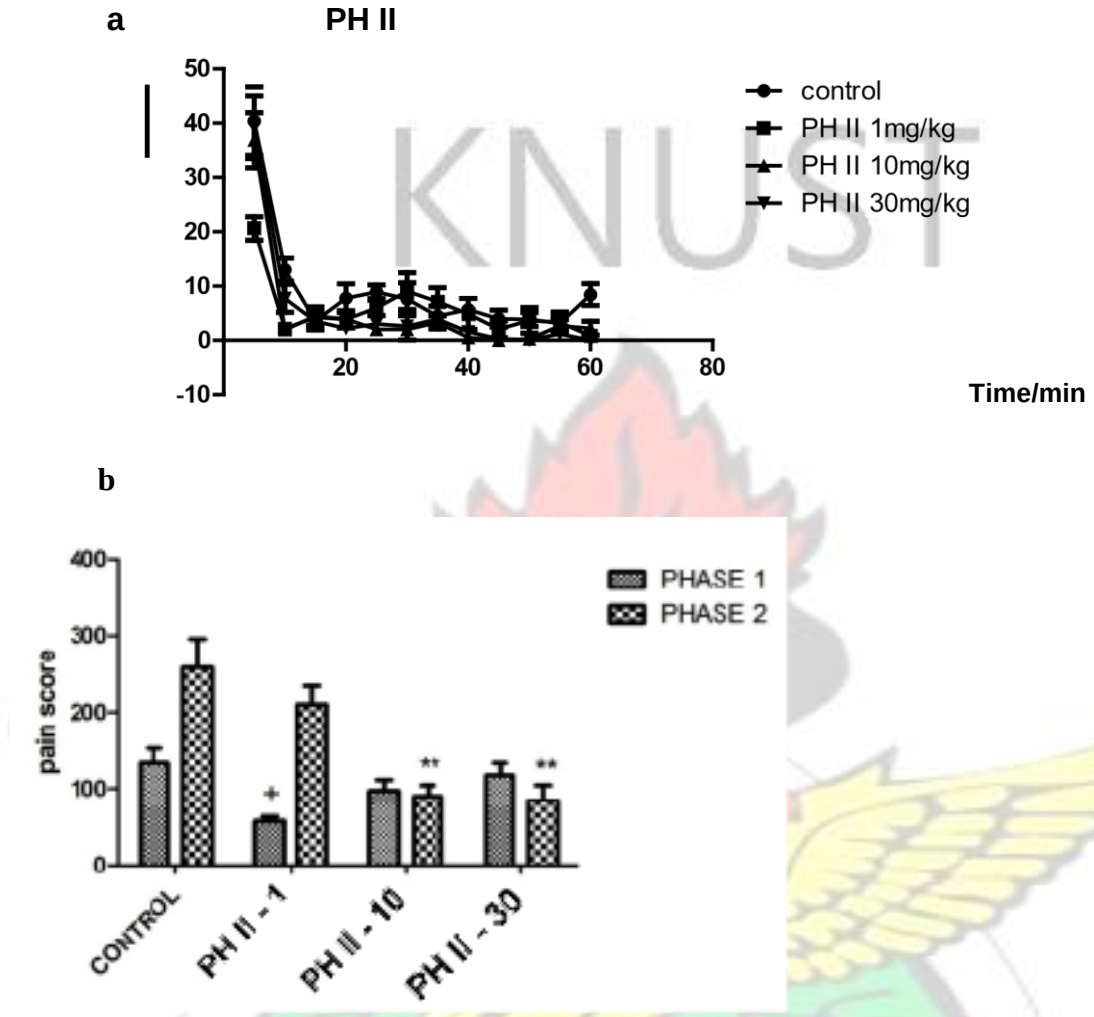


Figure 4.23 Effect of PH II (1-30 mg kg^{-1} *i.p.)*on the time course of formalin induced pain in mice. Nociceptive/pain scores are shown in 5 min blocks up to 60 min post formalin injection. Each point represents Mean ± S.E.M (n = 5). $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ compared to respective controls (two-way repeated measures ANOVA; the AUC (total response) for phase 1 and phase 2. Each column in b represents the mean ± S.E.M. $^{+}P \leq 0.05$, $^{++}P \leq 0.01$, $^{+++}P \leq 0.001$ (one-way ANOVA followed by Newman-Keuls *post hoc* test)

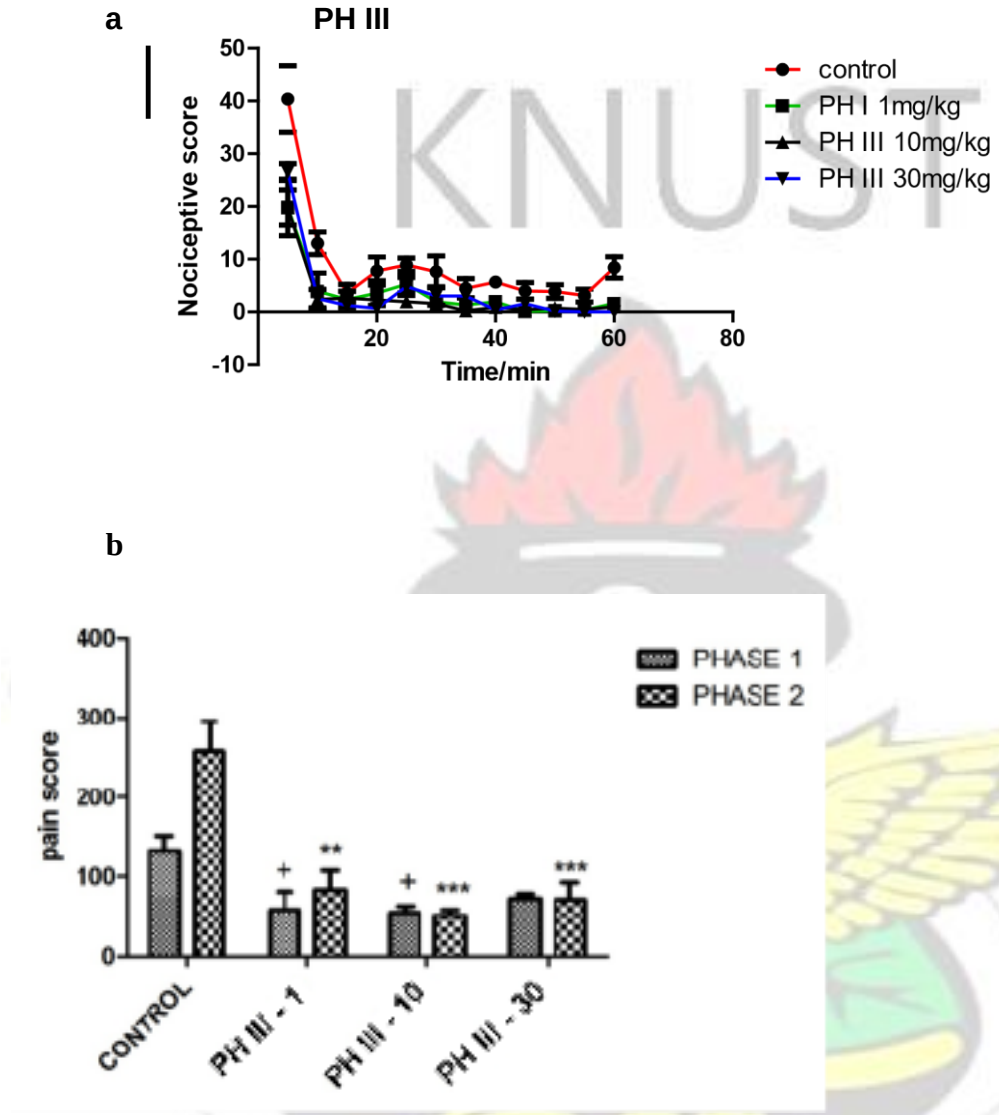


Figure 4.24 Effect of PH III (1-30 mg kg^{-1} *i.p.)* on the time course of formalin induced pain in mice (a). Nociceptive/pain scores are shown in 5 min blocks up to 60 min post formalin injection. Each point represents Mean ± S.E.M (n = 5). $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$ compared to respective controls (two-way repeated measures ANOVA; the AUC (total response) for phase 1 and phase 2. Each column in (b) represents the mean ± S.E.M. $^{+}P \leq 0.05$, $^{++}P \leq 0.01$, $^{+++}P \leq 0.001$ (one-way ANOVA followed by Newman-Keuls *post hoc* test)

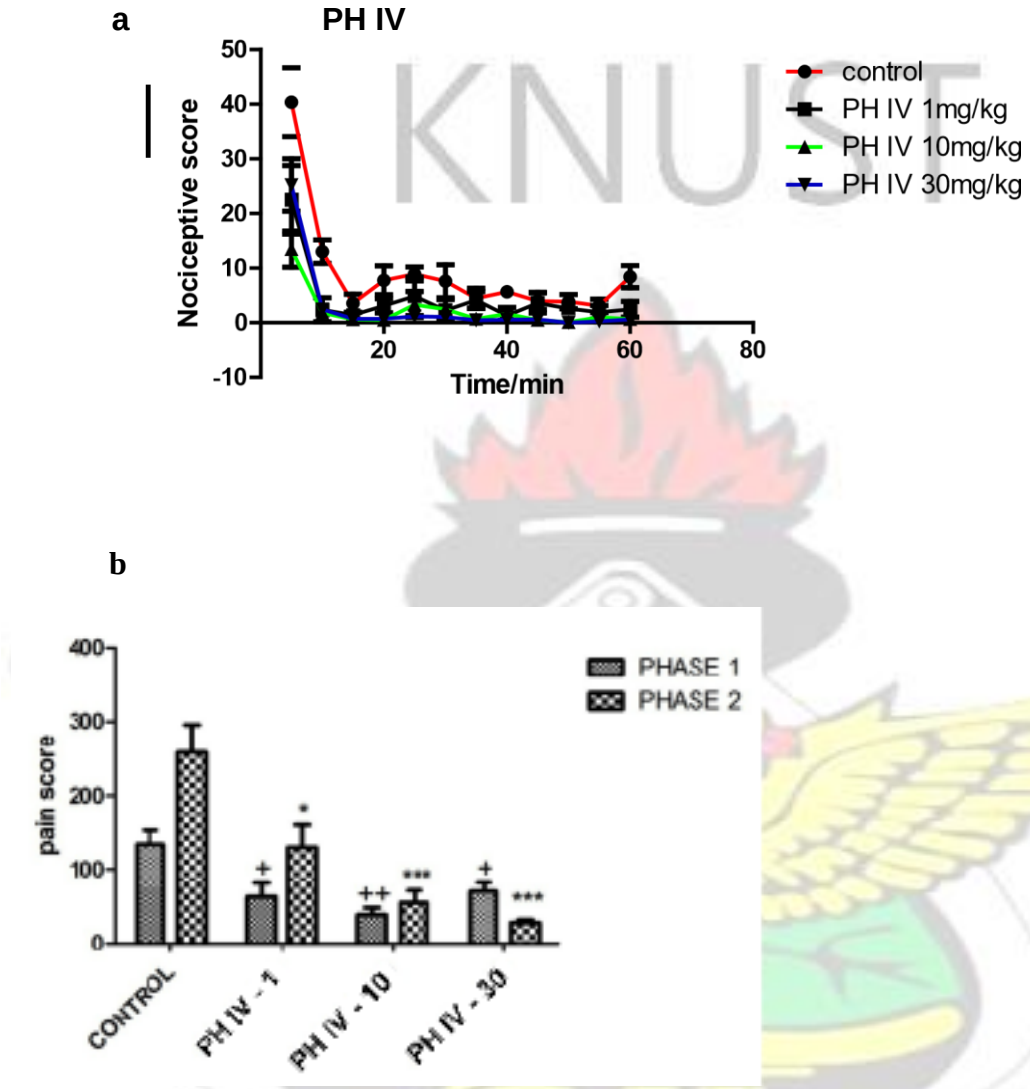


Figure 4.25 Effect of PH IV (1-30 mg kg^{-1} *i.p.)* and on the time course of formalin induced pain in mice (a). Nociceptive/pain scores are shown in 5 min blocks up to 60 min post formalin injection. Each point represents Mean ± S.E.M (n = 5). $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ compared to respective controls (two-way repeated measures ANOVA; the AUC (total response) for phase 1 and phase 2. Each column in (b) represents the mean ± S.E.M. $^{+}P \leq 0.05$, $^{++}P \leq 0.01$, $^{+++}P \leq 0.001$ (one-way ANOVA followed by Newman-Keuls

post hoc test)

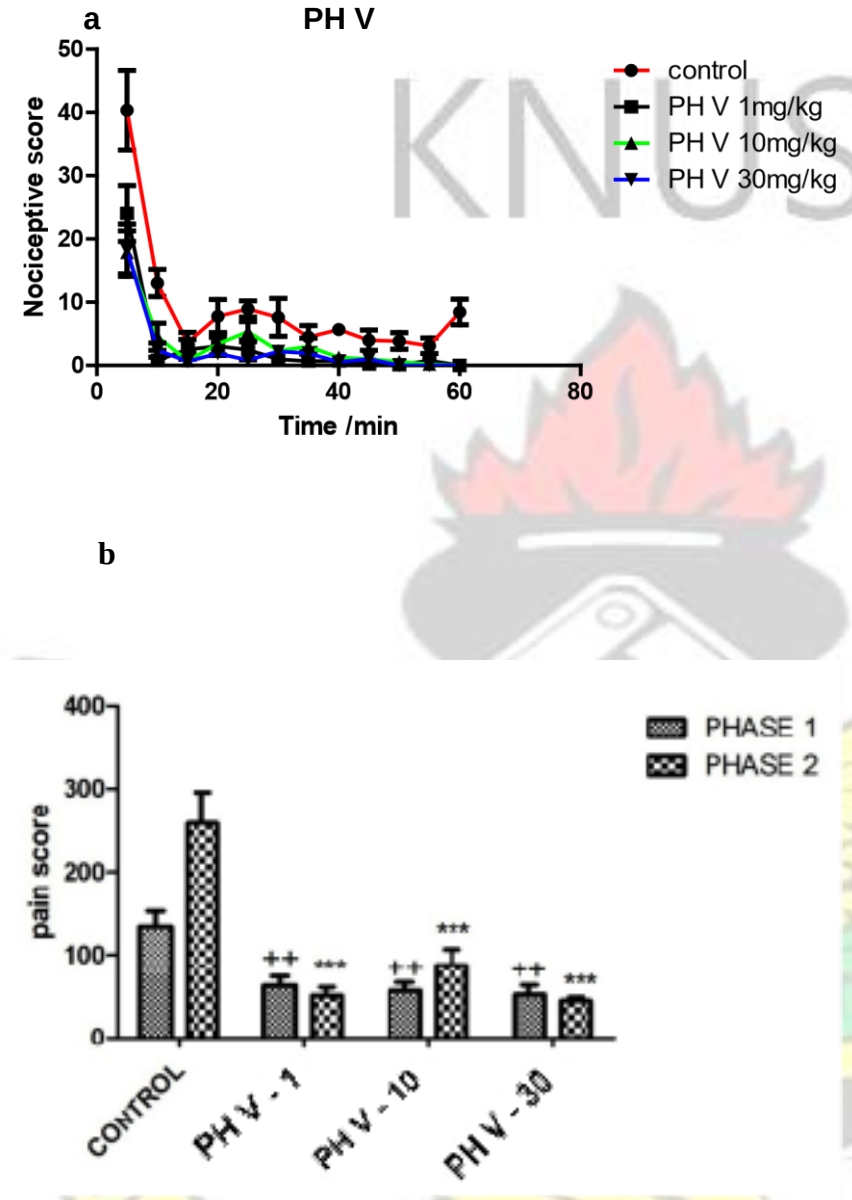


Figure 4.26Effect of PH V (1-30 mg kg^{-1} *i.p.)*on the time course of formalin induced pain in mice (a). Nociceptive/pain scores are shown in 5 min blocks up to 60 min post formalin injection. Each point represents Mean ± S.E.M (n = 5). *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$ compared to respective controls (two-way repeated measures ANOVA; the AUC (total

response) for phase 1 and phase 2. Each column in (b) represents the mean ± S.E.M. $^{+}P \leq 0.05$, $^{++}P \leq 0.01$, $^{+++}P \leq 0.001$ (one-way ANOVA followed by Newman-Keuls *post hoc* test)

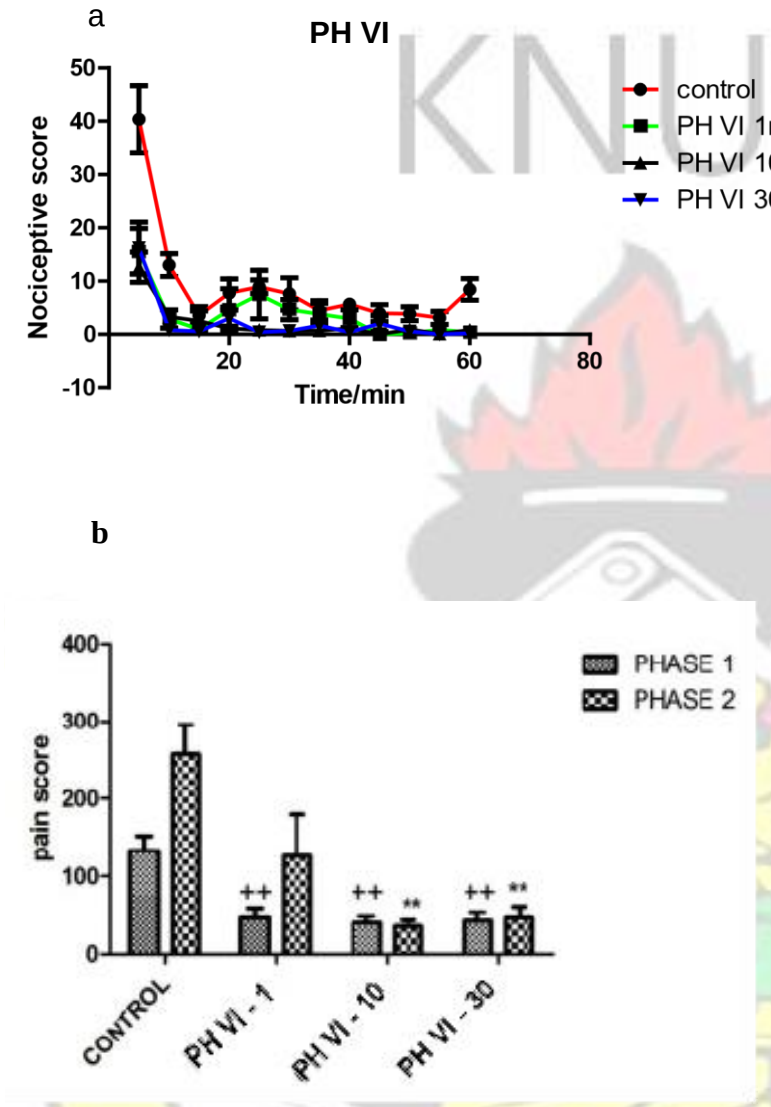


Figure 4.27 Effect of PH VI (1-30 mg kg^{-1} *p.o*) on the time course of formalin induced pain in mice (a). Nociceptive/pain scores are shown in 5 min blocks up to 60 min post formalin injection. Each point represents Mean ± S.E.M (n = 5). $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ compared to respective controls (two-way repeated measures ANOVA; the AUC (total response) for phase 1 and phase 2. Each column in (b) represents the mean ± S.E.M. $^{+}P \leq 0.05$, $^{++}P \leq 0.01$, $^{+++}P \leq 0.001$ (one-way ANOVA followed by Newman-Keuls *post hoc* test)

4.5 PHYTOCHEMICAL ANALYSIS

4.5.1 Preliminary Phytochemical screening

The phytochemical analysis of powdered root material revealed the presence of flavonoids, terpenoids, steroids (sterols), reducing sugars (glycosides) and alkaloids with glycosides (anthracene) and flavonoids being the most dominant (Table 4.4).

Table 4.4 Chemical constituents of root extract of *P. hirsuta*

TESTS	RESULTS
Tannins:	
Ferric chloride test	-
Lead acetate test	-
Flavonoids	+++
Alkaloids:	
Dragendorff's test	-
Glycoside general test	+
Saponins (Frothing test)	-
Anthracene glycosides	+++
Steroids	+
Lieberman's test	++
Terpenoids test	++
Glycosides general test	+++

–: Not detected, +: Present in low concentration, ++: Present in moderate concentration, +++: Present in high concentration.

4.5.2 Characterization of isolates

4.5.2.1 Identification of PH I, II and III by GC/MS analysis

GC/MS was used to identify some of components of the isolates which were oily or fatty in nature because simple column chromatography and preparative TLC could not be used to fully

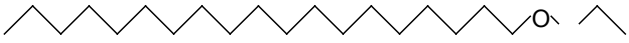
resolve all the components of the fatty acid mixture. The isolates; PH I, II and III were subjected to GC/MS to identify the components. Compounds were identified by their mass spectra, interpretation of their fragmentation patterns and comparing retention times of their peaks to those of standard compounds analysed previously in literature (National Institute of Standards and Technology (NIST), 2011).

Table 4.5 GC/MS data for PH I, II and III

Isolates	Component(s)	RT (minutes)	No of carbons
PH I	Eicosanoic acid	63.68	20
	Ethyl Octadecanoate	14.58	20
PH II	Trimethyl benzene	9.68	9
	Cymene	12	10
	Ethyl hexanoate	28.85	8
PH III	Methyl hexanoate	17.5	7
	Octadecanoic acid	56.74	18

- NIST (2011)

Ethyl Octadecanoate(ethyl stearate)



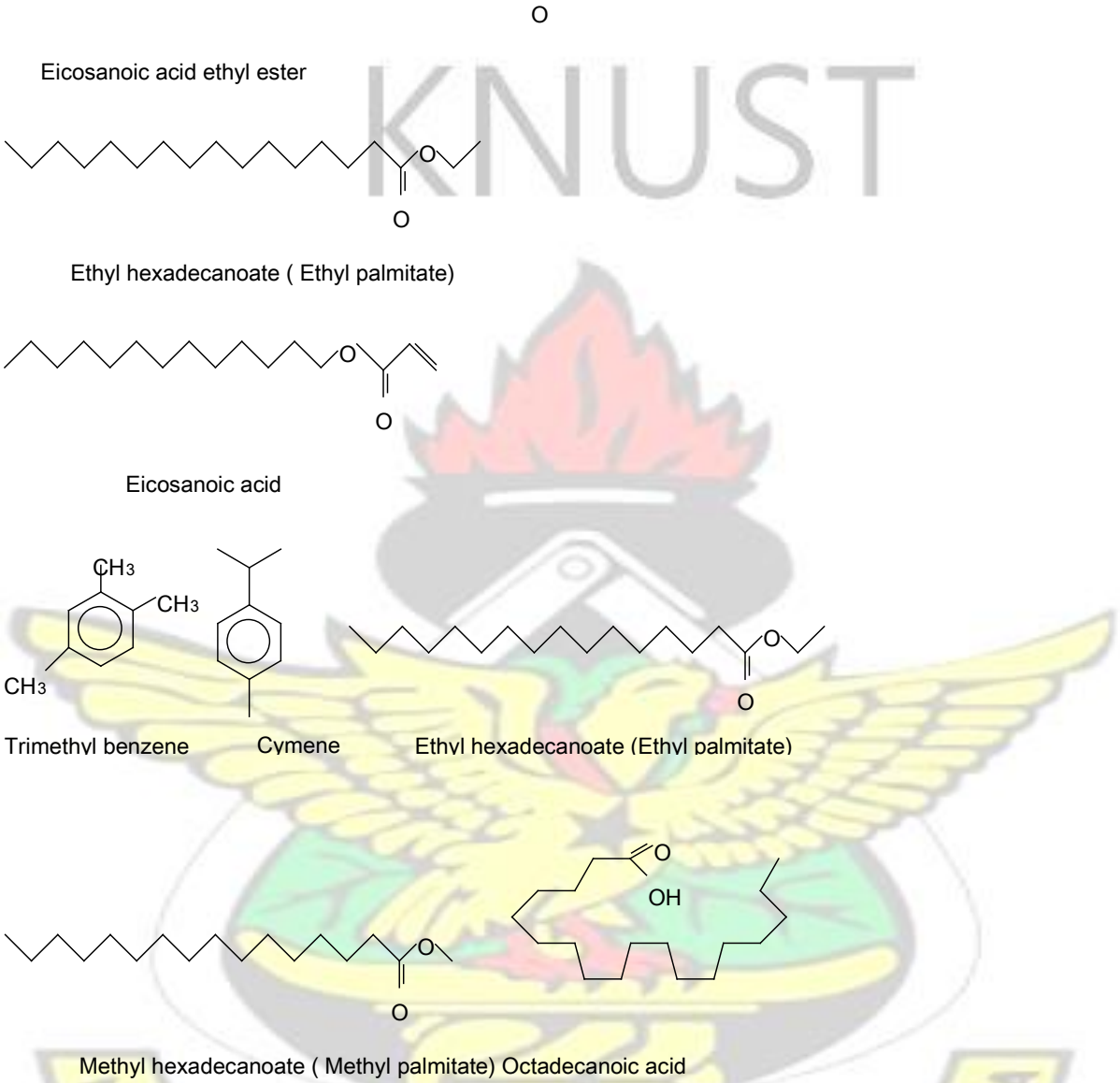


Fig 4.28 Structures of fatty acids identified in various fractions by GC-MS.

4.5.3 Identification of PH IV as 20-hydroxyecdysone

PH IV was isolated as an off white amorphous solid with a melting point of 240-242°C

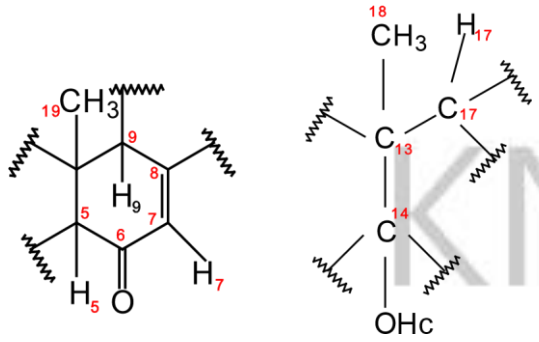
[Literature: 237.5-239.5°C, (Hikino and Hikino, 1970)]. It showed positive Liebermann's test. The Electron Spray Ionization Mass Spectrometer(ESI-MS) revealed a molecular ion peak $[MH]^+$ at m/z of 479 which corresponded to the molecular formular $C_{27}H_{44}O_7$, indicating six degrees of unsaturation, ascribed to α, β-unsaturated ketone and four steroid rings (Yun-Song *et al.*, 2006).

The ^{1}H-NMR revealed the presence of five methyl singlets at δ 0.76 (H-18), 0.83 (H-19), 1.06 (H-21), 1.05 (H-26) and 1.07 (H-27). The relatively downfield resonance of the last three signals revealed the presence of hydroxyl groups at C-20 and C-25 positions (i.e. OH_d and OH_f respectively). Other hydroxyl groups occurred at C-2, C-3, C-22 and the quaternary carbon C14 (i.e. OH_a, OH_b, OH_e and OH_c respectively).The presence of the OH groups caused the methine protons H-2, H-3 and H-22 to occur more downfield (at δ 3.52,3.76 and 3.11) relative to other methine protons at δ 2.19 (H-5),3.02 (H-9) and 2.23 (H-17). The cluster of signals in the region 1.09-2.01 ppm (Figure 4.28) revealed the presence of methylene protons of the steroid framework.

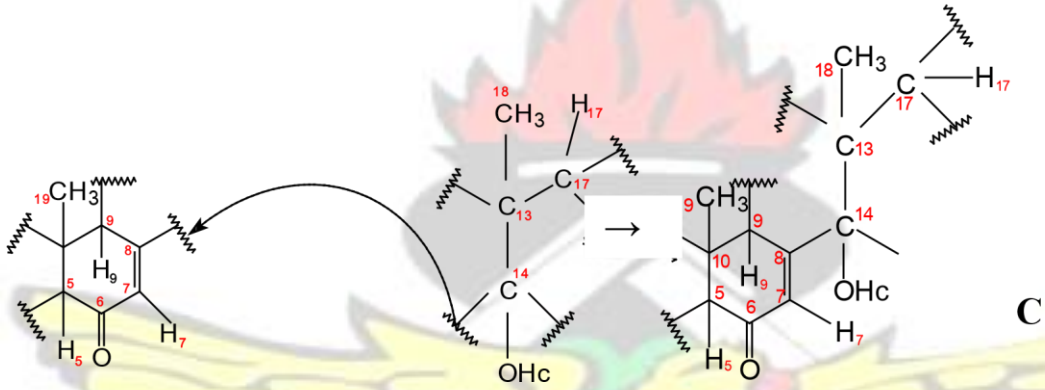
The ^{13}C-NMR spectrum revealed the presence of twenty-seven (27) carbon resonances(Figure 4.29; Table 4.6). Five methyl signals occurred at δ 17.6 (C-18), 24.3 (C-19), 21.4 (C-21), 30.8 (C-26) and 30.5 (C-27). A carbonyl(ketone) group occurred at δ 203.13 (C-6). The signals at 165.89 and 120.94 ppm were assigned to the olefinic carbons C-8 and C-7 respectively. The presence of 27 carbon resonances including five methyl signals suggested that PH IV may be an analogue of cholesterol. Other carbons present in the spectrum included five quaternary carbon signals at δ 83.5, 76.28, 69.17, 47.35 and 38.1. The signals occurring more downfield

at δ 83.5, 76.2 and 69.17 were assigned to the oxygenated carbons C-14, C-20 and C-25 respectively. Other oxygenated carbon resonances occurred at δ 76.8 (C-22), 67.25 (C-2), and 67.0 (C-3). Eight methylene carbon signals occurred at δ 37.1, 32.0, 21.4, 31.34, 29.5, 20.6, 26.6 and 41.9 ppm which were assigned to C-1, C-4, C-11, C-12, C-15, C-16 C-23 and C-24. Three methine signals occurred at δ 50.6 (C-5), 33.5 (C-9) and 49.2 (C-17). All of the protonated carbons were assigned by heteronuclear single quantum correlation(HSQC) experiment.

In the HMBC spectrum (Appendix II), the olefinic proton H-7 had ^{3}J correlations with C-5, C9, and C-14. The proton signal H-5 had ^{2}J correlation with the carbonyl C-6. This suggested the presence of a 7-en-6-one chromophore, characteristic of ecdysteroids (Mamadalieva *et al.*, 2003). H-5 also coupled to C-9 (^{3}J); H-19 to C-5 (^{3}J), C-9 (^{3}J) and H-9 to C-8 (^{3}J) in HMBC, giving the partial structure **A**. The methyl proton singlet δ 0.78 (H-18) had ^{2}J correlation with C-13 and ^{3}J correlation with C-14 and C-17 (Table 4.8). The hydroxyl signal at δ 4.62 (OH_c)coupled in HMBC to both C-14 (^{2}J) and C-13 (^{3}J). Also the proton H-17 had a ^{2}J and ^{3}J correlations with C-13 and C-18; H-17 (^{3}J) with C-14 in long range correlation. These data suggested the partial structure **B**. Combining structures A and B gave partial structure C.

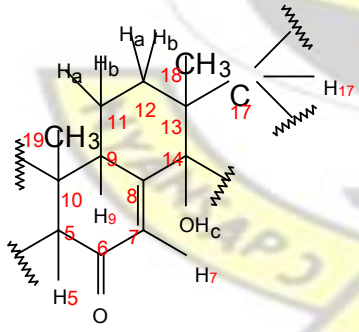


A B



C

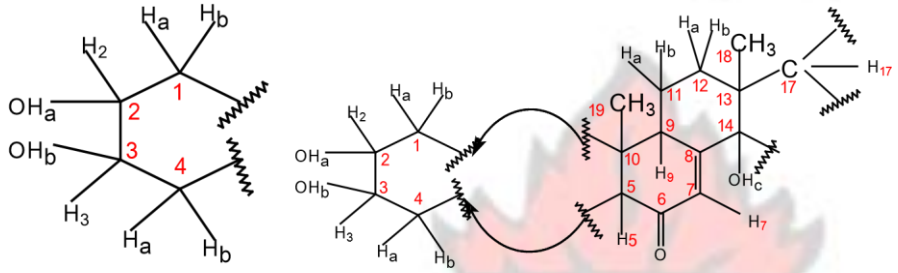
The methylene proton H-12$_b$ coupled to C-18 and C-13 while three other methylene protons in the region 1.09- 2.01 ppm coupled to C-10, C-13 and C-9in heteronuclear multiple bond correlation (HMBC) . This gave the partial structure D.



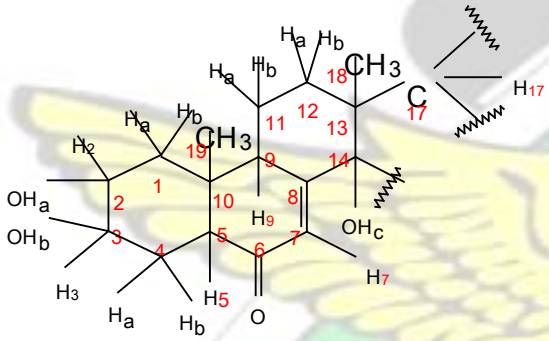
D

The hydroxyl proton OH$_a$ coupled to both C-3 and C-1 through a ^3J correlation. The hydroxyl proton OH$_b$ also had a ^3J correlation with C-4 and C-2. The proton H-3 also coupled to C-5 (^{3}J);

methylene protons H-1_{a-b} and H-4_{a-b} in the region (1.09 – 2.01) ppm to C-2 and C-3 (^3J); H-5 to C-1(^3J) and methylene proton H-1 to C-10 in HMBC correlation. This gave the partial structure E and merging D and E gave partial structure F.

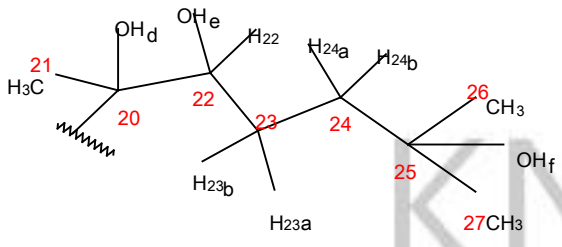


E



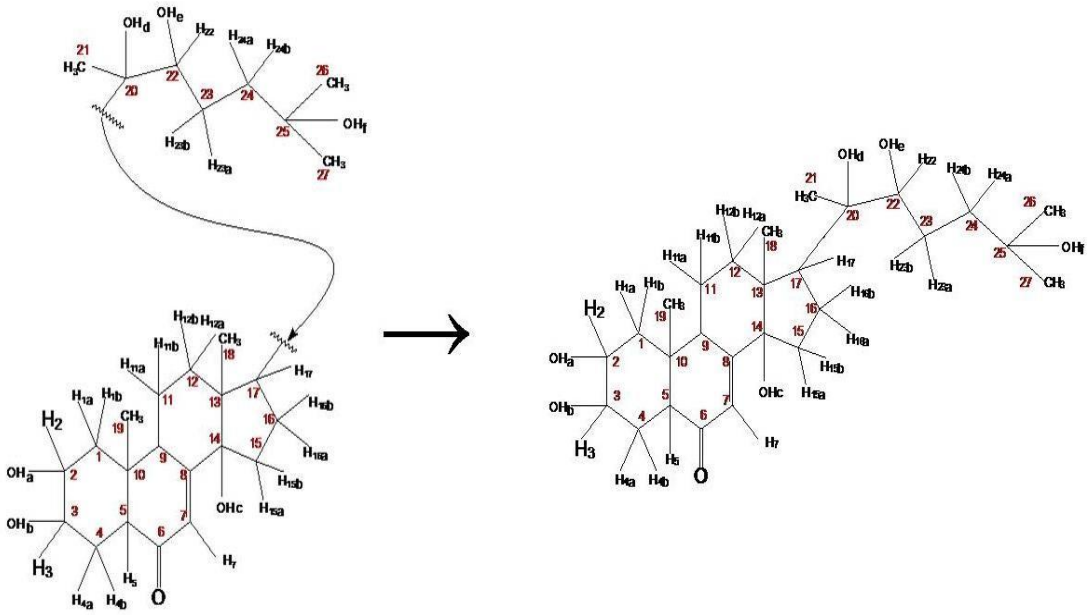
F

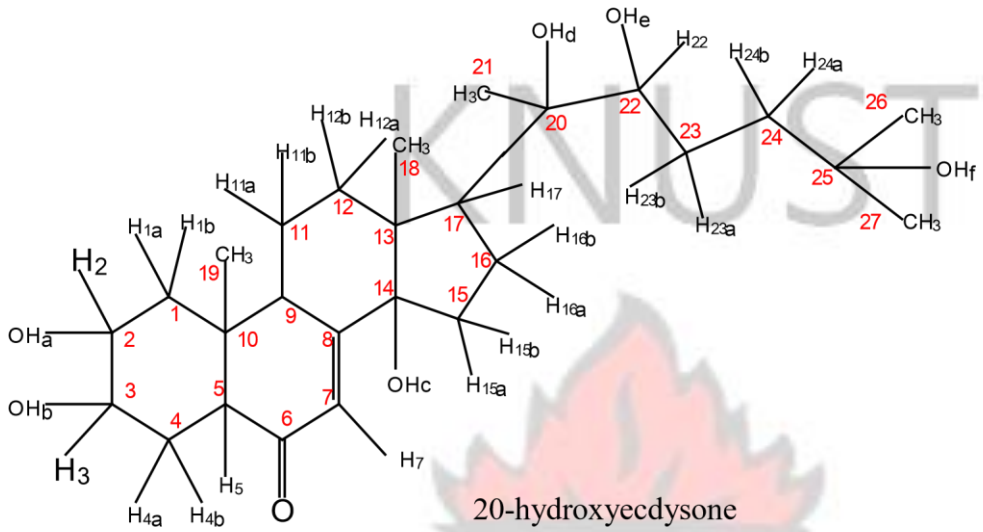
The hydroxyl signal OH_f had ^3J correlations with C-26 and C-27 and ^2J correlation with C-25. The methyl singlet H-26 also coupled to C-27 (^3J) and C-25 (^2J).H-27 also couples to C-26 (^3J), C-25 (^2J) and C-24 (^3J). Also OH_d coupled to C-20 (^2J), C-22(^{3}J) and C-17 (^{3}J); methyl singlet H-21had ^2J and ^3J correlation with C-20 and C-17 respectively. OHe also coupled in HMBC to C-23 (^3J), C-20 (^3J) and C-22 (^2J). This established the partial side chain structure G.



G

Joining structure G to F established the complete structure of PH IV as 20-hydroxyecdysone. ^{3}J correlation of OH_d and H-21with C-17 suggested that the side chain was attached to the steroidal nucleus at C-17 (Isaac *et al*, 1983).





20-hydroxyecdysone

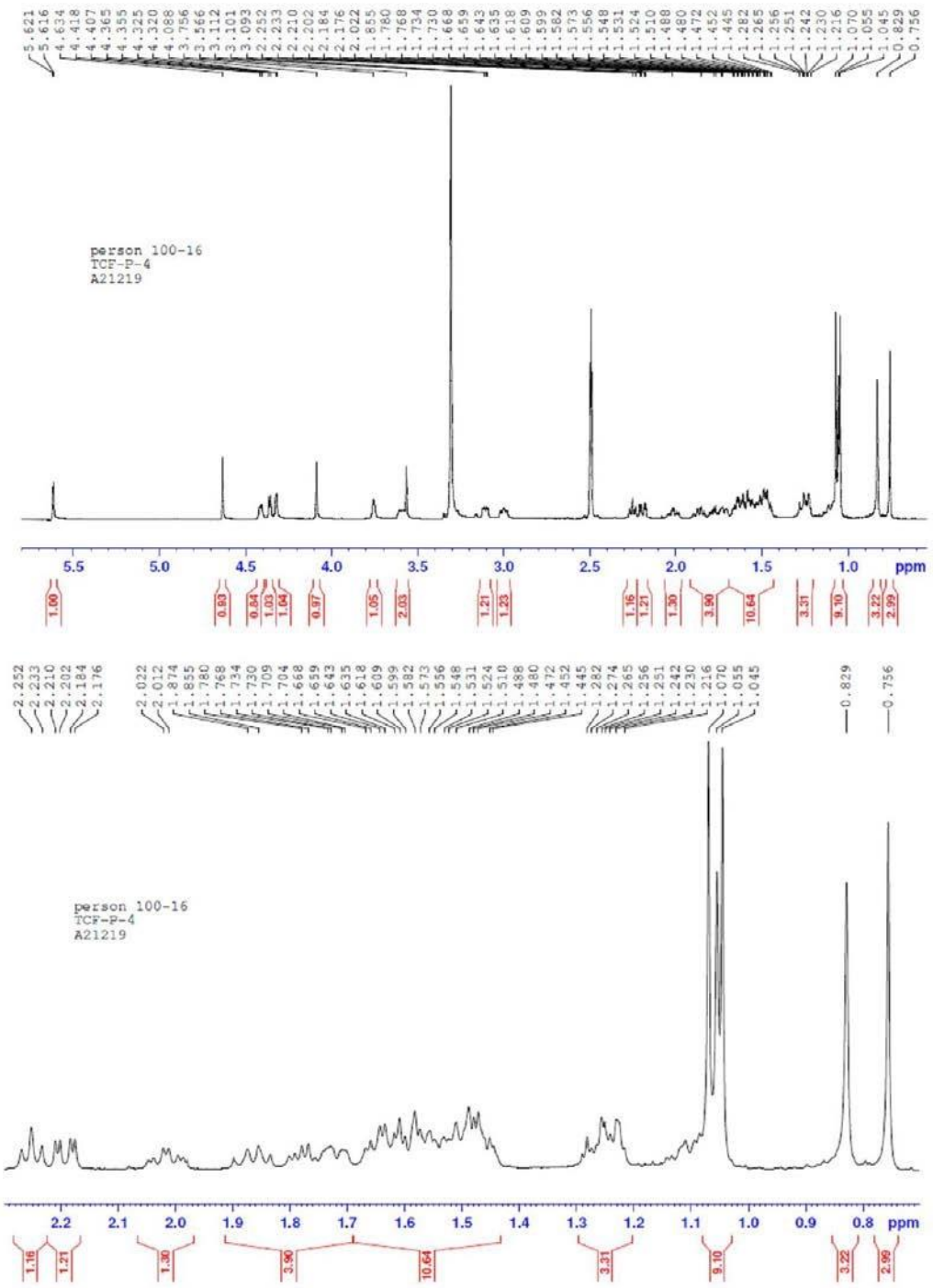


Figure 4.28[1]H-NMR spectra of PH IV

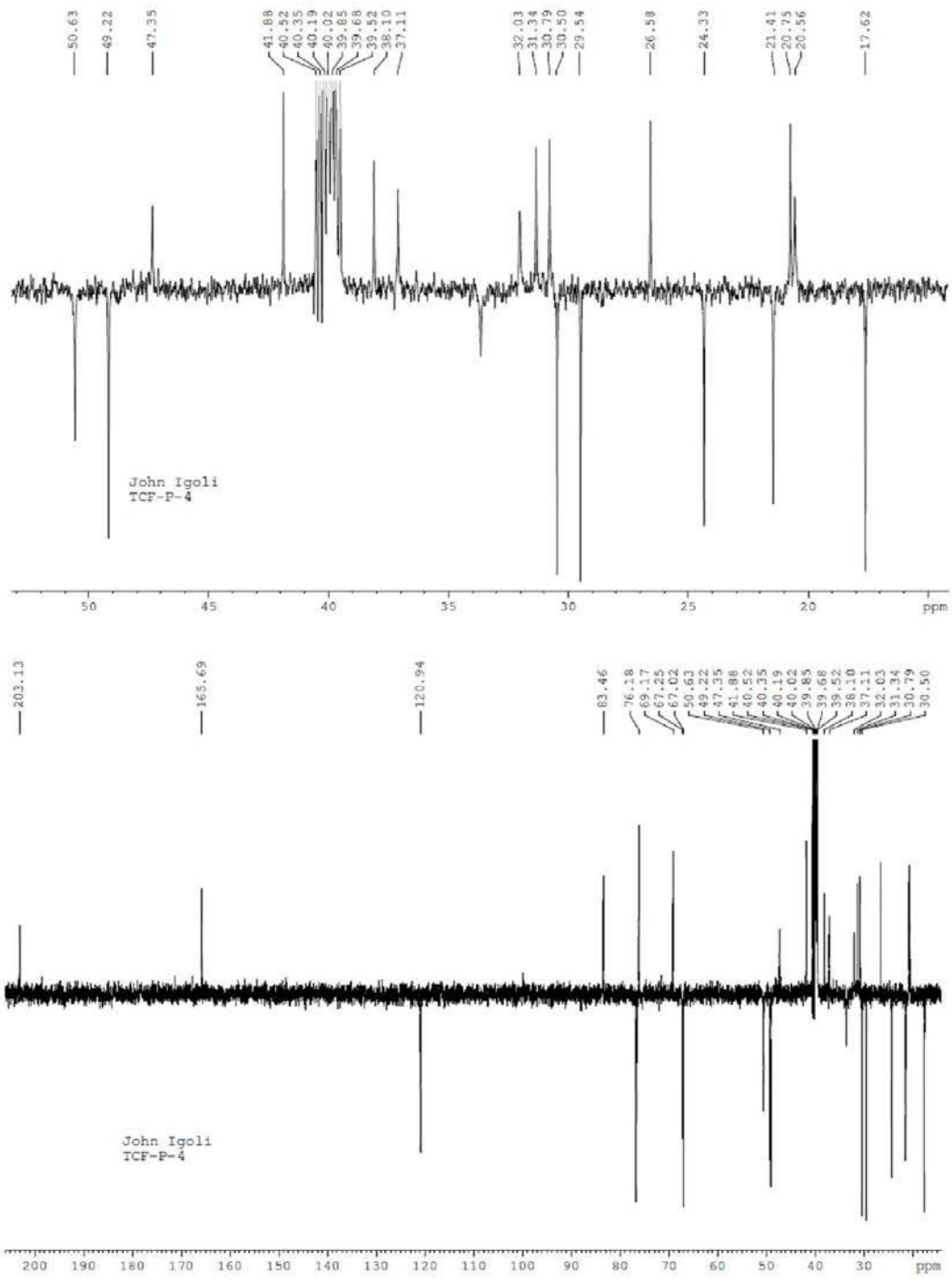

50.63
49.22
47.35
41.88
40.52
40.35
40.19
40.02
39.85
39.68
39.52
38.10
37.11
32.03
31.34
30.79
30.50
29.54
26.58
24.33
21.41
20.75
20.56
17.62
John Igoli
TCF-P-4
50
45
40
35
30
25
20
ppm
203.13
165.69
120.94
83.46
76.18
69.17
67.25
67.02
50.63
49.22
47.35
41.88
40.52
40.35
40.19
40.02
39.85
39.68
39.52
38.10
37.11
32.03
31.34
30.79
30.50
John Igoli
TCF-P-4
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
ppm

Figure 4.29[13]C-NMR spectra of PH IV

Table 4.6 [13]C-NMR data of PH IV in DMSO at 400MHz

Carbon position	**Chemical shift δppm**	**Carbon position**	**Chemical shift δppm**
C-1	37.1	C-15	29.5
C-2	67.3	C-16	20.6
C-3	67.0	C-17	49.2
C-4	32.0	C-18	17.6
C-5	50.6	C-19	24.3
C-6	203.1	C-20	76.2
C-7	120.9	C-21	21.4
C-8	165.7	C-22	77.0
C-9	33.5	C-23	26.6
C-10	38.1	C-24	41.9
C-11	20.8	C-25	69.2
C-12	31.3	C-26	30.8
C-13	47.4	C-27	30.5
C-14	83.5		

Table 4.7HSQC correlation data of PH IV

Carbon position	H-NMR δ ppm	Protons
C-1	1.25 1.58	H-1a H-1b
C-2	3.51 4.41	H-2 OHa
C-3	3.76 4.32	H-3 OHb
C-4	1.46 1.57	H-4a H-4b
C-5	2.19	H-5
C-6	-	-

C-7	5.62	H-7
C-8	-	-
C-9	3.02	H-9
C-10	-	-
C-11	1.52	H-11a
	1.66	H-11b
C-12	1.72	H-12a
	2.01	H-12b
C-13	-	-
C-14	4.62	OHc
C-15	1.49	H-15a
	1.78	H-15b
C-16	1.53	H-16a
	1.86	H-16b
C-17	2.23	H-17
C-18	0.76	H-18 (3H)
C-19	0.83	H-19 (3H)
C-20	3.57	OHd
C-21	1.06	H-21 (3H)
C-22	3.11	H-22
	4.36	OHe
C-23	1.09	H-23a
	1.46	H-23b
C-24	1.23	H-24a
	1.64	H-24b
C-25	4.09	OHf
C-26	1.05	H-26 (3H)
C-27	1.07	H-27 (3H)

1

Table 4.8 HMBC correlation of PH IV in DMSO at 600MHz

Carbon position	Protons	
C-2	OHb	
C-3	OHa	
C-4	OHb	
	C-5	H-7
		H-3
		H-19
	C-6	H-5

C-1	OHa H-5

C-7	
C-8	H-9
C-9	H-7
	H-5
	H-19
C-10	
C-11	C-19
C-12	H-18
C-13	H-1
	OHc
	H-17
	H-19
C-14	H-7
	OHc
	H-18
C-15	
C-16	OHd
C-17	H-21
	H-18
C-18	H-17
	H-12b
C-19	
C-20	OHe
	OHd
	H-21
C-21	H-17
C-22	OHd
	OHe
C-23	OHe

C-24 Carbon position	
	OHf
Protons	
	H-27
	H-26

C-25 OHf

H-26

H-27

C-26 OHf **C-27** OHf

H-27

H-26

Chapter 5

5.1 GENERAL DISCUSSION

The carrageenan-induced acute footpad oedema in laboratory animals has been used extensively to screen new anti-inflammatory drugs and it is still acceptable in the preliminary screening for anti-inflammatory activity Winter *et al*., 1962; Niemegeers *et al*., 1975; Singh *et al*., 2000). The model is commonly used to evaluate non-steroidal antiinflammatory drugs [NSAID] as used by Rosa and Willoughby, 1975 and in this current study, chicks were used instead of rodents commonly used for such experiments. Carrageenan-induced oedema in the chicks (Roach and Sufka, 2003) is more economical than rodent models and has the advantage that chicks are easier to handle. The intraplantar injection of carrageenan in the 7-day-old chick elicits measurable, reliable and relatively short-lasting state of oedema, that is differentially attenuated by the systemic administration of typical anti-inflammatory compounds as shown by studies(Roach and Sufka, 2003). The results obtained from such studies compare favourably with the more commonly used rodent models (rat and mice) in the screening of drugs with anti-inflammatory activities. The dose-dependent inhibition of carrageenan-induced foot edema by the crude extract and the fractions thereof as well as the isolates (PH I, II, III, IV, V and VI) in this model of acute inflammation depicts the anti-inflammatory potential of the root extract, the various fractions and the isolates in acute inflammation.

Even though the actual mechanism of action of the extract of *P. hirsuta* and for that matter the compounds isolated from the extract has not been determined experimentally, it is possible that, the anti-inflammatory activity exhibited by these could be attributed to the

inhibition of the synthesis, release or action of inflammatory mediators that are known to be involved in carrageenan-induced inflammation which include cytoplasmic enzymes and serotonin from mast cells and also bradykinin, prostaglandins and other cyclooxygenase products (Boakye-Gyasi, 2010).

The extract, fractions and isolates were compared to the standard drugs diclofenac and dexamethasone which both showed a dose-dependent inhibition of carrageenan-induced edema. The anti-inflammatory effect of diclofenac, a non steroidal anti-inflammatory drug (NSAID), is mediated chiefly through inhibition of the cyclooxygenase pathway (COX 1 and COX 2) and thus inhibit the release of arachidonic acid metabolites particularly prostaglandins which are well known mediators of inflammation (Al-Majed *et al*, 2003; Seibert *et al*, 1994; Wise *et al*, 2008). The anti-inflammatory effect of dexamethasone, a steroidal anti-inflammatory drug (NSAID), is mediated through their suppressive effects on the inflammatory cytokines and on other lipid and glucolipid mediators of inflammation (Enomoto *et al*, 2007; Kaur *et al*, 2004; Li *et al*, 2007; Masferrer *et al*, 1994). The immediate two statements above go to support the suggestion that the root extract of *P. hirsuta* and the isolates might exhibit their anti-inflammatory activities through similar pathways. The effect before the induction of inflammatory (pre-emptive protocol) paradigm was used for the assessment of inflammation.

The isolate PH IV (20-hydroxyecdysone) is one of the ecdysteroids which have been primarily certified as moulting hormones regulating metamorphosis and also several other important life-cycle processes in insects (Dinan,2001). It is generally accepted that ecdysteroids in plants play an important part in the protection against insect predators and soil nematodes, either as a consequence of their antifeedant activity or by inducing the

developmental disruption and even the death of non-adapted phytophagous insects or soil nematodes (Dinan, 2001). The anabolic effect of ecdysteroids on muscle size has been demonstrated in a wide range of animals. Increased body, organ and muscle weight and protein synthesis have been reported in cases of *p.o.* or *i.p*. administration of ecdysteroids in several animal species. The first observed and classical pharmacological activity of ecdysteroids is their protein-synthesis stimulatory effect and the effect of ecdysteroids on protein synthesis in the mouse liver has been reported (Okui *et al*, 1968; Otaka *et al,* 1968).

The effect of 20-hydroxyecdysterone on rat muscle fibres *in vivo* has been studied and reported to modify muscle fibre size in normal and regenerating muscles even after 7 days administration in a slightly higher dose than anabolic steroids. And that in the case of the regenerating muscle, the effect of 20-hydroxyecdysone was different in two applied doses which might indicate a dose-dependent action. This effect on muscle fibre could also have effect in man in enhancing analgesic and anti-inflammatory effect as well as improve growth by enlarging muscles. Ecdysterone has been demonstrated to exhibit potent oxytocic activity in the guinea pig uterus assay besides inhibiting nicotine and serotoninginduced contractions during guinea pig ileum assay (Parameswaran *et al*, 2001) and may have some implication in the folkloric use to treat infertility. There is ample evidence of the varied biological activities of the ecdysteroids especially 20-hydroxyecdystone, however this is the first time the anti-inflammatory activity has been established. The magnitude of anti-inflammatory activity of 20-hydroxyecdysone (1-30 mg/kg) was comparable to the standard drug Diclofenac (1-30 mg/kg) and even better than the corticosteroid dexamethasone (0.1-3 mg/kg) used in the experiment. Again, it has also been justified that the anti-inflammatory activity of PH IV (20-hydroxyecdysone) is dose-dependent. This might justify the folkloric use in Ghana and the other West African

countries for treating painful inflammatory conditions. This exceptional compound has been found for the first time in the roots the plant *Palisota hirsuta*. The other isolates PH V and VI showed dose dependent anti-inflammatory activity and by their molecular masses and phytochemical tests proved to be ecdysteroids. PH I, II and III were oils that contained some mixtures of fatty acids. PH I contained eicosanoic acid and ethyl octadecanoic acid whereas PH II contained the following; trimethyl benzene, cymene and Octadecanoic acid. PH III had methyl hexadecanoate (methyl palmitate) and octadecanoic acid as the constituents. These proved to have anti-inflammatory activity and such activity could be justified by likening to that of omega-3 fatty acids. The omega -3 fatty acids are anti-inflammatory and they achieve such activity by their metabolic pathway to form eicosanoid which has antiinflammatory activity (William, 1992; Ruggiero *et al*, 2009).

In the formalin test, the early phase is considered to be produced by direct activation of nociception neurons by formalin, whereas the late phase reflects pain generated in acutely injured tissue (Hunskaar and Hole, 1987; Tang *et al*, 2007). The licking response induced as a result of formalin injection, is due to a combination of peripheral input and spinal cord sensitization (Tjolsen et al., 1992). There is a release of EAAs, prostaglandin 2($PGE_{2)}$, nitrous oxide(NO) and kinins in the spinal cord (Tjolsen et al., 1992) and the antinociception of *Palisota hirsuta* could be dependent on either peripheral or central sites of action or both. Drugs, such as opioids that act centrally, inhibit both phases of pain by equally inhibiting the effect produced by prostaglandins released response to inflammation (Ferreira, 1981; Hunskaar and Hole, 1987; Shibata *et al*, 1989) and by endogenous opioids through their action on the central nervous system. Tjolsen *et al.,* (1992) demonstrated that the late phase in formalin test depends on an inflammatory reaction in peripheral tissue.

Peripheral acting drugs such as diclofenac (Rosland *et al*., 1990) which block prostaglandin synthesis reduce nociception mostly in the late phase but can also affect the early stage (Ortiz *et al.*, 2008). In fact, the anti-nociceptive effects of PHC, PHM, PHE and PH I-VI as exhibited in the formalin test suggest an involvement at both central and peripheral levels, which further implies that the extract and the isolates possess not only anti-nociceptive but also anti-inflammatory activity. The highest doses, the various extracts, fractions and isolates caused maximal inhibitions are indicated correspondingly below;

Material	**Maximal Inhibition**
PHC	40.89±24.10% and 66.25±32.08%
PHE	45.02±29.81% and 80.50±4.62%
PHM	31.72±15.29% and 61.0±41.97%
PH I	55.08±14.90% and 63.57±25.91%
PH II	90.86±6.40% and 67.47±20.84%
PH III	57.87±8.436 and 79.90±7.05%
PH IV	71.39±9.19% and 89.19±3.81%
PH V	61.34±11.07% and 82.89±3.97%
PH VI	70.14±8.60% and 86.18±7.42%

These indicate maximal inhibitions or reductions of the licking time in the early and late phase, respectively (Fig. 4.19a&b -4.27a&b). Similarly, morphine (1-10 mg kg^{-1}, i.p.) produced marked inhibition of both the neurogenic ($P<0.0001$) and inflammatory ($P<0.0001$) pain phases (Fig. 4.18a and b). Morphine, reduced the duration of formalin evoked nociceptive behavior by a maximum percentage of 92.49±4.67% in the early phase and 95.16±5.497% in the late phase of the formalin test (Fig. 4.18a and b). The antinociceptive actions of the extracts, fractions and various isolates of the roots of *Palisota hirsuta* are being reported for the first time. From the results obtained, the analgesic properties are not in doubt and these as well as anti-inflammatory activity might be accounted for by the steroidal nature of some of the compounds found in the plant. The combined actions might account for the pain reduction. However the anti-nociceptive action is not dose dependent for the extract, fractions and the isolates used for the study.

The ability of electron-rich compounds to donate electrons in reactions with oxidizing agents like hydrogen peroxide and superoxide which are produced in considerable quantities during inflammation when polymorphonuclear leukocytes and macrophages are stimulated, and still form stable species have been reported to be linked to their antioxidant properties (Kang *et al*, 2005; Ozkan *et al*, 2007). The anti-inflammatory effect of several compounds is closely related to their antioxidant properties; for example their abilities to protect against lipid peroxidation (Costa *et al*, 2004).This report buttresses the assertion that the antioxidant properties of the constituents may contribute in part to their antiinflammatory activities.

The GC/MS and NMR analyses confirmed PH IV to be 20-Hydroxyecdysone. PH I was a mixture of fatty acids and was resolved by GC/MS to contain eicosanoic acid and ethyl octadecanoic acid (ethyl stearate). PH II resolved with GC/MS, contained the mixture of

trimethyl benzene, cymene and ethyl hexadecanoic (ethyl palmitate) acid which are fatty acids. PH III was also resolved to contain the fatty acids methyl hexadecanoate (methyl palmitate) and octadecanoic acid. The preliminary phytochemical screening revealed PH V and VI to be steroids and the molecular masses from the GC/MS spectral analyses suggest them to be ecdysteroids. However their structures could not be elucidated for lack of adequate NMR information.

Chapter 6

CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS

The results as revealed by the study show the following conclusions which are ample evidence to support the local use of the roots of *Palisota hirsuta* for treating pain and

inflammatory conditions. The isolates (especially PH IV) could be used to treat pain individually (after their toxicities have been determined) or in combination with other antiinflammatory and analgesic agents. They could also be used as 'lead' substances for further drug development to find more potent analgesic/anti-inflammatory agents. The conclusions are:

- The ethanolic root extract, the methanolic fraction and the petroleum ether fraction and the isolates (PH I, II, III, IV, V and VI) of *Palisota hirsuta* have antiinflammatory activities.

- The ethanolic root extract, methanolic and petroleum ether fractions and the isolates (PH I, II, III, IV, V and VI) of *Palisota hirsuta* have anti-nociceptive/analgesic activities.

- That the isolates have demonstrated potent antioxidant activity that might contribute in part to the anti-inflammatory activity exhibited by same.

- That the isolate PH IV (20-Hydroxyecdysone) has been isolated for the first time from the roots of *Palisota hirsuta.*

- That 20-Hydroxyecdysone has been proven for the first time to have a potent antiinflammatory activity.

- That 20-hydroxyecdysone has been demonstrated in this study to have antinociceptive action.

Many medicinal plants used traditionally have proven through phytochemical studies to have varied class of compounds and this may contribute to their pharmacologic

actions. The study has proven scientifically the use of the roots of *Palisota hirsuta* to control pain and inflammatory diseases by the indigenous people of West Africa where the plant is found.

6.2 **RECOMMENDATIONS**

It is recommended that toxicological studies are performed first, then further work be done on 20-hydroxyecdysone to formulate it into a suitable dosage form(s) or standardize the extract of the plant for clinical trials.

That further work is done on the unresolved structures of the other ecdysteroids as they could hold the potential to be used as anti-inflammatory and analgesics products.

That further work is done to use these compounds as 'lead substances' to develop or design new compounds for anti-inflammatory analgesics that are more potent but with less undesirable side-effects of the traditional NSAID's especially gastric ulceration.

That if the methods used in this study could be improved to include High Performance Liquid Chromatography (HPLC) to help isolate more compounds.

That further work is done to use these compounds as "lead substances" to develop or design new compounds for anti-inflammatory analgesics that are more potent but with less undesirable side-effects of the known NSAID's especially gastric ulceration.

That further investigation be conducted on 20-Hydroxyecdysone to determine if it has insect repellant or insecticidal activity.

REFERENCES

Abbiw, D. K. (1990). "Useful Plants of Ghana." Intermediate Technology Publications and Royal Botanic Gardens, Kew.

Abdel-Salem, O. M. and S. El-Batran (2005). "Pharmacological investigation of trimetazidine in models of inflammation, pain and gastric injury in rodents." Pharmacology **75**(3): 122-132.

Actis-goretta, L., Mackenzie, G. G. Oteiza, P.I. and Fraga, C.G. (2002). "Comparative study on the antioxidant capacity of wines and other plant-derived beverages." Alcohol and Wine in Health and Disease **957**: 279-283.

Agarwal, A. B. and V. D. Rangari (2003). "Phytochemical investigation and evaluation of anti-inflammatory and anti-nociceptive activities of essential oil of *Stronbilanthus ixiocephala* Benth." Indian J Exp Biol **41**(8): 890-894.

Agarwal, N., Pacher, P., Tegeder, I., Amaya,F., Constantin, C.E., Brener, G.J. *et al.* (2007)."Cannabinoids mediate analgesia largely via peripheral type 1 cannabinoid receptors in nociceptors." Nat Neurosci **10**(7): 870-879.

Ainslie, J. R. (1937). "List of Plants Used in Native Medicine in Nigeria." Imperial Forestry Institute, Oxford: 71.

Ajila, C. M., Naidu, K.A., Bhat, S.G. and Prasada Rao, U.J.S (2007). "Bioactive compounds and antioxidant potential of mango peel extract." Food Chemistry **105**: 982988.

Akobundu, I. and C. W. Aggakwa (1987). "A handbook of West African Weeds." International Institute of Tropical Agriculture: Ibadan, Nigeria.

Ali,S.S., Kasoju, N., Luthra, A., Singh, A., Sharanabasava, H. Sahu, A., Bora, U., Blandino, A., Al-Aseeni, M.E., Pandiella, S.S., Cantero, D. and Webb, C.(2008) Indian medicinal herbs as sources of antioxidants. Food Research International, 41, 1-15.

Al-Majed, A. Khattab,M., Raza, M., Al-Shabanah, O.A. and Mostafa, A.M. (2003). "Potentiation of diclofenac-induced anti-inflammatory response by aminoguanidine in carrageenan-induced acute inflammation in rats: the role of nitric oxide." Inflamm Res **52**(9): 378-382

Almeida, I. F., Ferriandes, E., Lima, J., Costa, P. and Fernandabahia, M. (2008). "Walnut(*Juglans reglia)* leaf extracts are strong scavengers of pro-oxidant reactive species." Food Chemistry **106**: 1014-1020.

Amado, L. L., Jaramillo, M.D, Rocha, A.M., Ferreira, J.L.R. Garcia, L.M., Ramos, P.B., Urrutia, C., Beltran, C and Montesserat, J.M (2007). "A new method to evaluate total antioxidant capacity against reactive oxygen and nitrogen species (RONS) in aquatic organisms." Comparative Biochemistry and Physiology P.A: Molecular and Integrative Physiology **148**: S75-S76.

Amarowicz, R., Trosynska, A., Agnieszka, T. and Shahidi, F. (2005). "Antioxidant activity of almond seed extract and its fractions." J. Food Lipids **12**: 344-358.

Anani, K., Hudson, J.B, de Souza, C., Alepagana, K., and Towers, G.H.N. (2000). "Investigation of Medicinal Plants of Togo for Antiviral and Antimicrobial Activities." Pharmaceutical-Biology **38**: 40-45.

Andrejus, K. (1992). "Chemotherapy of arthritis induced in rats by mycobacterial adjuvant." J Assoc. Acad. Minor. Phys **3**(3): 70-77.

Anseloni, V. C. and M. S. Gold (2008). "Inflammation-induced shift in the valence of spinal GABA-A receptor-mediated modulation of nociception in the adult rat." J. Pain **9**(8): 732-738.

Argoff, C. E. (2002). "Pharmacologic management of chronic pain." J. Am. Osteopath Assoc. **102**: S21-7.

Arouma, O. I. (1998). "Free radicals, oxidative stress and antioxidants in human health and disease." Journal of the American Oil Chemists Society**75**: 199-212.

Arteel, G. E. (2003). "Oxidants and antioxidants in alcohol-induced liver disease." Gastroenterology **124**: 778-790.

Aruoma, A. M., Butler, M.J., Murcia, A. and Halliwell, B. (1993). "Evaluation of the antioxidant and prooxidant actions of gallic acid and its derivatives." Journal of Agricultural and Food Chemistry **41**: 1880-1885.

Auddy, B., Ferreira, M., Blasina, F., Lafon, L., Arredondo, F., Dajas, F., Tripathi, P.C., Seal, T. and Mukherjee, B. (2003). "Screening of antioxidant activity of three Indian medicinal plants, traditionally used for the management of neurodegenerative diseases." Journal of Ethnopharmacology **84**(2-3): 131-138.

Bagchi, D., and Sen, C, K (2003). "Molecular mechanisms of cardioprotection by a novel grape seed proanthocyanidin extract." Mutation Research **9462**: 1-11.

Balasubashini, M. S., Rukkumani, R., Viswanathan, P. and Venugopal, P.M. (2004). "Ferulic acid alleviates lipid peroxidation in diabetic rats." Phytotherapy Research **18**: 310314.

Baron, R. and R. D. Treede (2007). "Diagnosis of neuropathic pain." Dtsch Med Wochenscher **132**(41): 2139-2144.

Bass, W. B. and M. J. Vanderbrook (1952). "A note on an improved method of analgesic evaluation." J Am Pharm Assoc Sci Ed **41**: 569-70.

Bellomaria, B. and P. Kacou (1995). "Plants et Medicine Populaire d'Agboville(Cote D'Ivoire)." Fitoterapia **LXVI**(1995): 117-141.

Ben-Bassat, J., Peretz, E. and Sulma, F.G (1959). "Analgesimetry and ranking of analgesic drugs by the receptacle method." Arch Int.Pharmacodyn Ther **122**: 434-47.

Benson, B. B., Bekro, Y. A., Mamyrbekova-Bekro, J.A., Coulibaly, W.K. and Ehile, E.E. (2008). "Assessment of Sexual stimulant potential of Total flavonoids Extracted from Leaves of *Palisota hirsuta* Thunb. K Schum(Commelinaceae)." European Journal of Scientific Research **22**(4): 533-538

Benzie, F. F. and J. J. Strain (1999). "Ferric reducing antioxidant power assay: Direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration." Methods in Enzymology **299**: 15-27.

Berrino, L., Oliva, P., Massimo, F., Aurilio, C.,Maione, S. *et al*. (2003). "Antinoceptive effect in mice of intraperitoneal N-methyl-D-aspartate receptor antagonists in the formalin test." Eur. J Pain **7**: 131-7.

Boakye-Gyasi, E., Woode, E., Ainooson, G.K., Ansah, C. and Duwiejua, M. (2009b). "Anti-Nociceptive Effects and Mechanism of *Palisota hirsuta* K.Schum Leaf Extract in Murine Models." International Journal of Pharmacology **5**(2): 101-113.

Boakye-Gyasi, E., Woode, E., Ainooson, G.K., Obiri, D.D., Ansah, C., Duwiejua, M. and Donkor, A. (2008). "Anti-inflammatory and antipyretic effects of an ethanolic extract of *Palisota hirsuta* K. Schum roots." African Journal of Pharmacy and Pharmacology **2**(9): 191-199.

Boakye-Gyasi, E., Woode, E., Ainooson, G.K., Ansah, C., and Duwiejua, M. (2009a). "Anti-arthritic effects of *Palisota hirsuta* K.Schum, Leaf Extract in Freund's AdjuvantInduced Arthritis in Rats." International Journal of Pharmacology **5**(3): 181-190.

Boakye-Gyasi, E (2010).Anti-inflammatory and Ethopharmacological Effects of an Ethanolic Leaf Extract of *Palisota hirsuta* (PhD Thesis): 115,172.

Bolton, J. L., Trush, M.A., Penning, T.M., Dryhurst, G. and Monks, T.J. (2000). "Role of quinones in toxicology." Chemical Research in Toxicology **13**: 1357.

Brand-Williams, W., Cuvelier, M. and Berset, C. (1995). "Use of a free radical method to evaluate antioxidant activity." Food Science and Technology **28**: 25-30.

Branen, A. L. (1975). "Toxicology and biochemistry of butylated hydroxyanisole and butylated hydroxytoluene." Journal of American Oil Chemists Society **52**: 59-63.

Burkill, H. M. (1985). "The Flora of West Africa, Second edn." Royal Botanic Gardens, Kew.

Burton, G. W. and Ingold, K.U (1981). "Antioxidation of biological molecules 1: Antioxidant activity of Vit. E and related chain-breaking phenolic antioxidants in vitro." Journal of the American Chemical Society **103**: 6472-6477.

Burton, G. W. and Ingold, K.U. (1981). "Antioxidation of biological molecules 1: Antioxidant activity of Vit E and related chain-breaking phenolic antioxidants in vitro." Journal of the American Chemical Society **103**: 6472-6477.

Calvino, B., Besson, J.M., Mounier, F., Kordon, C. and Bluet-Pajot, M.T. (1992). "Chronic Pain induces a paradoxical increase in growth hormone secretion without affecting other hormones related to acute stress in the rat." Pain **49**(1): 27-32.

Calvino, B., and Courand, J.Y. (1988). "Increased reactivity to chemical but not to heat noxious stimuli in mice producing anti-idiotypic antibodies for substances." P.Brain Res. **460**: 389-93.

Chatterjee, S., Niaz, Z., Gautam, S.,Adhikari, S., Variyar, P.S. and Sharma, A.(2007). "Antioxidant activity of some phenolic constituents from green pepper (*Piper nigrum.L*) and fresh nutmeg mace (*Myristica fragrans*)." Food Chemistry **101**: 515-523.

Clavin, M., Gorzalczany, S., Macho, A., Munoz, E., Ferraro, C., Acevedo, C. and Martino, V. (2007). "Anti-inflammatory activity of flavonoids from *Eupatorium arnottianum*." J.Ethnopharmacol **112**(3): 585-589.

Coderre, T. J. and P. D. Wall (1987). "Ankle joint urate arthritis (AJUA) in rats: an alternative animal model of arthritis to that produced by Freund's adjuvant." Pain **28**: 37993.

Cornu, M. A. (1896). "Name String Source, Catalogue of Life 2009 Species Fungorum." Bot.Gaz **21**: 326.

Costa, B., Colleoni, M., Conti, S., Parolaro, D., Franke, C., Trovato, A.E. and Giagnoni, (2004). "Oral anti-inflammatory activity of cannabidiol, a non-psychoactive constituent of cannabis in acute carrageenan-induced inflammation in the rat paw." NauynSchmiedeberg's Arch Pharmacol **369**: 294-299.

Cuendet, M., Hostettmann, K. and Potterat, T (1997). "Iridoid glucosides with free radical scavenging properties from *Fragneae blumei*." Helvetica Chimica Aca **80**: 1144-1152.

Cui, K., Lou, X., Xu, K. and Ven Murthy, M (2004). "Role of oxidative stress in neurodegeneration: recent developments in assays methods for oxidative stress and nutraceutical anti-oxidants." Progress in Neuro-Psychopharmacology and Biological Psychiatry **28**: 771-799.

Curtis, M., Page, C., Hoffman, B., Sutter, M. and Walker, M. (2002). "Integrated Pharmacology." Mosby London: 272.

Dahl, J. B. and S. Moiniche (1998). "Why Zebras don't get ulcers: An updated guide to stress, stress-related diseases and coping." New York: W.H Freeman and Co.

Dastmalchi, K., Dorman, H.J.D., Laakso, I. and Hiltman, R. (2007). "Chemical composition and antioxidative activity of Moldavian balm (*Dracocephaum moldavica*) extracts." Food Science and Technology **40**: 1655-1663.

Degood, D. E., Manning, D.C., Middaugh, S. and Davies, T.C. (1997). "The Headache and Neck Pain Workbook." New Harbinger Publications, Oakland, California.
DeLeo, J. A. (2006). "Basic Science of Pain." J.Bone Joint Surg **Am 88**(Suppl 2): 58-62.

Dokosi, O. B. (1998). "Herbs of Ghana." Ghana Universities Press; Accra.

Dubinsky, B., Gebre-Mariam, S., Capetola, R.J. and Rosenthale, M.E. (1987). "The analgesic drugs: human therapeutic correlates of their potency in laboratory animal models of hyperalgesia." Agents Actions **20**: 50-60

Dubner, R. and K. Ren (1999). "Assessing transient and persistent pain in animals. In: Wall,P.D, Melzack,R.(Eds).Textbook of Pain. Edinburgh. Churchill Livingstone,." 359369.

Dubuisson, D. and S. G. Dennis (1977). "The formalin tests: a quantitative study of the analgesic effects of morphine, meperidine and brain stem stimulation in rats and cats." Pain **4**: 161-74.

Dudley, H. and I. Fleming (1995). "Spectroscopic methods in organic chemistry(5th edn)." McGraw-Jill Publishing Co. England: 211.

Dufour, D., Pichette, A., Mshvildadze, V., Bradette-Hebert, M-E., Lavoie, S., Longtin, A., Laprise, C. and Legault, J. (2007). "Antioxidant, anti-inflammatory and anticancer activities of methanolic extracts from *Ledum groerdandicum* Rethzho." Journal of Ethnopharmacology **111**: 22-28.

Duwejua, M. and Zeitlin, I.J. (1993). "Plants as sources of anti-inflammatory compounds: In: Drugs from Natural Products." Pharmaceuticals and Agrochemicals Harvey A.L(ed) London: Ellis Horwood.

Duwiejua, M., Woode, E. and Obiri, D.D. (2002). "Pseudo-akuammigine, an alkaloid from *Picralima nitida* seeds, has anti-inflammatory and analgesic actions in rats." J.Ethnopharmacol **81**(1): 73-79.

Duwiejua, M., Zetlin, I.J. and Waterman, P.G. (1994). "Anti-inflammatory activity of *Polygonum bistorta,Guiacum officinale* and *Hamamelis virginiana* in rats." J.Pharmacol **46**(4): 286-290.

Dwivedi, S. (2007). "*Terminalia arjuna*, Wight &Arn - A useful drug for cardiovascular disorders." Journal of Ethnopharmacology **114**: 114-129.

Egger, K. and H. Kleinig-Voigt (1968). "Carotenoids of Commelinaceae *Palisota barteri*." Z.Naturforsch **B23**: 1105-8.

Enomoto, R., Suzuki, C. Lee, E., Ohno, M., Ohashi, T., Miyauchi, A., *et al*. (2007). "Wogonin-prevents immuno-suppressive action but not anti-inflammatory effect induced by glucocorticoid." Ann N Y Acad Sci **1095**: 412-

Fabianska, M. J. (2004). "GC-MS investigation of distribution of fatty acids in selected Polish brown coals " Chemometrics and intelligent laboratory systems **72**: 241-244.

Feinstein, B., Langton, J.N.K, Jameson, R.M. and Schiller, F. (1954). "Experiments on pain referred from deep somatic tissues." J.Bone Joint Surg **36 A(5)**: 981-97.

Fereidoni, M., Ahmadiani, A., Simnaman, S. and Javan, M. (2000). "An accurate and simple method for measurement of paw edema." J Pharmacol Toxicol Methods **43**(1): 1114.

Ferreira, S. H. (1981). "Local control of inflammatory pain." Agents Actions **11**(6-7): 636638.

Fiorucci, S., Antonelli, E., Burgaud, J.L. and Morelli, A. (2001). "Nitric oxide-releasing NSAID's: a review of their current status." Drug Safety **24**: 801-11.

Foltz, E. L. and L. E. White (1962). "Pain "relief" by frontal cingulotomy." Journal of Neurosurgery **19**(89-100).

Fridovich, I. (1997). "Superoxide anion radical(Obardo.2), superoxide dismutases and related matters." Journal of Biological Chemistry **272**: 18515-18517.

Garcia-Larrea, L. and M. Magnin (2008). "[Pathophysiology of neuropathic pain:review of experimental models and proposed mechanisms]." Presse Med **37**(2Pt2): 315-340.

Glazer, A. N. (1990). "Phycoerythrin flurescence-based assay for reactive oxygen species." Methods in Enzymology **186**: 161-168.

Goldstein, E. M. (2001). "Spasticity management :an overview." J Child Neurol **16**: 16-23.

Govindarjan, R., Rastogi, S., Vijayakumar, M., Shirwaikar, A.K.S., Rawat, S., Mehrota, S and Palpu, P. (2003). "Studies on the Anti-oxidant Activities of *Desmodium gangeticum*." Biol Pharm Bull **26**: 1424-1427.

Greco, A., Minghetti, L., Sette, G., Fieschi, C. and Levi, G. (2000). " Isoprostanes, novel markers of oxidative injury, help understanding the pathogenesis of neurodegenerative diseases." Neurochemical Research **25**: 1357.

Green, A. F, Young, P.A. and Gregory, E.I. (1951). "A Comparison of heat and pressure analgesimetric methods in rats." Br.J Pharmacol **6**: 572-85.

Grumbach, L. (1964). "The prediction of analgesic activity in man by animal testing. In Knighton,R.S;Dumke,P.R.,editors."Pain, 15th International Symposium, Detroit, Boston: Little Brown **1966**: 163-82.

Guidi, I., Galimberti, D., Lonati, S., Novembrino, C., Bamonti, R. and Tiritico, M. (2006). "Oxidative imbalance in patients with mild cognitive impairement and Alzheimer's disease." Neurobiology of Ageing **27**: 262-269.

Guilianao, F. and T. D. Warner (2002). "Origins of prostaglandins E2: involvements of cyclooxygenase(COX)-1and COX-2 in human and rat systems." J Pharmacol Exp Ther **303**: 1001-6.

Gunasekera, S. P., Zuleta, I.A., Longley, R.E., Wright, A.E., and Pomponi, S.A. (2003). "Discorhabins S, T and U, new cytotoxic pyrroloimidinoquinones from a deep-water Caribbean sponge of the genus Batzella." Journal of Natural Products **66**: 1615-1617.

Gutteridge, J. M. and J. Mitchell (1999). "Redox imbalance in the critically ill." Br.Med.Bull **55**(1): 49-75.

Halliwell, B. and J. M. C. Gutteridge (1999). "Free radicals in biology and medicine (3rd ed.)." Oxford University Press.

Hammond, D. L. (1989). "Inference of Pain and its modulation from simple behaviours. In: Chapman, C.R., Loeser, J.D., editors. Issues in pain research and therapy." New York: Raven Press: 69-91.

Harvey, V. L. and A. H. Dickenson (2008). "Mechanisms of pain in non-malignant disease." Curr Opin Support Palliat Care **2**(2): 133-139.

Hayashida, K., Takeuchi, T., Shimizu, H., Ando, K. and Harada, E. (2003). "Lactoferrin enhances peripheral opioid-mediated anti-nociception via nitric oxide in the rat spinal cord." Am.J.Physiol-Regul Integr and Comp Physiol **285**: R306-R12.
Heapy, C. G., Jameson, A. and Russell, N.J.W (1987). "Afferent C-fibre and A-delta activity in models of inflammation." Br.J Pharmacol **90**: 164.

Higgs, G. A. (1989). "Use of Implanted sponges to Study the Acute Inflammatory Response." In:Pharmacological Methods in the Control of Inflammation, Chang, J, Lewis, A.J (eds) New York: Alan R Liss: 151-171.

Hikino, H and Hikino, Y (1990). Arthropod moulting hormones, Progress in Chemistry of Organic Natural Products, **28**:256-312

Hudson, J. B., Anani, K., Arnason, J.T. and Gbeasor, M. (2000). "Further Investigations on the Antiviral Activities of Medicinal Plants of Togo." Pharmaceutical Biology **38**: 46-50.

Humnskaar, S., Fasmer, O.B.and Hole, K. (1985). "Formalin test in mice, a useful technique for evaluating mild analgesics." J Neurosci Methods **14**: 69-76.

Hunskaar, S. and K. Hole (1987). "The formalin test in mice: dissociation between inflammatory and non-inflammatory pain." Pain **30**(1): 103-114

Hwang, A. S. and G. L. Wilsox (1986). "Intradermal hypertonic saline-induced behaviour as a nociceptive test in mice." Life Scie **38**: 2389-96.

Iadarola, M. J. Brady, L.S., Draisici, G. and Dubner, R. (1988). "Enhancement of dynorphin gene expression in spinal cord following experimental inflammation: Stimulus specificity, behavioural parameters and opioid receptor binding." Pain **35**: 313-26.

Isaac, R.E., Milner, N. P. and Rees, H.H. (1983) Identification of ecdysonoic acid and 20hydroxyecdysonoic acid isolated from developing eggs of *Schistocera gregaria* and pupae of *Spodoptera littoralis*. Biochem. J. (1983) **213**(61-265)

Issekutz, C. A. and T. B. Issekutz (1989). "Quantitation of Blood Cell Accumulation and Vascular Responses in Inflammatory Reactions." In: Chang J.Y, Lewis A.J editors: Pharmacological Methods in the Control of Inflammation, New York, Alan R. Liss Inc: 129-50.

Ito, N., Fukushima, Hasegawa, A., Shibata, M and Ogiso, T (1983). "Carcinogenicity of butylated hydroxyanisole inF344 rats." Journal of National Cancer Institute **70**: 343-347.
Janero, D. R. (1990). "Malondialdehyde and thiobarbituric c acid reactivity as diagnostic indices of lipid peroxidation and per- oxidative tissue injury." Free Radic Biol Med **9**: 515540.

Jha, P., Flather, M., Lonn, E., Farkouh, M. and Yusuf, S. (1995). "The antioxidant vitamins and cardiovascular disease. A critical view of epidemiologic and clinical trial data." Annals of Internal Medicine **123**: 860.

Johnson, G. L. and C. A. Lange-Carter (1994). "Ras-dependent growth factor investigation of MEK kinase in PC12 cells." Science **265**: 1458-61.

Kahkonen, M., Kylli, P., Ollilairen, V., Salminen, J-P., and Heinoren, M. (1999). "Antioxidant activity of plant extracts containing phenolic compounds." Journal of Agricultural and Food Chemistry **47**: 3954-3962.

Kang, K., Lee, K. H., Chae, S., Koh, Y.S., Yoo, B.S., Kim, J.H., Ham, Y.M., Baik, J.S. Lee, N.H and Hyun, J.W. (2005). "Triphlorethol-A from Ecklonia cava protects V79-4 lung fibroblast against hydrogen peroxide induced cell damage." Free Radic Res **39**(8): 883-892.

Kaur, G., Hamid, H., Ali, A., Alam, S.A. and Athar, M. (2004). "Anti-inflammatory evaluation of alcoholic extract of galls of *Quercus infectoria*." J.Ethnopharmacol **90**(2-3): 285-292.

Kendler, B. S. (1995). "Free radicals in health and disease: Implications for the primary health care providers." Nuase Pract. **20**(7): 29.

Kerr, J., Winterford, C.M. and Harmon, B.U. (1994). "Morphological criteria for identifying apoptosis." In Cell Biology: A Laboratory Handbook Vol 2. J Celis(London:Academic Press): 319-330.

Kilmartin, P. A. and C. F. Hsu (2003). "Characterisation of polyphenols in green, oolong and black teas and coffee using cyclic voltammetry." Food Chemistry **82**: 501-512.

Kohen, R. and Nyska, A. (2002) Oxidation of biological systems: oxidative stress phenomena, antioxidants, redox reactions and methods for their quantification. Toxicol Pathol 30(6):620-50.

Kupeli, E. and E. Yesilada (2007). "Flavonoids with anti-inflammatory and antinociceptive activity from *Cistus laurifolius* L-leaves through bioassay-guided procedures." J.Ethnopharmacol **112**(3): 524-530.

Kusamba, C., Nicoletti, M., Federici, E. and Federici, E. (1995). "Isolation of ecdysteroids from three species of Palisota." Fitoterapia **66**: 175-178.

Kweifio-Okai, G., Bird, D., Field, B., Ambrose, R., Caroll, A.R., Smith, P. and Valdes, R. (1995). "Anti-inflammatory activity of a Ghanaian anti-arthritic herbal preparation III." J.Ethnopharmacol **46**(1): 7-15.

Lariviere, W. R. and R. Melzack (1996). "The bee venom test: a new tonic-pain test." Pain **66**: 271-7.

LeBars, D., Gozariu, M. and Cadden, S.W. (2001). "Animal models of Nociception." Pharmacol Rev **53**(4): 597-652.

Lee, S. C., Kuan, C.Y., Yang, C.C. and Yang, S.D. (1998). "Bioflavonoids commonly and potentially induce tyrosine dephosphorylation/inactivation of oncogenic proline-directed protein kinase FA in human prostrate carcinoma cells." Anticancer Research **1**: 1117-1121.

Levites, Y., Amit, T., Mandel, S and Youdin, M.B.H. (2003). "Neuroprotection and neurorescue against amyloid Beta toxicity and PKC dependent release of nonamyloidogenic soluble precursor protein by green to polyphenol(-)-epigallocatechin 3gallate." FASEB Journal **17**: 952-954.

Levites, Y., Weinreb, O, Maor, G., Youdin, M.B.H. and Mandel, S. (2001). "Green tea polyphenol(-)-epigallocatechin 3-gallate prevents N-methyl-4-phenyl-

1,2,3,6tetrahydropyridine-induced dopaminergic neuro-degeneration." Journal of Neurochemistry **78**: 1073-1082.

Li, C. Q., He, L.C., Dong, H.Y. and Jin, J.Q. (2007). "Screening for the anti-inflammatory activity of fractions and compounds from *Atractylodes macrocephala* Koidz." J.Ethnopharmacol **114**(2): 212-217.

Lim, Y. Y. and J. Murtijaya (2007). "Antioxidant properties of *Phyllantus amanus* extracts as affected by different drying methods." Food Science and Technology **40**: 1664-1669.
Liu, L., Buchner, E., Beitze, D.,Weber, C.B.S. Kaever, J., Emmrich, F. and Kinne, R.W. (1996). "Amelioration of rat experimental arthritides by treatment with the alkaloid sinomenine." Int J Immunopharmacol **18**(10): 529-543.

Loux, J. J., Smith, S and Salem, H. (1978). "Comparative analgesic testing of various compounds in mice using writhing techniques." Arzneim Forsch **28**: 1644-7.

Lucas, S. M., Rothwell, N.J., and Gibson, R.M. (2006). "The role of inflammation in CNS injury and disease." Br.J Pharmacol **147**: S232-S40.

MacCaffery, M. and C. Pasero (1999). "Pain." Clinical Manual.St.Louis.

MacLean, D. D., Murr, D.P. and DeEll, J.R. (2003). "A modified total oxyradical scavenging capacity assay for antioxidants in plant tissues." Postharvest Biology & Technology **29**: 163-194.

MacPherson, R. D. (2002). "New directions in pain management." Drugs Today **38**: 13545.

Malmberg, A. B. and T. L. Yaksh (1995). "Cyclooxygenase inhibition and the spinal release of prostaglandin E2 and amino acids evoked by paw formalin injection: a microdialysis study in unanaesthetized rats." J.Neurosci **15**: 2768-2776.

Masferrer, J. L., Zweifel, J.L., Manning, P.T., Hauser, S.D., Leahy, K.M., Smith, W.G., Isakson, P.C. and Seiber, K. (1994). "Selective inhibition of inducible cyclooxygenase 2 in vivo is anti-inflammatory and non-ulcerogenic." Proc Natl Acad Sci.USA **91**(8): 32283232.

Mbagwu, H. O., Anene, R.A. and Adeyemi, O.O. (2007). "Analgesic, antipyretic and antiinflammatory properties of of *Mezoreuron benthamianum* Baill(Caesalpiniaceae." Nig J Hosp Med **17**(1): 35-41.

McCarffery, M. (1968). "Nursing practice theories related to cognition, bodily pain and man-environment interactions." Los Angeles; UCLA Students Store.

McCormack, K., Prather, P. and Chapleo, C. (1998). "Some new insights into the effects of opioids in phasic and tonic nociceptive tests." Pain **78**: 79-98.

McDermott, J. H. (2000). "Antioxidant nutrients: Current dietary recommendations and research update." J.Am Pharmacol.Assoc **40**(6): 785-99.

Melzack, R., and Casey, K.L. (1968). "Sensory, motivational and central control determinants of chronic pain: A new conceptual model." The Skin Senses (Springfield, Illinois, Thomas): 423-443.

Merskey, H. (1964). "An investigation of pain in Psychological Illness,." DM Thesis,Oxford.

Merskey, H. (1994). "Logic,truth and language in concepts of pain." Qual Life Re **3**(Suppl 1): S69-76.

Messeguer, A., Planells-Cases, R., and Ferrer-Montiel, A. (2006). "Physiology and Pharmacology of the vanilloid receptor." Curr Neuropharmacol **4**(1): 1-15.

Miller, N. J., Rice-Evans, C., Davies, M.J., Gopinathan, V. and Milner, A. (1993). "A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates." Clinical Science **84**: 407-412.

Mitsuda, H., Yasumoto, K. and Iwani, K. (1967) Antioxidant action of indole compounds during the autoxidation of linoleic acid. Eiyo To. Shodury ou. 19(3): 210.

Monnier, D. G. and J. Chaudiere (1996). "Metabolism and antioxidant function of glutathione." Pathologie Biologie(Paris) **44**: 77-85.

Mshana, N. R., Abbiw, D.K., Addae-Mensah, I., Adjanohoun, E., Ahji, M.R. *et al*. (2000). "Traditional Medicine and Pharmacopoeia. Contribution to revision of Ethnobotanical and Floristic Studies in Ghana." Scientific, Technical and Research Commission of Organization of African Unity: 199.

Muniappar, M. and T. Sundararaj (2003). "Anti-inflammatory and antiulcer activities of *Bambusa arundinacea*." J.Ethnopharmacol **88**(2-3): 161-167.

Narendhirakannan, R. T., Subramanian, S. and Kandaswamy, M. (2007). "Antiinflammatoryand lysosomal stability actions of *Cleome gynandra* L. studied in adjuvant induced arthritic rats." Food and Chemistry Toxicol **45**(6): 1001=1012.

Netzel, M., Netzel, G., Tian, Q., Schwartz, S. and Konczak, I. (2007). "Native Australian fruits-A novel source of antioxidants for food." Innovative Food Science and Emerging Technologies **8**: 339-346.

Niemegeers, C. J., F. M. Lenaerts, and Janssen, P.A. (1975). "The antipyretic-effect of suprofen in rats with yeast-induced fever." Arzneimittelforschung **25**(10): 1519-1524.

O'Callaghan, J. P. and S. G. Holzman (1975). "Quantification of the analgesic activity of narcotic antagonists by a modified hot plate procedure." J.Pharmcol Exp Ther **192**: 497505.

Ohkawa, H., Oshishi, N. and Yagi, K. (1979). "Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction." Anal Biochem **95**(2): 351-358.

Okoli, I. C. and Anunibi, M.O. (2003). "Studies on selected browses of Southeastern Nigeria with particular reference to their proximate and some endogenous anti-nutritional constituents." Livestock Research for Rural Development **15**(9).

Okui, S., Otaka, T., Uchiyama, M., Hikino, H., Ogawa, S. and Nishimoto, N. (1968). Chem. Pharm. Bull (Tokyo) **16**: 2426-2429.

Ornstein, R. and D. Sobel (1988). "The Healing Brain.";Simon and Schuster Inc. New York: 98-99.

Ortiz, M. I., Lozano-Cuenca, J., Granados-Soto, V. and Castaneda-Hernandez, G. (2008). "Additive interaction between peripheral and central mechanisms involved in the antinociceptive effect of diclofenac in the formalin tests in rats." Pharmacol Biochem Behav., **91**(1): 32-37.

Otaka, T., Uchiyama, M., Okui, S., Hikino, S., Nabetani, K. and Nomoto, K. (1983). Phytoecdysteroids of plants. Chem. Pharm. Bull (Tokyo) **16**: 384-387.

Otsuki, T., Nakahama, I., Nuzuma, H. and Suzuki, J. (1986). "Evaluation of the analgesic effects of capsaicin using a new rat model for tonic pain." Brain Res **365**: 235-40.

Oyaizu, M. (1986). "Studies on products of browning reaction: Antioxidant activities of browning reaction prepared from glucosamine." Jpn J Nutr **44**(6): 307-315.

Parameswaran, P. S., Naik, C.G., Gonslaves, C. and Achunthankathy, M. (2001). Isolation of 2-deoxyecdysterone, a novel oxytoxic agent from Marine Zoanthus sp. J. Indian Inst. Sci. **81**: 169-173.

Popov, I., and Lewin, G. (1987). "Photochemiluminescent detection of antiradical activity; I: Assay of superoxide dismutase." Biomed Biochem Acta **46**: 775-776.
Popov, I. N. and G. Lewin (1994). "Photochemiluminescent detection of antiradical activity II; Testing of non-enzymic water-soluble antioxidants." Free Radical Biology and Medicine **17**: 267-271.

Popov, I. N. and G. Lewin (1996). "Photochemiluminescent detection of antiradical activity: IV, Testing of lipid-soluble antioxidants." Journal of Biochemical and Biological Methods **31**: 1-8.

Prieto, P., Pineda, M. and Aguila, M. (1999). "Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybedum complex: specific application to the determination of vitamin E." Anal Biochem **269**: 337-341.

Radisky, D. C., Radisky, E.S., Barrows, L.R., Copp, B.R., Kramer, R.A. and Ireland, C. (1993). "Novel cytotoxic topoisomerase II inhibiting pyrroloiminoquinones from Fijian sponges of the genus Zyzzya." Journal of American Oil Chemical Society **115**: 1632-1638.

Rainville, P., Duncan, G.H., Price, D.D., Carnier, B. and Bushnell, M.C. (1997). "Pain effect encoded in human anterior cingulate but not somatosensory cortex." Science **277**(5328): 968-971.

Ramakrishna, B. S., Varghese, R., Jayakumar, S., Mathan, M. and Balasubramamian, K.A. (1977). "Circulating antioxidants in ulcerative colitis and their relationship to disease severity and activity." Journal of Gastroenterology and Hepatology **12**: 490-494.

Randall, L. and J. Selitto (1957). "A method for measurement of analgesic activity on inflammed tissue." Arch Int. Pharmacodyn **4**: 409-19.

Rang, H. P., Dale, M. M., Ritter, J.M. and Moore, P.K. (2003). "Pharmacology 5 edition." Churchill Livingstone.

Raymundo, M. S., Paula, M.M.S.,Chatterjee,S. and Nias, Z. (2007). "Quantitative determination of the phenolic antioxidants using voltammetric techniques." Food Science and Technology **40**: 1133-1139.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M. and Rice-Evans, C. (1999). "Antioxidant activity an improved ABTS radical cation decolorization assay." Free Radical Biology and Medicine **26**: 1231-1237.

Rice-Evans, C. and N. J. Miller (1994). "Total antioxidant status in plasma and body fluids." Methods in Enzymology **234**: 279-293.

Roach, J. T. and K. J. Sufka (2003). "Characterization of the chick carrageenan response." Brain Res **994**(2): 216-225.

Rosa, M. D. (1972). "Biological Properties of Carrageenan." J.Pharm Pharmacol **24**(2): 89-102.

Rosa, M. D. and D. A. Willoughby (1971). "Screens for anti-inflammatory drugs." J.Pharm Pharmacol **23**(4): 297-298.

Rosland, J. H., Tjolsen, A., Maehle, B. and Hole, K. (1990). "The formalin test in mice: effect of formalin concentration." Pain **42**(2): 235-242.

Rozenberg, O., Howell, A and Aviram, M. (2006). "Pomegranate juice sugar fraction reduces macrophage oxidative state, whereas white grape juice sugar fraction increases it." Artherosclerosis **188**: 68-76.

Ruggiero, C., Lattanzio, F., Lauretani,F., Gasperini, B., Andres-Lacueva, and Cherubini, A. (2009), Fatty acids from fish; the anti-inflammatory potential of long chain Omega-3 fatty acids. Curr. Pharm. Des, **15**(36) 4135-48.

Sakai, A., Hirano, T., Okazai, R., Okimoto, N., Tanaka, K. and Nakamura, T. (1999). "Large-dose ascorbic administration suppresses the development of arthritis in adjuvantinjected rats." Arch Orthop Trauma Surg **119**: 121-126.

Sakurada, T., Katsumata, K., Tan-No, K.,Sakurada, S. and Kisara, K. (1992). "The capsaicin test in mice for evaluating tachykinin antagonists in the spinal cord." Neuropharm **31**: 1279-85.

Sanchez-Moreno, C., Larrauri, C.A. and Saura-Calixto, F (1998). "A procedure to measure the antiradical efficiency of phenols." Journal of Food and Agricultural **76**: 270-276.

Sapolsky, R. M. (1968). "Why zebras don't get ulcers, an updated guide to stress, stressrelated disease and coping." W.H. Freeman & Co, New York.

Scheibmeir, H. D., Christensen, K. and Harath, D. (2005). "A review of free radicals and antioxidants for critical care nurses." Intensive Care and Critical care nursing **21**: 24-28.

Schim, J. D. and P. Stang (2004). "Overview of pain management." Pain Pract.**4**(Suppl 1): S4-18.

Schlesier, K., Harwat, M., Bohm, V. and Bitsch, R. (2002). "Assessment of antioxidant activity by using different in vitro methods." Free Radical Research **36**: 177-187.

Seibert, K., Zhang, Y., Leahy, K., Hauser, S., Masferrer, J., Perkins, W. *et al* (1994). "Pharmacological and biochemical demonstration of the role of cyclooxygenase 2 in inflammation and pain." Proc Natl Acad Sci.USA **91**(25): 12013-12017.

Serhan, C. N. (2004). "A search for endogenous mechanism uncovers novel chemical mediators ,missing link to resolution." Histochem Cell Biol **122**: 305-21.

Shahin, S. A., Kasoju, N., Luthra, A., Singh, A., Sharanabasava, H., Sahu, A. and Bora, U. (2008). "Indian Medicinal herbs as sources of antioxidants." Food Research International **41**: 1-15.

Shaunbaum, G. R. and B. Chance (1976). "Catalase." In P.D.Boyer(Ed), the enzymes. New York Academy **VIII(2nd ed)**: 363-408.

Shibata, M., Ohkubo, T., Takahashi, H. and Inoki, R. (1989). "Modified formalin test: characteristic biphasic pain response." Pain **38**(3): 347-352.

Siems, W. G., Gruna, T., and Esterbauer, H. (1995). "4-Hydroxynonenal formation during ischaemia and reperfusion of rat small intestine." Life Science **57**: 785-789.

Singh, B., Sahu, P.M. and Sharma, M.K. (2002). "Anti-inflammatory and anti-microbial activities of triterpenoids from *Strobilanthes callosus* nee " Phytomedicine **9**(4): 355-359.

Singh, H., Kumar, S., Dewan, S. and Kumar, V.L. (2000). "Inflammation induced by latex of *Caleotropis procera*-a new model to evaluate anti-inflammatory drugs." Journal of Pharmacological and Toxicological Method **43**: 219-224.

Smith, M. A., Perry, G., Richly, P.L., Sayrec, L.M., Anderson, V.E. Beal, M.F. and Kowall, N. (1996). "Oxidative damage in Alzheimer's (letter)." Nature **382**: 120.

Smith, M. A., Rottkamp, C.A., Nunomora, A., Raina, A.K. and Perry, G. (2000). "Oxidative stress in Alzheimer's disease." Biochimica et Biophysica Acta **1502**: 139-144.

Sofowora, A. (1993). "Phytochemical screening of medicinal plants trditional medicine in Africa 2nd edn." Spectrum Books Limited Nigeria: 150-156.

Srinivasan, R., Chandrasekar, M.J.N., Nanjan, M.J. and Suresh, B. (2007). "Antioxidant activity of (*Caesalpina digyna*) root." Journal of Ethnopharmacology **113**: 284-291.

Stadtman, E. R. (2004). "Role of oxidant species in aging." Current Medical Chemistry **11**: 1105-1112.

Stanner, S. A., Hughes, J., Kelly, C.N. and Buttriss, J. (2004). "Review of the epidermiological evidence for the 'antioxidant hypothesis'." Public Health Nutrition **7**: 407422.

Straube, S., Andrew, M.R., Derry, S. and McQuay, H.J. (2009). "Vitamin D and Chronic Pain." Pain **141 (1-2)**: 10-13.

Stvrtinova, V., Jahnova, E. Weissova, S., Horvathova, M. and Ferencik, M. (2001). "Inflammatory mechanisms involving neutrophils in chronic venous insufficiency of lower limbs." Bratish Lek Listy **102**(5): 235-239.

Suardiaz, M., Estivill-Torrus, G., Goicoechea, C., Bilbao, A. and De Fonseca, R. (2007). "Analgesic properties of oleoylethanolamide (OEA) in visceral and inflammatory pain." Pain **133**(1-3): 99-110.

Sundquist, E. A., Briviba, K., and Sies, H. (1994). "Singlet oxygen quenching by carotenoids." Methods in Enzymology **234**: 354-566.

Tang, L., Chen, Y., Chen, Z., Blumberg, P.M., Kozikowski, A.P. and Wang, Z.J. (2007). "Anti-nociceptive pharmacology of N-(4-chlorobenzyl)-N-(4-hydroxy-3-iodo-5methoxybenzyl) thiourea, a high-affinity competitive antagonist of the transient receptor potential vanilloid 1 receptor." J Pharmacol Exp Ther **321**(2): 791-798.

Teiger, D. G. (1976). "A test for antinociceptive activity of narcotic and narcotic antagonist analgesics in the guinea pig" J Pharmacol Exp Ther **197**: 311-6.

Tepe, B. and A. Soknen (2007). "Screening of antioxidative properties and total phenolic contents of three endemic *Tanacetum* subspecies from Turkish flora." Bioresource Technology **98**: 3076-3079.

Tjolsen, A., Berge, O.G., Hunskaar, S., Rosland, J.H. and Hole, K. (1992). "The formalin test: an evaluation of the method " Pain **51**(1): 5-17.

Tonussi, C. R. and S. H. Ferreira (1992). "Rat knee-joint carrageenan incapacitation test: an objective screen for central and peripheral analgesics." Pain **48**: 421-40.

Trease, G. E. and W. C. Evans (1983). "Pharmacognosy 12th edn." Bailliere Tindal: London: 343-383.

Trevisani, M., Siemens, J., Materazzi, S., Bautista, D.M., Nassini, R., Campi, B. *et al.* (2007). "4-Hydroxynonenal, an endogenous aldehyde causes pain and neurogenic

inflammation through activation of the irritant receptor TRPA-1." Proc Natl Acad Sci.USA **104**(33): 13519-13524.

Ugochukwu, N. H. and M. K. Cobourne (2003). "Modification of renal oxidative stress and lipid peroxidation in streptozocin-induced diabetic rats treated with extracts from *Gongronema latifolium* leaves." Clinica Chimica Acta **336**: 73-81.

Ursini, F., Maiorino, M., Brigelius-Flohe, R., Aumann, K.D., Roveri, A., Schomburg, D and Flohe, L. (1995). "The diversity of glutathione peroxidase." Methods in Enzymology **252**: 38-53.

Valko, M., Leibfritz, D., Moncol, J., Cronin, M.T., Mazur, M and Telser, J. (2007). "Free radicals and antioxidants in normal physiological functions and human disease." The International Journal of Biochemistry and Cell Biology **39**: 44-84.

Valko, M., C. J. Rhodes, C.J., Moncol, J. Izakovic, M. and Mazur, M. (2006). "Free radical metals and antioxidants in oxidative stress-induced cancer." Chemico Biological Interaction **160**: 1-40.

Valkonen, M. and Y. Kuusi (1997). "Spectrophotometric assay for total peroxyl radicaltrapping antioxidant potential in human serum." Journal of Lipid Research **38**: 823-833.

Vinegar, R., J. F. Truax, J.F. and Selph, J.L. (1987). "Quantitative comparison of the analgesic and anti-inflammatory activities of aspirin, phenacetin and acetaminophen in rodents." Eur J Pharmacol **37**(1): 23-30.

Vlaeyen, J. W. S. and S. Morley (2005). "Cognitive-Behavioural Treatments for Chronic Pain; What Works for Whom?" Clinical Journal of Pain Special Topic Series: CognitiveBehavioural Treatments for Chronic Pain. **21(1)**: 1-8.

Wang, M., Tsao, R., Zhang, S., Dong, Z., Yang, R., Gong, J and Pei, Y. (2006). "Antioxidant activity, mutagenicity/antimutagenicity and clastogenicity/anticlastogenicity of lutein from marigold flowers." Food and Chemistry Toxicity **44**: 1522-1529.

Watson, G. S., Sufka, S.J. and Coderre, T.J. (1997). "Optimal scoring strategies and weights for the formalin test in rats." Pain **70**: 53-8.

Watson, L. and Dallawitz, M.J. (1992). "The families of flowering plants: descriptions, illustrations, identification and information retrieval."

Wayner, D. D. M., Burton, G.W., Ingold, K.U. and Locke, S. (1985). "Quantitative measurement of the total, peroxyl radical-trapping antioxidant capacity of human blood plasma by controlled peroxidation." FEBS letter **187**: 33-37.

Whitehouse, M. W., Fairlie, D.P. and Thong, Y.H. (1994). "Anti-inflammatory activity of the isoquinoline alkaloid, tetradrine, against established adjuvant arthritis in rats." AgentsActions **42**(3-4): 123-127.

William, E.M (1992). Biochemistry and Physiology of n-fatty acids. FASEB Journal **6**(8) 2530-2536.

WHO (Dec. 2008). "Report on Traditional Medicine Practice." Fact Sheet **No.134**.

Wilson, S. G., Chesler, E.S., Hain, H., Rankin, A.J. Scharwz, J.Z and Call, S.B. (2002). "Identification of quantitative trait loci for chemical/inflammatory nociception in mice." Pain **96**(3): 385-91.

Wilson, S. G. and Mogil, J.S. (2001). "Measuring pain in the (knockout) mouse: big challenges in a small mammal." Behav. Brain Res **125**: 65-73.

Winston, G. W., Regoli, P., Dugas, A.J., Fong, J.H. and Blanchard, K.A. (1998). "A rapid gas chromatographic assay for determining oxyradical capacity of antioxidants and biological fluids." Free Radical Biology and Medicine **24**: 480-493.

Winter, C. A., E. A. Risley, E.A. and Nuss, G.W. (1962). "Carrageenan -induced edema in hind paw of the rat as an assay for anti-inflammatory drugs." Proc Soc Exp Biol Med **111**: 544-547.

Wise, L. E., Cannavaccinlo, R., Cravatt, B.F., Martin, B.F. and Lichtmann, A.H. (2008). "Evaluation of fatty acid amides in the carrageenan-induced paw oedema model." Neuropharmacology **54**(1): 181-188.

Wolfe, K., Xianzhong, W.U. and Liu, R.H. (2003). "Antioxidant activity of apple peels." Journal of Agricultural and Food Chemistry **51**: 609-614.

Wollin, S. D. and P. J. Jones (2001). "Alcohol, red wine and cardiovascular disease." Journal of Nutrition **131**: 1401-1404.

Woode, E., Ainooson, G.K., Boakye-Gyasi, E., Ansah, C., Obiri, D.D., Koffour, G.A. Mensah, A.Y. and Duwiejua, M. (2008). "Anti-arthritic and anti-oxidant properties of the ethanolic extract of *Newbouldia laevis*(P.Beauv) Seaman ex.Bureau(Bignoniaceae)." Journal of Medicinal Plants Research **2**(8): 180-188.

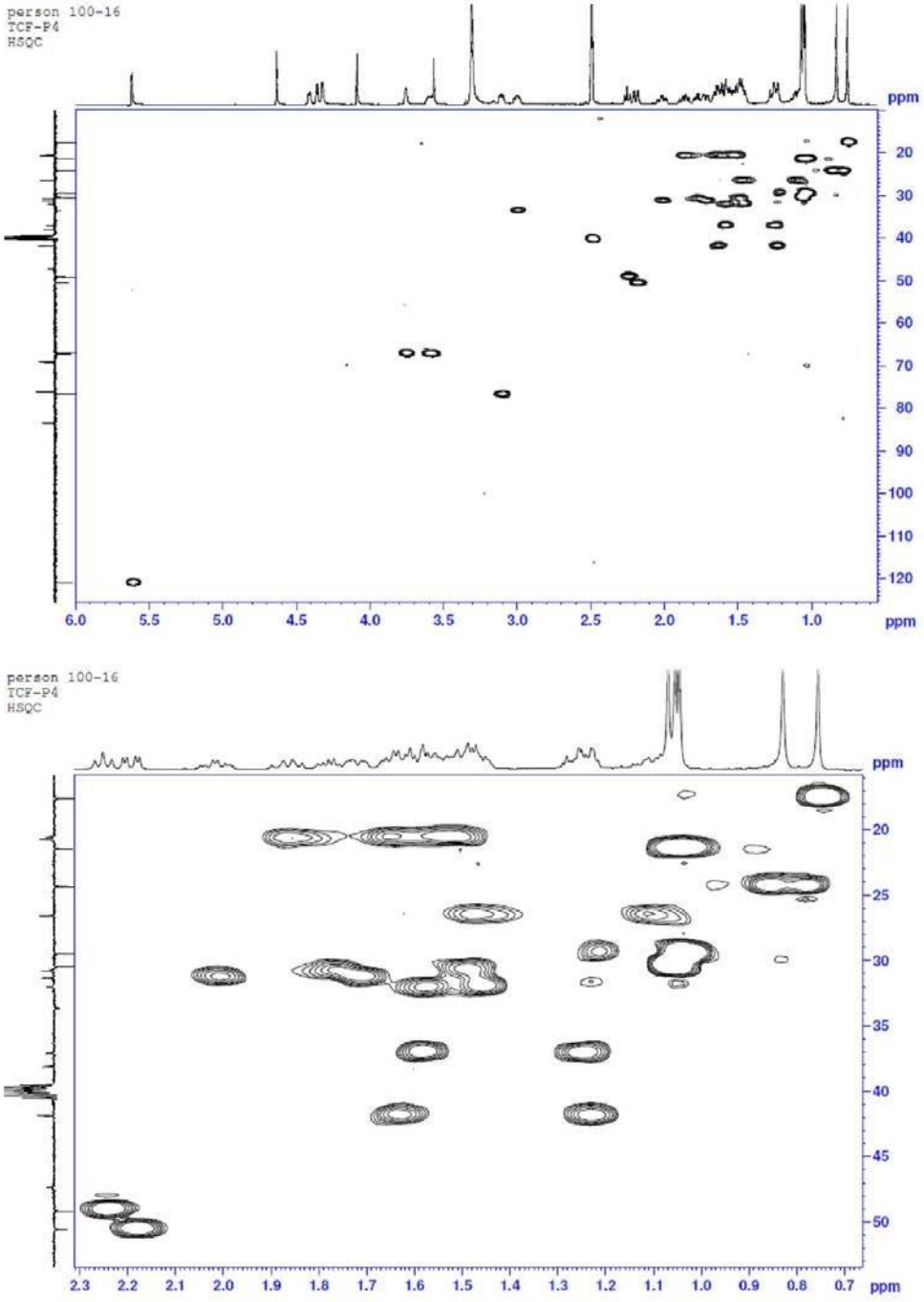
Woode, E., Obiri, D.D. and Duwiejua, M. (2006). "Total alkaloidal extract of *Picralima nitida* (Fam.Apocynaceae) seeds has anti-inflammatory actions." J.Ghana Sci Assoc.

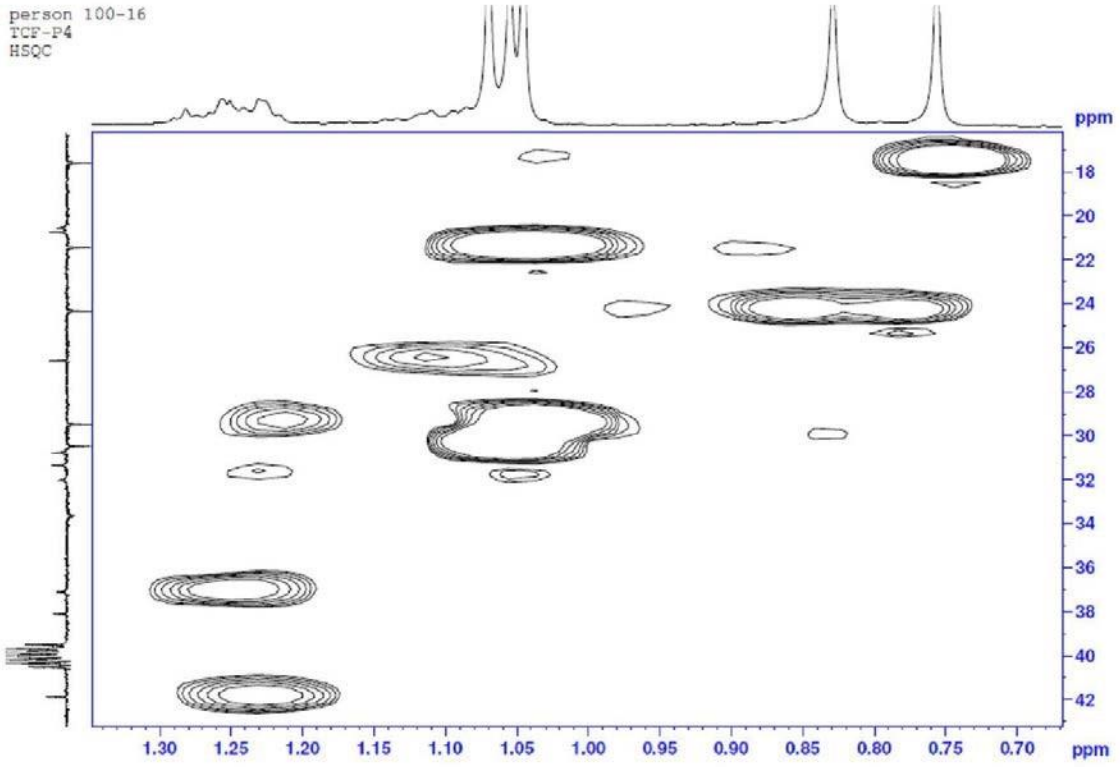
Yaksh, T. L. (1999). "Central Pharmacology of nociceptive Transmission. In: P.Wall and P.Melzack, (editors)." Textbook of Pain 3E Churchill Livingstone.

Yun-Song, W., Ying-Tong, D., Hong-Ping,H., Liang-Bo, L., Yang, L., Jian-Bo, G., Xin, F., Ning-Chuan, K., Shun-Lin, L., Hua-Jie, Z. and Xiao-Jiang, H. (2007) Isolation, X-ray crystallography and computational studies of calydaphninone, a new alkaloid from *Daphniphyllum calycillum*. Organic Letters 9(7): 1355-1358.

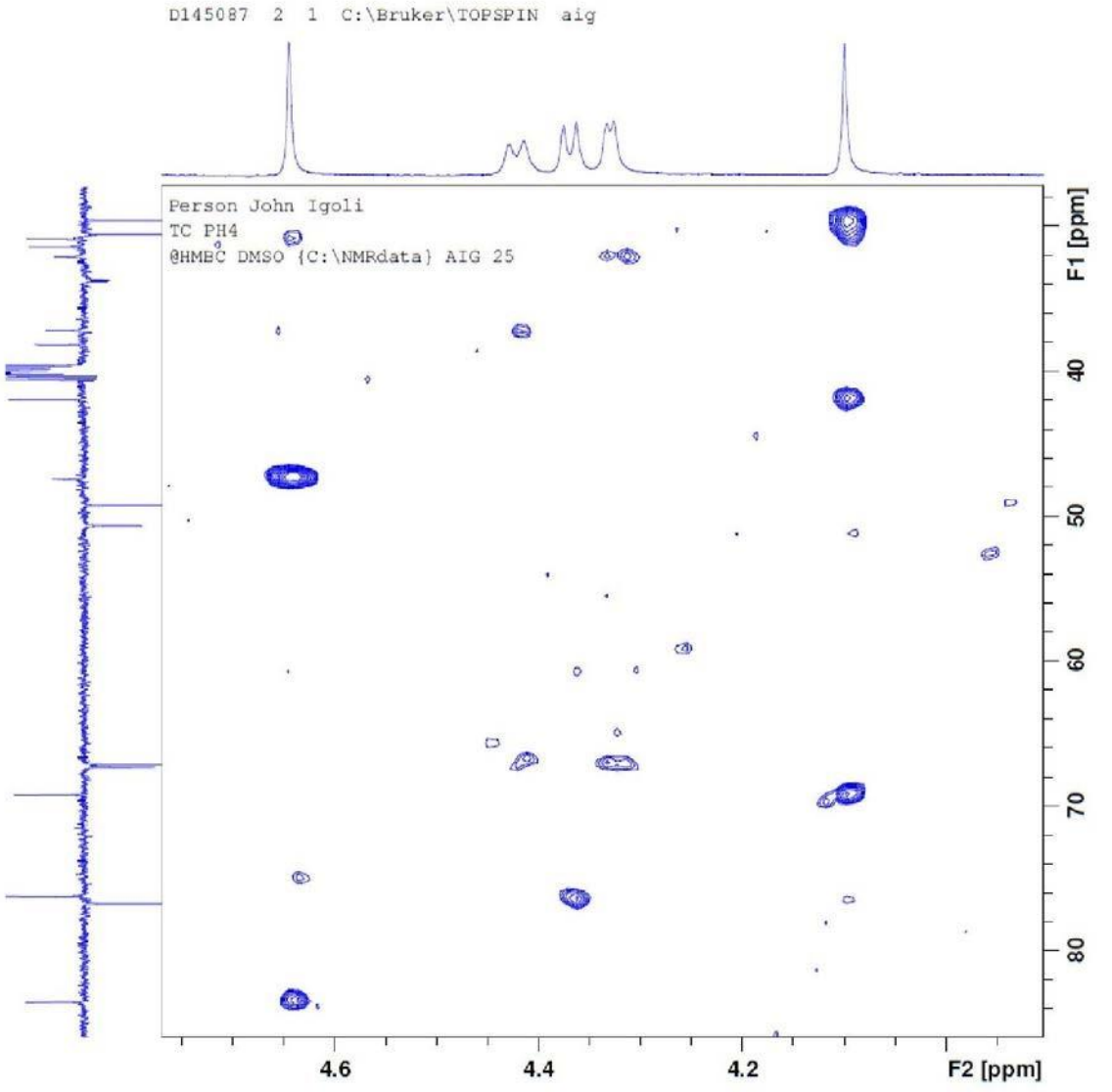
APPENDICES

APPENDIX I: HSQC SPECTRA OF PH IV IN DMSO





APPENDIX II: HMBC SPECTRA OF PH IV IN DMSO



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