ANTI-INFLAMMATORY AND ETHOPHARMACOLOGICAL EFFECTS OF AN ETHANOLIC LEAF EXTRACT OF *PALISOTA HIRSUTA* K. SCHUM. (COMMELINACEAE)

KNUST

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by

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DECLARATION

The experimental work described in this thesis was carried out at the Department of Pharmacology, KNUST. This work has not been submitted for any other degree.

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Dr. Eric Woode

ABSTRACT

Leaves of *Palisota hirsuta* are used in Ghana and other West African states for various painful and inflammatory conditions. This study is aimed at evaluating the anti-inflammatory and ethopharmacological properties as well as toxicity profile of an ethanolic leaf extract of *Palisota hirsuta* using animal models.

Preliminary phytochemical screening showed that the powdered leaves contained tannins, reducing sugars, flavonoids, steroids and terpenoids with traces of alkaloids.

Effect of the extract on acute inflammation was assessed in the carrageenan-induced foot edema in 7-day-old chicks with diclofenac and dexamethasone as reference drugs. Pre treatment with the extract (30-300 mg kg^{-1}; *p.o.*) significantly, inhibited foot edema in the chicks comparable to the NSAID diclofenac with maximal inhibition of 54.71±11.04%. Diclofenac and dexamethasone also dose-dependently inhibited carrageenan-induced foot edema.

The anti-arthritic effect of the ethanolic leaf extract was assessed in the Freund's adjuvant induced-arthritis model in rats. *Palisota hirsuta* extract (PHE) as well as dexamethasone and methotrexate, used as positive controls, showed significant dose-dependent anti-arthritic properties when administered prophylactically, curatively and also in combination therapy. PHE (30-300 mg kg^{-1}) significantly reduced the arthritic edema in the ipsilateral paw with the highest dose used giving a maximum inhibition of 13.02±8.77%. PHE (300 mg kg^{-1}) also significantly prevented the spread of the edema from the ipsilateral to the contralateral paw indicating inhibition of systemic spread. Dexamethasone (0.3-3 mg kg^{-1}) and methotrexate (0.1-1.0 mg kg^{-1}) significantly and in a dose dependent manner also inhibited polyarthritis edema. PHE in combination with methotrexate did not show significant effect. However there was a significant inhibition of arthritis in both the acute and the polyarthritic phases when PHE was combined with

dexamethasone. Dexamethasone in combination with methotrexate caused the greatest inhibition of both phases with an extreme level of significance as expected. Overall, the present results demonstrate that PHE has anti-arthritic effect which could be similar to that exhibited by methotrexate.

P. hirsuta (30-300 mg kg^{-1}; *p.o.*) also dose-dependently decreased baker's yeast induced fever in rats when Paracetamol (10-100 mg kg^{-1}; *p.o.*) was used as the reference drug.

The *in vitro* antioxidant properties of the extract were evaluated using the reducing power test; 2, 2-diphenyl-1-picrylhydrazyl hydrate (DPPH) radical scavenging assay and the lipid peroxidation assay. In all tests, *n*-propyl gallate was used as the reference antioxidant. The extract (0.1-3.0 mg ml^{-1}) showed a reducing power potential (EC_{50}; 133.7±7.59 mg ml^{-1}) but was less than that of the reference antioxidant *n*-propyl gallate (EC_{50} 3.77±0.07 mg ml^{-1}) in the reducing power test. The relative anti-oxidative activity in the DPPH de-colorization assay (defined by the EC_{50}) was in the order: *n*-propyl gallate ($8.02±0.01 \times 10^{-4}$) > extract ($1.77±0.40 \times 10^{-1}$). The extract (0.1-1.0 mg ml^{-1}) and *n*-propyl gallate (0.01-0.1 mg ml^{-1}) exhibited a concentration dependent inhibition of lipid peroxidation. The rank order of potency (defined by ED_{50} in mg ml^{-1}) was found to be: *n*-propyl gallate ($1.31±3.00 \times 10^{-2}$) > extract ($4.29±0.95 \times 10^{-1}$). These findings present the extract with potent antioxidant properties which may account in part for its anti-inflammatory and analgesic activities.

In the analgesic assay, the leaf extract of *P. hirsuta* (PHE) (30, 100 and 300 mg kg^{-1} *p.o*) as well as morphine and diclofenac (positive controls), caused significant dose-dependent anti-nociceptive activity in all the pain models used. In the tail withdrawal test, PHE (300 mg kg^{-1}) increased withdrawal latencies significantly by 43.83±11.62%. Also, PHE (300 mg kg^{-1}) completely reversed the inflammatory-induced mechanical hyperalgesia with a maximum percentage effect of 154.79±15.84%. PHE significantly reduced the number of acetic acid induced

writhing in mice. In the formalin test, PHE (10–300 mg kg^{-1}, *p.o.*) caused a marked and dose-related inhibition of both phases of formalin-induced nociception. The anti-nociceptive effect exhibited by PHE in the formalin test was reversed by the systemic administration of the non-selective opioid antagonist, naloxone, the NO synthase inhibitor, N^{G}-nitro-arginine methyl ester (L-NAME) and the ATP-sensitive K^{+} channel inhibitor, glibenclamide. However, theophylline a non-selective adenosine receptor antagonist did not reverse the effect. PHE, unlike morphine, did not induce tolerance to its anti-nociceptive effect in the formalin test after chronic administration and also morphine tolerance did not cross-generalize to PHE. Overall, the present results demonstrate that the central and peripheral anti-nociceptive of PHE may partially or wholly be due to the stimulation of peripheral and/or central opioid receptors through the activation of the nitric oxide-cyclic GMP- ATP-sensitive K^{+} (NO/cGMP/K^{+}ATP)-channel pathway.

As part of the present study, the ethopharmacological properties of the ethanolic leaf extract, in multiple behavioral paradigms of anxiety and depression— the open field test, the light/dark box, the elevated plus maze (EPM), the forced swimming test (FST) and tail suspension test (TST) was evaluated. *P. hirsuta* treated mice (30-300 mg kg^{-1}) exhibited anxiolytic activity similar to diazepam in all the anxiety models used. PHE significantly increased the percentage number of center entries and the percentage time spent in the center of the open field. It also significantly increased the time spent in the lit area in relation to the time spent in the dark area of the light/dark box as well as significantly increasing open arm activity in the EPM. These effects were completely reversed in the presence of flumazenil (3 mg kg^{-1}), a specific antagonist of the benzodiazepine site in the $GABA_A$ benzodiazepine receptor complex. The extract also dose-dependently reduced the duration of immobility in both the FST (ED_{50}: 114.55±72.69 mg kg^{-1}) and TST (70.42±0.06 mg kg^{-1}). Pretreatment with •-methyldopa (400 mg kg^{-1}; 3 h; *p.o.*), to reduce brain NE and DA tissue content or reserpine (1 mg kg^{-1}; 24 h; s.c.) for the disruption of vesicular storage of brain NE, DA and 5-HT tissue content or

a combination of the two drugs to deplete both newly synthesized and vesicular components of NE and DA transmission attenuated the anti-immobility effects of both imipramime and the extract but not fluoxetine. Neither the extract nor the standard drugs used modified motor performance on the rota rod test at all doses tested. Collectively, these results suggest that the extract has anxiolytic and antidepressant-like effects in the models employed possibly by GABAergic activation and/or modification of monoamine transport and/or metabolism.

In the toxicological study, there were no significant differences found in almost all of the hematological, serum biochemical parameters and organ/body weight ratio. No abnormality of any organ was found during histopathological examination. The results showed that the no-observed adverse- effect level (NOAEL) of *P. hirsuta* extract (PHE) was >3000 mg kg^{-1} body weight per day in rats, which can be regarded as virtually non-toxic. In conclusion, PHE had no overt organ specific toxicity and hence has a high safety profile in rats.

Putting all together, these novel findings provide some pharmacological evidence and basis for the traditional use of the leaves of *P. hirsuta* in traditional medicine to manage various painful and inflammatory conditions.

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ABBREVIATIONS

5 – HT	5 hydroxytryptamine
AGES	Advanced glycation end product
AIA	Adjuvant induced arthritis
AIDS	Acquired immunodeficiency syndrome
AMPA	α-Amino-3-hydroxyl-5-methylsoxazole-4-propionic acid
COX	Cyclooxygenase
DA	Dopamine
DEX	Dexamethasone
DMARD	Disease-modifying anti-rheumatoid drugs
DNA	Deoxyribonucleic acid
DPN	Diabetic peripheral neuropathy
DPPH	2, 2-diphenyl-1-picrylhydrazyl hydrate
ED_{50}	Dose of drug which elicits 50% of the maximum response
EDTA	Ethylene diamine tetra acetic acid
EFIC	European federation of IASP chapter
EPM	Elevated plus maze
FST	Forced swimming test

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GABA	Gamma-aminobutyric acid
GAFCO	Ghana Agro Food company
IASP	International Association for the Study of pain
IL	Interleukin
LBP	Lipopolysaccharide-binding protein
LD_{50}	Dose of drug which kills 50% of the population
L-NAME	N^{G}-nitro-arginine methyl ester
MAOI	Monoamine oxidase inhibitor
MDA	Malondialdehyde
NADPH	Reduced nicotinamide-adenine dinucleotide phosphate
NE	Norepinephrine
NET	Norepinephrine transporter
NF	Nuclear factor
NIH	National Institute of Health
NMDA	*N*-methyl *D*-aspartate
NO	Nitric oxide
NOAEL	No-observed adverse- effect level
NSAID	Nonsteroidal anti-inflammatory drugs
OA	Osteoarthritis

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p.o	Per ox
PG	Prostaglandin
PHE	*Palisota hirsuta* extract
PHN	Post-herpetic neuralgia
PUFA	Poly unsaturated fatty acids
PWT	Paw withdrawal threshold
RA	Rheumatoid arthritis
SERT	Serotonin transporter
SNRI	Serotonin and noradrenaline reuptake inhibitors
SSRI	Selective serotonin reuptake inhibitor
STAT-3	Signal transducer and activation of transcription – 3
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substance
TCA	Trichloroacetic acid
TCA	Tricyclic antidepressant
TNF	Tissue necrosis factor
TST	Tail suspension test
VMAT-2	Vesicular monoamine transporter-2

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Chapter 1
INTRODUCTION

1.1 GENERAL INTRODUCTION

Medicinal uses of natural products can be traced back for at least 5000 years, while Western medicine has a relatively short history of a few hundred years (Goldman, 2001). At present, there are more than 85,000 plant species that have been acknowledged for medical use globally (Balunas and Kinghorn, 2005). The World Health Organization (WHO) estimates that almost 75% of the world's population has therapeutic experience with herbal remedies (Liu *et al.*, 2007). This is principally because of a belief that herbal remedies may have fewer side effects and can enhance the effects of conventional agents or be an alternative treatment (Desai *et al.*, 2003). WHO estimates conservatively that sixty to ninety (60-90) percent of the population of developing countries rely on medicinal plants either totally or partially in their healthcare needs (Ghana Herbal Pharmacopoeia, 1992). In Ghana, it is anticipated that there is one (1) traditional doctor to approximately four hundred (400) people as opposed to one allopathic or orthodox doctor to every twelve thousand (12,000) people (Ghana Herbal Pharmacopoeia, 1992).

Between 1983–1994, about 40% of the new drugs accepted in North America were derived from natural compounds (Simmonds, 2003), and approximately 70% of the new chemical entities reported between 1981 and mid-2006 resulted from studies on natural products (Newman *et al.*, 2007). A recent survey of the medical literature shows that a majority of clinical research on botanical drugs in the 21st century involves modernization and globalization, focusing on the efficacy and safety (Liu *et al.*, 2007).

Palisota hirsuta is a common plant used widely in West African folklore medicine for treating several diseases. There is the need for scientific research on the pharmacological activities of *Palisota hirsuta* since it has been reported that just the traditional use of a substance may not necessarily carry with it any scientific

assessment but may just infer community knowledge of existence and application of such substances (Zhang, 2000).

1.2 PALISOTA HIRSUTA.

***Botanical name**: Palisota hirsuta* (Thunb.) K. Schum. (Sny. *P. thyrsiflora* Benth.)

Family: Commelinaceae.

Local names: Twi – *Mpɛntemi, Akwabe, Sommenini*

Fante – *Sombanyin*

Ewe – *Sumbe, Klugbogbo*

Guan (Borada) – *Ofonugba*

Dan. (Krobo) – *Nakutso Kpo*

1.2.1 Description

Palisota hirsuta is named after the French botanist and traveller Joseph Palisot de Beauvois. This tropical West African plant is a member of the spiderwort family. This species is the most commonly used of the Commelinaceae (Akobundu *et al.*, 1987).

It is a robust herb in forest regrowths about 2-4 m high, reproducing from the seeds. The stem is rigid, more or less fleshy or woody at the base and covered with dense, softly hairy, brown hairs. The internodes reach up to 30 cm long with swollen nodes made larger by short ragged sheaths of the nodal whorl of leaves. This character is noted in the Liberian Bassa name meaning 'swollen knee' and Nigerian Igbo 'sheep's knee' and Yoruba 'knee-cap'. The leaves are arranged in rosettes, mostly at the terminal of the stem, and are obovate to oblanceolate, about 15-30 (~40) cm long and 4-11.5 cm broad. They are acute at the apex and narrow to the base terminating in flat, densely hairy petioles that are about 3 cm long. The

margins and the midribs have brown soft hairs and the under surface of the blade is dark green and hairy. The inflorescence is a loose and spreading panicle about 10-30 cm long that has many slender whitish-pink lateral branches 1-2 cm long. It bears many small flowers that are whitish, pinkish or purple in colour and they open from 4 pm until dusk and have three sepals, three petals, three stamens and two or three staminodes or rudimentary stamens. The fruits are glossy and black (Akobundu *et al.*, 1987).

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Figure 1.1 Leaves of Palisota hirsuta plant

1.2.2 Ecological and Geographical distribution

Palisota hirsuta is a weed of regrowths and farm lands in the forest zone. It is common along roadsides and in forest clearing in closed forest in southern Ghana and distributed from Senegal to Cameroon; also in Fernando Po and the Congo (Dokosi, 1998).

1.2.3 Traditional Uses

1.2.3.1 Non-Medicinal Uses

The plant is grown as an ornamental and is sometimes used as a component of hedges (Burkill, 1985). The young leaves are also eaten in Nigeria (Dokosi, 1998).

1.2.3.2 Medicinal Uses

The whole plant and its various parts are used extensively in West African traditional medicine and these uses may be broadly classified into:

Gastro-intestinal tract disorders: The dried powered leaves dissolve in water are used as an anti-dysentery enema (Burkill, 1985). The roots put in warm water are used as enema for constipation (Burkill, 1985). The whole plant is also used in Ghana for stomach pains (Burkill, 1985).

Nutritional disorders: The leaves cooked with groundnuts are taken by suckling mothers to cleanse their milk (Burkill, 1985). Roots are added to soup taken by pregnant women (Burkill, 1985).

Disorders of the respiratory system: In Ghana and Nigeria, the stem is chewed as a sedative for cough (Burkill, 1985; Dokosi, 1998) and also a draught of plant sap is taken for cough, bronchitis and chest pains (Burkill, 1985).

Disorders of the central nervous system: In Ghana, leaf decoction of this plant is also taken orally for general CNS disorders (Abbiw, 1990).

***Disorders of the genito-urinary system*:** Pieces of the stem, after exposure to the sun are made into draught for urethral discharge (Burkill, 1985). In Sierra Leone, the roots washed, cut up and boiled with lime are used as a cure for gonorrhea in three days (Ayensu, 1978; Burkill, 1985). A decoction of the dry leaves is given as a drink for gonorrhea in Ghana (Dokosi, 1998).

***Eye, Ear, Nose, and throat disorders*:** In Liberia, the plant is used for treating deafness (Burkill, 1985). Sap from the roasted leaves is also instilled in the ear for earache (Burkill, 1985).

***Infectious diseases and infestations*:** Stem or the sap in compressed form is applied as a dressing to furuncles and whitlow sores (Burkill, 1985). The plant sap is also applied to yaws and guinea worm sores (Burkill, 1985).

***Musculoskeletal, joint and inflammatory disorders*:** Heated leaves are applied over the lumbar region for kidney pains (Burkill, 1985). The roots are also used in the management of rheumatism. The stem sap is applied for fractures, adenitis and arthritic pains (Burkill, 1985). The Igbo of Obompa in Nigeria prepare an ointment of the plant for gunshot wounds and swellings (Burkill, 1985). The stem is beaten and the juice squeezed into a bullet wound and the fibre used as bandage and facilitates the extraction of the bullet (Dokosi, 1998). The leaves infusion is taken for piles and given to babies to heal the navel (Burkill, 1985; Dokosi, 1998). Stem shavings are also used in Ghana to promote healing of wounds particularly the umbilicus. The roots pounded up with peppercorns of *Piper guineense* are applied externally to sprains. Dried leaves are also smoked for toothache (Burkill, 1985). In Ghana, a leaf poultice is locally applied to protruding piles (Dokosi, 1998)

1.2.4 Some Identified Chemical Constituents of Palisota hirsuta

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 the plant *Palisota hirsuta*. The leaves contain 93.60% dry matter, 15.34% crude protein, 10.90%crude fibre, 2.10% ether extract, 10.80% ash extract, 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).

1.2.5 Previous Studies On the Activities of Palisota hirsuta

1.2.5.1 Antiviral Activity

The methanol extract of the leaves of the plant *Palisota hirsuta* has antiviral activity against herpes simplex, sindbis virus and poliovirus (Anani *et al.*, 2000; Hudson *et al.*, 2000).

1.2.5.2 Sexual Stimulant Effect

Benson and colleagues (Benson *et al.*, 2008) have recently reported that the total methanolic extract as well as the total flavonoids isolated from the leaves of *Palisota hirsuta* has sexual stimulant effects in rats.

1.2.5.3 Anti-inflammatory and Antipyretic Effects

Ethanolic extract of the roots of *Palisota hirsuta* exhibited both anti-inflammatory and antipyretic effects (Boakye-Gyasi *et al.*, 2008).

1.3 INFLAMMATION

Inflammation is basically a protective response the ultimate goal of which is to rid the organism of both the initial cause of cell injury (e.g., microbes, toxins) and the penalty of such injury (e.g., necrotic cells and tissues) (Weiss, 2008). A Roman writer of the first century, Celsus was the first to list the four cardinal signs of inflammation as *rubor, tumor, calor,* and *dolor* representing redness, swelling, heat, and pain respectively (Brenner *et al.*, 2006; Hunter, 1794). A fifth clinical sign, loss of function (*functio laesa*), was later added by Virchow (Hunter, 1794). In 1793, the Scottish surgeon John Hunter noted what is now considered an obvious fact: - that inflammation is not a disease but a nonspecific response that has a helpful effect on its host (Hunter, 1794). Without inflammation, infections would go unchecked, wounds would never heal, and injured organs might remain permanent festering sores. Inflammation and repair may be potentially harmful, however. Inflammatory reactions, for example, underlie life-threatening hypersensitivity reactions to insect bites, drugs, and toxins as well as some common chronic diseases, such as rheumatoid arthritis, atherosclerosis, and lung fibrosis. For this reason, anti-inflammation drugs are produced, which ideally would enhance the effects of inflammation yet control its harmful sequelae. Inflammatory process is divided into acute and chronic patterns.

1.3.1 Acute Inflammation

Acute inflammation is of relatively short duration, lasting for minutes, several hours, or a few days, and its main characteristics are the exudation of fluid and plasma proteins (edema) and the emigration of leukocytes, predominantly neutrophils (Hurley and Willoughby, 1973; Pulichino *et al.*, 2006). Acute inflammation is the immediate and early response to an injurious agent. Vascular phenomena are known to play a major role in acute inflammation since the two major defensive components against microbes--antibodies and leukocytes--are normally carried in the bloodstream (Schmid-Schönbein, 2006). Therefore, acute inflammation has three major components: (1) alterations in vascular caliber that

lead to an increase in blood flow, (2) structural changes in the microvasculature that permit the plasma proteins and leukocytes to leave the circulation, and (3) emigration of the leukocytes from the microcirculation and their accumulation in the focus of injury (Pulichino *et al.*, 2006; Schmid-Schönbein, 2006).

The vascular phenomena are characterized by increased blood flow to the injured area, resulting mainly from arteriolar dilation and opening of capillary beds. Increased vascular permeability results in the accumulation of protein-rich extravascular fluid, which forms the exudates. Plasma proteins leave the vessels, most commonly through widened inter-endothelial cell junctions of the venules or by direct endothelial cell injury. The leukocytes, initially predominantly neutrophils, adhere to the endothelium via adhesion molecules, transmigrate across the endothelium, and migrate to the site of injury under the influence of chemotactic agents. Phagocytosis of the offending agent follows, which may lead to the death of the microorganism. During chemotaxis and phagocytosis, activated leukocytes may release toxic metabolites and proteases extracellularly, potentially causing tissue damage.

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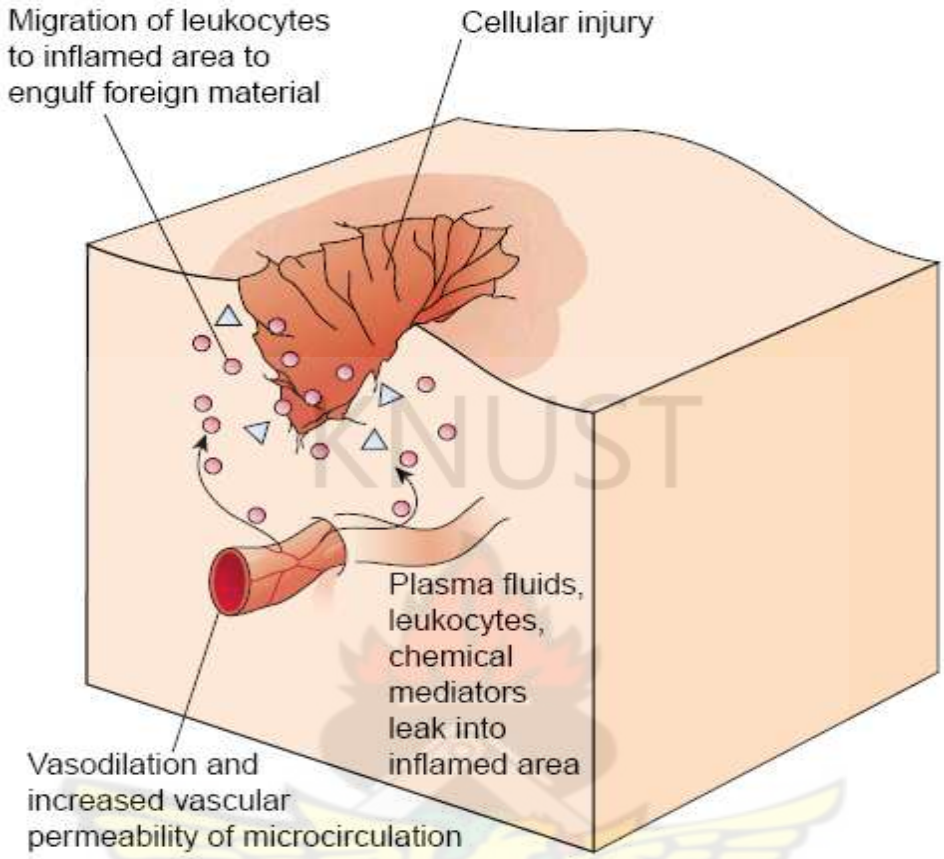

Figure 1.2 The acute inflammatory response (Adapted from Smeltzer and Bare, 2003).

1.3.2 Chronic Inflammation

Chronic inflammation is considered to be inflammation of prolonged duration (weeks or months) in which active inflammation, tissue destruction, and attempts at repair are proceeding simultaneously (Weiss, 2008). Although it may follow acute inflammation, chronic inflammation frequently begins insidiously, as a low-grade, often asymptomatic response. This latter type of chronic inflammation includes some of the most common and disabling human diseases, such as rheumatoid arthritis, atherosclerosis, tuberculosis, and chronic lung diseases (Agarwal and Brenner, 2006).

Rheumatoid arthritis (RA) is an autoimmune, chronic inflammatory disorder characterized by joint swelling, synovial inflammation, and joint destruction (Campo *et al.*, 2003; Feldmann *et al.*, 2005; Pakozdi et al., 2006). It usually causes pain, swelling, stiffness, and loss of function in the joints (Kroger *et al.*, 1999). In many patients, the disease is relentlessly progressive, resulting ultimately in joint destruction, leading to significant disability (Meyer *et al.*, 2006; Zhao *et al.*, 2006). RA develops as a result of interaction of many factors which include genetic (inherited) factors, environmental (viral or bacterial) factors and hormonal factors (Akaogi *et al.*, 2006; Weissmann, 2006).

Chronic inflammation occurs in the following settings as described by (Mullazehi *et al.*, 2007):

(1) Persistent infections by certain microorganisms such as tubercle bacilli, *Treponema pallidum*, and certain fungi;

(2) Prolonged exposure to potentially harmful agents, either exogenous like particulate silica or an endogenous agent. Atherosclerosis is thought to be a chronic inflammatory process of the arterial wall induced, at least in part, by endogenous toxic plasma lipid components.

(3) Autoimmunity: Under certain conditions, immune reactions are set up against the individual's own tissues, leading to autoimmune diseases. In these diseases, auto antigens evoke a self-perpetuating immune reaction that results in several common chronic inflammatory diseases, such as rheumatoid arthritis and lupus erythematosus.

Chronic inflammation is associated histologically with the presence of lymphocytes and macrophages, the proliferation of blood vessels, fibrosis, and tissue necrosis (Medzhitov, 2008). In terms of what is happening locally within the tissues, the changes can be divided into cellular and vascular events (Agarwal and Rangari, 2003; Issekutz *et al.*, 1989). The vascular events results 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 (Agarwal and Rangari, 2003).

1.3.3 Role of Reactive Oxygen Species in Inflammation

Oxygen derived free radicals and their products are known to play an important role in the pathogenesis of chronic inflammatory disorders (Adam and Kramer, 1995). These activated oxygen intermediates together with secondarily formed radicals, like the hydroxyl radicals (OH·) are able to destroy membrane lipids, proteins, DNA, hyaluronic acid, and cartilage (Valko *et al.*, 2007). The importance of oxygen free radicals and related activated oxygen intermediates in the pathogenesis of rheumatoid arthritis has been identified (Valko *et al.*, 2007).

The generation of oxygen metabolites is due to the rapid activation of an oxidase (NADPH oxidase), which oxidizes NADPH (reduced nicotinamide-adenine dinucleotide phosphate) and, in the process, reduces oxygen to superoxide anion (O^{2-}). Superoxide is then converted into H_2O_2, mostly by spontaneous dismutation:

$$2O_2 + e^- \longrightarrow 2O_2^- + NADP^- + H^+$$

Polymorphonuclear leukocytes and macrophages are stimulated, which results in the production of inflammatory mediators including large amount of superoxide and hydrogen peroxide (Ozkan *et al.*, 2007). Free radicals and, in particular, superoxide radicals cause cellular disruption due to peroxidation of membrane lipids (Ozkan *et al.*, 2007). The production of free radicals is essential for normal metabolism but they can be destructive if their activity is not controlled by intra/extra cellular defense mechanisms. A range of intracellular antioxidant systems limit the toxic potential of intermediates formed during the four electron reduction of oxygen to water (Vijayalakshmi *et al.*, 1997). Of particular importance is the enzyme superoxide dismutase which catalyzes the dismutation of O_2^- to H_2O_2. Once again, the cell is protected from the potential toxicity of H_2O_2 either by the haem enzyme catalase or by the seleno enzyme glutathione peroxidase.

The other non-enzymatic antioxidants include reduced glutathione, vitamin E, vitamin C and uric acid (Cuzzocrea, 2006).

1.3.4 Fever as a Systemic Effect of Inflammation

Fever is one of the most prominent systemic manifestations of acute inflammation, especially when an inflammation is associated with infection (Romanovsky *et al.*, 2005). Fever depends on humoral signals from the body. It is coordinated by the hypothalamus and involves the orchestration of a wide range of endocrine, autonomic, and behavioral responses (Saper and Breder, 1994). The components of this so-called acute-phase reaction include endocrine and metabolic processes. This involves the secretion of acute-phase proteins by the liver (including C-reactive protein) which increases the production of glucocorticoids, and activates a stress response. This decreases vasopressin secretion, thus reducing the volume of body fluid required to be warmed (Ohsugi, 2007). Autonomically, there is redirection in blood flow from cutaneous to deep vascular beds, thus minimizing heat loss through the skin and increasing pulse and blood pressure; and decreased

sweating. Behavioral responses include rigors (shivering), chills (search for warmth), anorexia, somnolence, and malaise.

The principal manifestation of fever is an elevation of body temperature, usually by 1 to 4°C. The elevation in temperature by even a few degrees may improve the efficiency of leukocyte killing and probably impairs the replication of many offending microorganisms.

Cytokines play a key role in signaling a fever (Conti *et al.*, 2004). IL-1, IL-6, and TNF-alpha are produced by leukocytes (and other cell types) in response to infectious agents or immunologic and toxic reactions and are released into the circulation. IL-1 acts directly and also by inducing IL-6, which has essentially similar effects in producing the acute-phase reactions. Among the cytokines, IL-1, IL-6, TNF-alpha, and the interferons can cause fever, thus functioning as primary endogenous pyrogens (Licinio and Wong, 1996). Peripheral cytokines signal the brain through four mechanisms to cause fever (Conti *et al.*, 2004): (1) They can enter the brain through regions lacking a blood-brain barrier (specialized areas along the cerebral ventricular surface); (2) they can cross the blood-brain barrier by specific transport mechanisms; (3) they can transmit a signal to the brain via the vagus nerve; and (4) they can activate brain vasculature stimulating release of mediators such as prostaglandins (PGE), NO, or cytokines (IL-1beta), which act on brain parenchymal cells. In contrast, during more significant sepsis, circulating cytokine levels are high, and the vascular route to brain activation becomes more prominent. Once generated, the signal is transmitted from the anterior through the posterior hypothalamus to the vasomotor center to induce the responses.

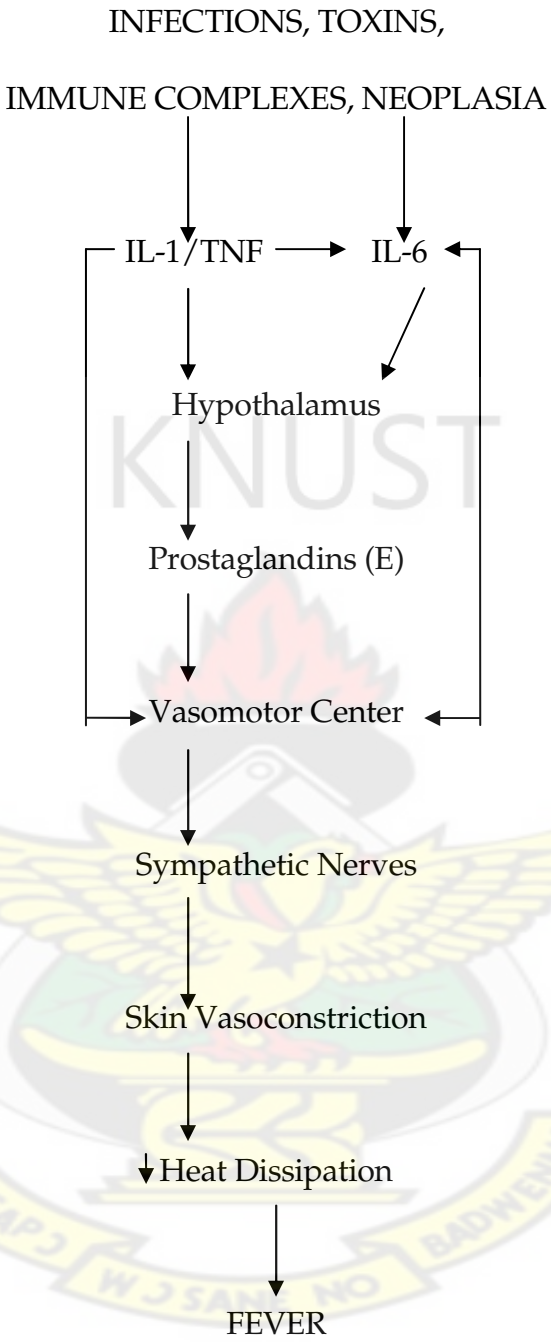

Figure 1.3 The Mechanism of fever

1.3.5 Experimental Models of Acute Inflammation

Acute inflammatory responses may be induced by injection of inflammatory agents such as heat killed bacteria example *Escherichia coli*, zymosan (Araico *et al.*, 2007; Lucas *et al.*, 2003), vasoactive agents (e.g. platelet activating factor and histamine (Vasudevan *et al.*, 2007), arachidonic acid (Kang *et al.*, 2008), carrageenan (Duwiejua *et al.*, 1994; Jeon *et al.*, 2008; Winter *et al.*, 1962), yeast (Ushiyama *et al.*, 2008; Zakaria *et al.*, 2007), dextran (Lima *et al.*, 2007; Melgar *et al.*, 2007), and latex (Higgs, 1989; Shivkar and Kumar, 2003) into various parts of the body. The effect can then be judged by such responses as the increase in foot volume produced by edema (e.g. in the rat's paw), detection of plasma markers in skin, the local rise in skin temperature, measurement of inflammatory mediators in plasma exudates, hyperemia, polymorphonuclear leukocyte accumulation, lymphocyte accumulation, monocyte infiltration, quantization of hemorrhage, platelet deposition and thrombosis using diverse techniques (Issekutz and Issekutz, 1989). Of these models, the carrageenan-induced acute edema in chicks (Roach and Sufka, 2003) was employed in this study.

Carrageenan is a polysaccharide derived from the Irish Sea moss. It is the phlogistic agent of choice for testing anti-inflammatory drugs as it is known to be antigenic and is devoid of apparent systemic effect (Di Rosa and Willoughby, 1971; Kaur *et al.*, 2004). It causes the release of more than one inflammatory mediator which is a useful tool in testing for anti-inflammatory effect. Carrageenan has been used because of its ability to induce an intense and reproducible inflammatory action and its sensitivity to inhibition by various anti-inflammatory drugs (Kaur *et al.*, 2004; Winter *et al.*, 1962).

1.3.6 Experimental models of Chronic Inflammation

The most frequently studied models of chronic inflammation have been models of arthritis, particularly the polyarthritis induced in the rat with Mycobacterium (Freund's adjuvant arthritis) (Brand, 2005; Escandell *et al.*, 2007; Hughes *et al.*, 1989; Wang *et al.*, 2008). Other models of arthritis have been developed over the last decade including the polyarthritis induced by type II collagen in rats and mice (Bajtner *et al.*, 2005; Griffiths *et al.*, 2007; Subramanian *et al.*, 2005; Williams, 2007), a condition resembling gout may be produced by the injection of urate crystals into the synovial fluids of joints (Kannan *et al.*, 2005; Scott *et al.*, 2006). Chronic inflammatory reactions can be produced by implantation of cotton wool pellets subcutaneously; these may be subsequently removed and weighed to determine the extent of granulation (Khanna and Sharma, 2001). The injection of turpentine oil (into pleural cavity or subcutaneous pouch) provides a long-standing inflammatory reaction (Singh *et al.*, 2007). Of these models, the adjuvant induced arthritis was employed in this study.

Adjuvant induced arthritis (AIA) in rats, a chronic inflammatory disease characterized by infiltration of the synovial membrane and associated with destruction of the joints, resembles rheumatoid arthritis in humans (Behar and Porcelli, 1995; Kumar *et al.*, 2002). AIA is induced by heat-killed cells of *Mycobacterium tuberculosis* and it mimics the immunological and biochemical features of Rheumatoid arthritis (RA) wherein self antigens are recognized as foreign bodies (Aota *et al.*, 1996; Ramprasath *et al.*, 2006). AIA in rats serves as an animal model for rheumatoid arthritis (RA). Its popularity stems from: its reproducibility; its relatively short time course 2-4weeks and its adaptability to drug screening paradigms (Ishikawa *et al.*, 2005). Although the clinical course of AIA is somewhat different, it shares important features with RA: - Poly synovitis, characteristic histological findings and ultimately erosive bone destruction (Aota *et al.*, 1996). In addition, the clinical response to anti-inflammatory agents is similar in RA and established AIA (Behar and Porcelli, 1995). The only real difference

between rheumatoid and adjuvant disease are the absence of rheumatoid factor and the lack of genital or skin lesions in RA which is a feature common to adjuvant disease (Bersani-Amado *et al.*, 1990; Dick, 1972; Pearson *et al.*, 1963). Although the experimental arthritis in animals has some but not all of the clinical and biochemical features of patients with polyarthritic diseases (Arnett and Viney, 2007), adjuvant-induced arthritis in rats has been widely used as a model for therapeutic and pathogenetic studies of chronic forms of arthritis (Pearson, 1963).

1.3.7 Management of Inflammation

In healthy states, inflammation is self-limiting; with many cell types and tissues involved in initiation and termination of the acute phase (Schwab and Serhan, 2006). However, inflammation often results in tissue injury due to a direct destructive action or the activation of a reparative process that alters tissue functions (Schmid-Schönbein, 2006). Among drugs used for the treatment of rheumatic diseases, include the non-steroidal anti-inflammatory drugs (NSAIDs), the disease modifying anti-rheumatoid drugs (DMARDs), and corticosteroids. The NSAIDs are a structurally diverse group of drugs that provide symptomatic relief in inflammatory joint and active osteoarthritic disorders— reducing swelling, joint pain and stiffness and improving joint mobility—but have little effect on the underlying tissue degenerative processes that lead to cartilage loss and bone damage (Day *et al.*, 1987). DMARDs, on the other hand, have little or no acute anti-inflammatory or analgesic properties, but act, usually over a period of weeks or months, to slow down or stop the progression of RA (Fries *et al.*, 1996). The mechanisms of action of DMARDs are complex and vary considerably, in many cases remaining unclear, but they all cause slowing of the progression of joint destruction and, in some patients, remission. Corticosteroids also have multiple sites of action in the treatment of rheumatic diseases. Although they inhibit disease progression (Kirwan, 1995), they are generally restricted to use in severe cases because of long-term side-effects. Side-effects are a disadvantage of the other groups of drugs as well. NSAID use is frequently associated with gastrointestinal

toxicity and DMARDs can cause skin, liver, kidney and gastrointestinal side-effects (Day *et al.*, 1987). For this reason, in mild cases of RA and particularly in OA (which is associated with less intense inflammatory lesions), simple analgesics, such as paracetamol, are frequently prescribed, predominantly in Anglo-Saxon countries (Hochberg *et al.*, 1995). Although the aetiology of OA is not entirely understood, it is associated predominantly with loss of joint cartilage. Repeated attempts have been made to develop drugs that either protect cartilage or stimulate cartilage repair and have few adverse effects. So far this approach has not met with success. In treating RA, a variety of approaches are used. Drugs may be used in different combinations and at different times during the course of the disease and are chosen according to the patient's individual situation (Chen *et al.*, 2005; Tlustochowicz, 2006). No matter what treatment approach is chosen, the goals are however the same; relieve pain, reduce inflammation, slow down or stop joint damage and improve a sense of well-being and ability to function (Atzeni and Sarzi-Puttini, 2007; Smolen *et al.*, 2005b). Current treatment strategies include pain-relieving drugs and medications that slow joint damage, a balance between rest and exercise allow most people with the disease to lead active and productive lives (Kirwan *et al.*, 2007; Lee *et al.*, 2006; Wells *et al.*, 2008). The person's general condition, the current and predicted severity of the illness, the length of time he or she will take the drug, and the drug's effectiveness and potential side effects are important considerations in prescribing drugs for rheumatoid arthritis (Suresh, 2007).

Scientists are also finding the genetic basis of rheumatoid arthritis by studying rats with a condition that resembles rheumatoid arthritis in humans (Kumar *et al.*, 2002). Researchers are searching for new drugs or combinations of drugs that can reduce inflammation and slow or stop the progression of rheumatoid arthritis with few side effects. New therapeutic strategies for chronic forms of arthritis have to aim at both, suppression of inflammation and bone protection. To achieve these goals it is important to understand the different stages of disease progression and

to identify relevant targets—that is, most relevant cell type(s) or cytokine(s) or both (Goldblatt and Isenberg, 2005; Moreland, 2005).

The new biologic response modifiers infliximab and etanercept are proving to be extremely effective for some people whiles others fail to respond to them (Efthimiou and Markenson, 2005; Mahajan *et al.*, 2006; Moreland, 2004; Ruderman and Pope, 2006). Studies show that these new treatments are more effective at slowing joint damage when used in combination with methotrexate, a conventional DMARD, than methotrexate alone (Fleischmann *et al.*, 2005). Combination treatment with etanercept and methotrexate or infliximab and methotrexate has been found even more effective than either of the new treatments alone (Hisadome *et al.*, 2004; Nordstrom *et al.*, 2006). Current therapies are often effective at relieving symptoms, although this benefit is attended by a significant risk of toxicity (Ruderman, 2005). It is therefore necessary to develop new agents that are effective for preventing joint destruction, as well as synovial inflammation, in RA which will be less toxic.

1.4 PAIN

Pain is a complex event that is uniquely experienced by each individual. The International Association for the Study of Pain (IASP) defines pain 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 an unpleasant subjective incident that is the net effect of a complex communication of the ascending and descending nervous systems involving biochemical, physiological, psychological, and neocortical processes (Clancy and McVicar, 1992; Seidel *et al.*, 2008). Pain can affect all areas of a person's life including sleep, thought, emotion, and activities of daily living. Since there are no reliable objective markers for pain, the patients are the only ones to describe the intensity and quality of their pain (Clancy and McVicar, 1992).

Pain is the most common symptom prompting patients to seek medical attention and is reported by more than 80% of individuals who visit their primary care provider (Schim and Stang, 2004b). Despite the frequency of pain symptoms, individuals often do not obtain satisfactory relief of pain. This has led to recent initiatives in health care to make pain the fifth vital sign, thus making pain assessment equally important as obtaining a patient's temperature, pulse, blood pressure, and respiratory rate (Chapman, 2005).

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1.4.1 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.*, 2001). 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 postherpetic neuralgia are however examples of pain described by etiology.

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.

1.4.1.1 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-Salam and El-Batran, 2005; Calvino *et al.*, 1992; Schim and Stang, 2004b).

1.4.1.2 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. It may be accompanied by severe psychological and social disturbance. It usually lasts longer than 6 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).

1.4.2 TYPES OF PAIN

Several distinct types of pain have been described based on their pathophysiology: nociceptive, inflammatory, neuropathic, and functional.

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. Nociceptors 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).

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 (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. Several conditions considered to have this abnormal sensitivity or hyper responsiveness include fibromyalgia and irritable bowel syndrome (Nielsen and Henriksson, 2007).

1.4.3 Mechanisms of Pain

1.4.3.1 Pain Transmission

The mechanisms of nociceptive pain are well-defined and provide a foundation for the understanding of other types of pain (Chen *et al.*, 2007; Paszcuk *et al.*, 2008; (Schim and Stang, 2004a). Following nociceptor stimulation, tissue injury causes the release of substances including bradykinin, serotonin, potassium, histamine, prostaglandins, and substance P that may further sensitize and/or activate nociceptors. Nociceptor activation produces action potentials that are transmitted by the peripheral nervous system along myelinated A-• fibers and unmyelinated C fibers to the spinal cord. The A-• fibers are responsible for first, fast, sharp pain and release excitatory amino acids that activate •-amino-3-hydroxy- 5-methylisoxazole-4-propionic acid (AMPA) receptors in the dorsal horn. The C fibers produce secondary pain which is described as dull, aching, burning, and diffuse. These nerve fibers synapse in the dorsal horn of the spinal cord, where several neurotransmitters are released including glutamate, substance P, and calcitonin gene-related peptide. Transmission of pain signals continues along the spinal cord to the thalamus, which serves as the pain relay center, and eventually to the cortical regions of the brain where pain is perceived (D'Mello and Dickenson, 2008).

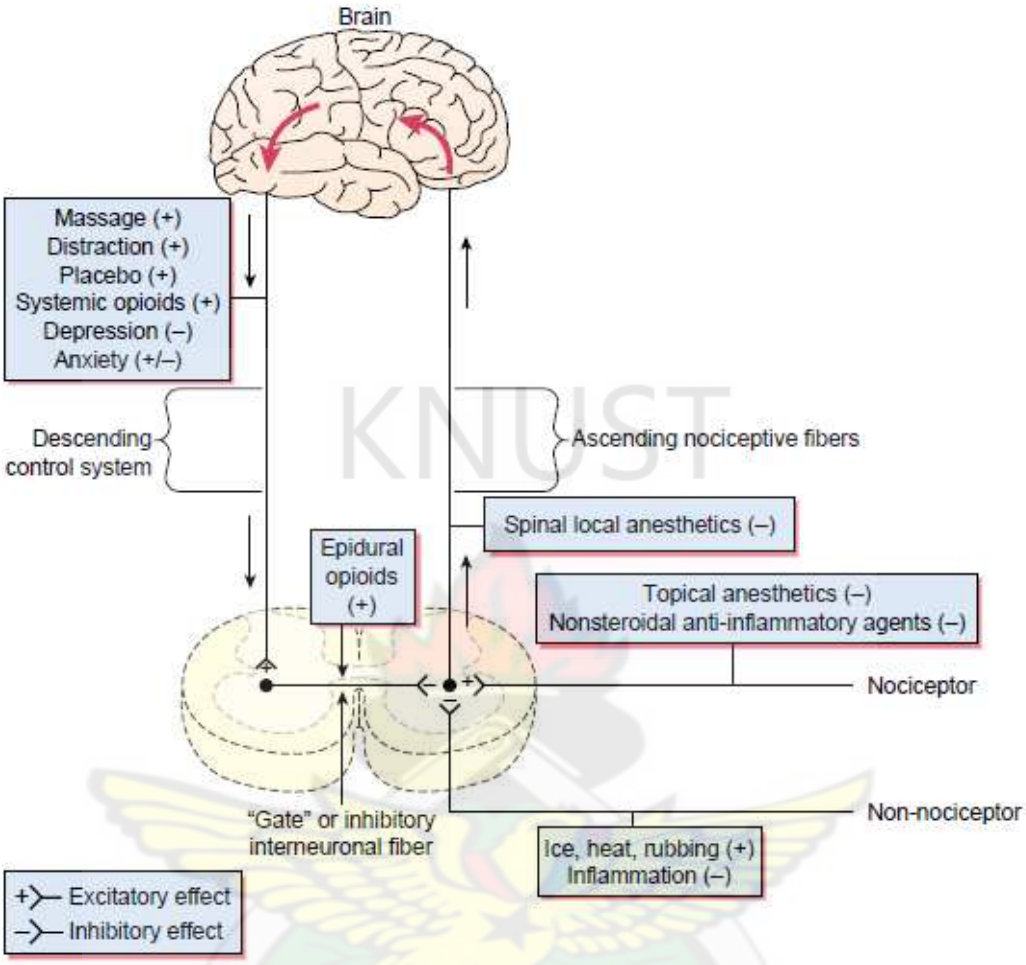

Figure 1.4 A schematic representation of the gate control system and aspects of the nociceptive system. (Adapted from Smeltzer and Bare, 2003)

1.4.4 MODELS OF PAIN

Analgesia is has often been described as a selective reduction in pain without altering sensitivity in other sensory modalities. This makes pain and analgesia essentially subjective experiences, and their existence in humans is typically assessed using verbal reports. However, verbal reports are obviously not suitable for measuring pain and analgesia in animals, and this presents a challenge to preclinical research (Yeomans *et al.*, 1996), since the usefulness of animal models of pain is not in doubt (Morgan *et al.*, 2006; Whiteside *et al.*, 2008). It has always been difficult to tell when an animal is in pain, and how does one tell if a candidate analgesic is effective in reducing that pain (Blackburn-Munro *et al.*, 2004)? Animal models of pain can be generally classified as either somatic pain models or models of visceral pain (such as gastrointestinal pain, urinary bladder dysfunction). Somatic pain models are the most widely used and include acute nociceptive models (hot-plate, tail-flick) and pathological pain models. Pathological pain models including persistent central pain induced by formalin or capsaicin, chronic inflammatory pain by carrageenan, turpentine, UV-irradiation or Freund's complete adjuvant or chronic neuropathic pain by damage or disturbance to a peripheral nerve. Pain models may also represent particular diseases that feature pain as a prominent symptom (e.g. diabetic neuropathy).

1.4.5 Models of somatic pain

1.4.5.1 Acute Nociceptive Models

Models of acute pain measure the behavioral responses of naive animals to noxious stimuli. Acute pain measurements in animals can be estimated only by examining their reactions but at the same time the existence of a reaction does not necessarily mean that there is concomitant sensation (Wheeler-Aceto *et al.*, 1990). A noxious stimulus can be defined by its physical nature, its site of application and what has previously happened to the tissues at this site (Clarke and Harris, 2004). For nearly a century, preclinical researchers interested in issues such as the genetics, neurobiology, and pharmacology of pain and analgesia have focused

largely on withdrawal responses or other nocifensive behaviors that increase in rate, frequency, or intensity following the presentation of an acute noxious stimulus (Bennett, 2001; Le Bars *et al.*, 2001). To be adequate, these stimuli have to be quantifiable, reproducible, and non invasive (Beecher, 1957; Martini *et al.,* 2000). Noxious heat is the most common stimulus used in these tests. Stimulus modality has also been extensively manipulated in assays of nociception. In addition to thermal stimuli, other commonly used modalities include electrical, mechanical, and chemical noxious stimuli (Bennett, 2001; Le Bars *et al.*, 2001). Electrical stimuli directly activate primary afferents, including nociceptors, whereas mechanical noxious stimuli physically deform tissue (e.g., by pinching or applying pressure via probes). As with thermal tests, the dependent measure in tests using electrical or mechanical stimuli is usually the latency to a withdrawal response or the threshold stimulus intensity for evoking a withdrawal response. Analgesic drugs, such as opiates, can modify the behavioral responses produced. Anaesthetic drugs are also effective in these models. However, many common 'painkillers', such as non-steroidal anti-inflammatory drugs (NSAIDs), are not effective in models of acute pain. These drugs interact with mechanisms that develop during pathological conditions. Therefore, relying on models of acute nociception alone might prevent the discovery of potentially important new classes of pain-relieving drugs.

1.4.5.2 Pathological Pain Models

Ideally, a drug that does not affect the nocifensive properties of the nervous system but that can reset the nociceptive thresholds to normal levels in pathological conditions provides the greatest clinical benefit. Persistent stimulation of nociceptive primary afferents produces sensitization in the central nervous system owing to the process of 'windup' (Bennett, 2001; Le Bars *et al.*, 2001). This fundamental spinal process involves a dominant N-methyl-D-aspartate (NMDA) receptor component and underlies the development of hyperalgesia. Commonly used chemical noxious stimuli include intraperitoneal injections of dilute acetic

acid or intraplantar injections of formalin. When chemical noxious stimuli are used, withdrawal from the stimulus is rarely possible, and the primary dependent variables are typically behaviors such as writhing or paw-flinching. The incidence of these behaviors can then be counted during a set observation period. It should also be noted that chemical noxious stimuli often produce initial features of acute pain followed by subsequent effects involving inflammatory processes. Chemical stimuli differ from those mentioned above by the progressive onset of their effectiveness, their duration of action and the fact that they are of an inescapable nature (Elmer *et al.*, 1998; Wood, 2004). Experimental models employing chemical stimuli are undoubtedly the most similar to acute clinical pain (Whiteside *et al.*, 2008). Mechanistic studies of the physiological and pharmacological mechanisms of chronic pain have depended largely on the use of the capsaicin and the formalin models of persistent pain. In particular, the formalin model has the advantage of easy assessment of spontaneous nocifensive behaviors (Tjolsen *et al.,* 1992).

More sophisticated pain models, which produce complex time-dependent pathomechanisms, include models of inflammatory pain. Pain that requires clinical intervention is often associated with inflammation, and one active area of research has been the development of procedures that model inflammatory pain (Luo, 2004). Inflammation can result from tissue damage (e.g., a surgical incision or burn), exposure to chemical stimuli (e.g., the chemical constituents of a bee sting), or autoimmune processes (e.g., some forms of arthritis). In each case, stimulation of the immune system results in the release of inflammatory mediators, such as bradykinin and prostaglandins. These mediators in turn produce numerous effects, including sustained activation and sensitization of both primary nociceptors and higher order neurons involved in the transmission of nociceptive input (Marchand *et al.*, 2005). This hypersensitivity of nociceptive pathways contributes to the behavioral phenomena of allodynia (pain-like responses to normally innocuous stimuli) and/or hyperalgesia (enhanced pain-like responses to normally noxious stimuli), and the goal of drug treatment is to normalize pain sensitivity.

For example, one model of acute inflammatory pain involves the subcutaneous administration of formalin into the hind paw of rats (Dubuisson and Dennis, 1977) which elicits an acute nociceptive response as well as an inflammatory second phase associated with plasma extravasation and is thought to involve the release of inflammatory mediators. Importantly, flinching during the second phase of the formalin response is decreased not only by morphine-like opioids but also by steroid and NSAID analgesics that have established clinical efficacy (Hunskaar and Hole, 1987; Taylor and Basbaum, 2000).

Carrageenan (a family of sulfated polysaccharides extracted from red seaweeds) is another substance commonly used in models of inflammatory pain (Vinegar *et al.*, 1976). Injection of carrageenan into the paw does not produce the robust flinching response observed with formalin; however, it elicits substantial paw swelling and both thermal and mechanical allodynia and hyperalgesia in the affected paw for up to 7 h. Injection of carrageenan or other compounds (e.g., iodoacetate, Freund's complete adjuvant) into the knee or ankle joint produces even more protracted allodynia/hyperalgesia, lasting for several days to weeks, and these joint injections of inflammatory compounds are used to model more chronic inflammatory conditions such as osteoarthritis. As with the second-phase formalin response, inflammation-associated allodynia/hyperalgesia can be attenuated, and nociceptive sensitivity can be normalized by morphine-like opioids as well as by steroid and NSAID analgesics (da Silva Filho *et al.*, 2004; Jett *et al.*, 1999; Whiteside *et al.*, 2005). As with assays of acute nociception, drug effects in these models of inflammatory pain may be influenced by the specific characteristics of the procedure. For example, NSAIDs are most effective in procedures that produce large amounts of edema and in which thermal or mechanical sensitivity are measured using low to medium intensity stimuli. Moreover, drugs may be more effective in models of acute inflammation than in models of chronic inflammation, because chronic inflammation may recruit sustained activity of both C-fibers and A • fibers (Smolen *et al.*, 2005a).

1.4.6 MANAGEMENT OF PAIN

Pain is among the most common complaints for which people seek medical care, yet pain is also among the most under treated patient complaints (Heit, 2003). Despite extensive progress in the scientific understanding of pain over the last decade, patients appear to continue to suffer needlessly and are paying a heavy price for the reduced quality of life, and economic costs (Glajchen, 2001). On October 11, 2004, during the Global Day Against Pain, the World Health Organization (WHO), the International Association for the Study of Pain (IASP) and the European Federation of IASP Chapters (EFIC) issued a joint declaration supporting that ''The Treatment of Pain Should be a Human Right' (Lynch *et al.*, 2008).

Good pain and symptom management require the intervention of all disciplines in a holistic approach. Unrelieved pain affects the patient's physical, psychological, social, and spiritual well-being (Dubois *et al.*, 2003).

Pain management strategies include both pharmacological and non pharmacological approaches. Most current protocols for the management of acute pain pharmacologically rely on using nonsteroidal anti-inflammatory drugs (NSAIDs) and opioid analgesics. Chronic pain is treated with combinations of NSAIDs and opioid analgesics as well as medications to reduce swelling and anxiety. Although effective in relieving pain and inflammation, traditional nonsteroidal anti-inflammatory drugs (NSAIDs), one of the most widely used medications, are associated with a significant increase in the risk for gastrointestinal adverse events, because of the non-selective inhibition on cyclooxygenase (COX)-1 and -2 (COX-2) (Wolfe *et al.*, 1999; Wong *et al.*, 2005). And the selective inhibitors of COX- 2, proved to induce fewer gastrointestinal toxicities compared to traditional NSAIDs, have been reported to raise a high risk for cardiovascular events that are associated with chronic use and higher doses (Clemett and Goa, 2000; Solomon *et al.*, 2005). Opioid drugs avoid the peripheral

toxicity of the NSAIDs, but their long-term use is also limited by side effects, such as nausea, constipation, confusion, respiratory depression, sedation, tolerance and possibly dependence are also a high priority when prescribing for chronic therapy (Dray and Urban, 1996).

Tricyclic antidepressants (TCA), especially amitriptyline, are useful in the treatment of neuropathic pain and also beneficial for patients with chronic musculoskeletal pain problems, such as fibromyalgia and low back pain (Maizels and McCarberg, 2005). They interact with the pain pathways in several ways (Arnold *et al.*, 2005). The analgesic effect of antidepressants is thought to be centrally mediated. The classical mechanism is the reinforcement of monoamine-containing bulbospinal pathways by either acting on the terminals of these fibres at the spinal level or reinforcing these pathways at the brain stem level (Ardid *et al.*, 2001; Ardid *et al.*, 1991; Ardid *et al.*, 1995). A supraspinal effect in the brain is also suspected to be involved (Anjaneyulu and Chopra, 2004; Anjaneyulu and Chopra, 2006; Marchand *et al.*, 2003b). Finally, some studies have demonstrated a peripheral action of antidepressants, which might be of primary relevance to localized delivery methods (e.g. topical application), but its involvement in the effect of systemically administered antidepressants has not been demonstrated (Sawynok, 2003).

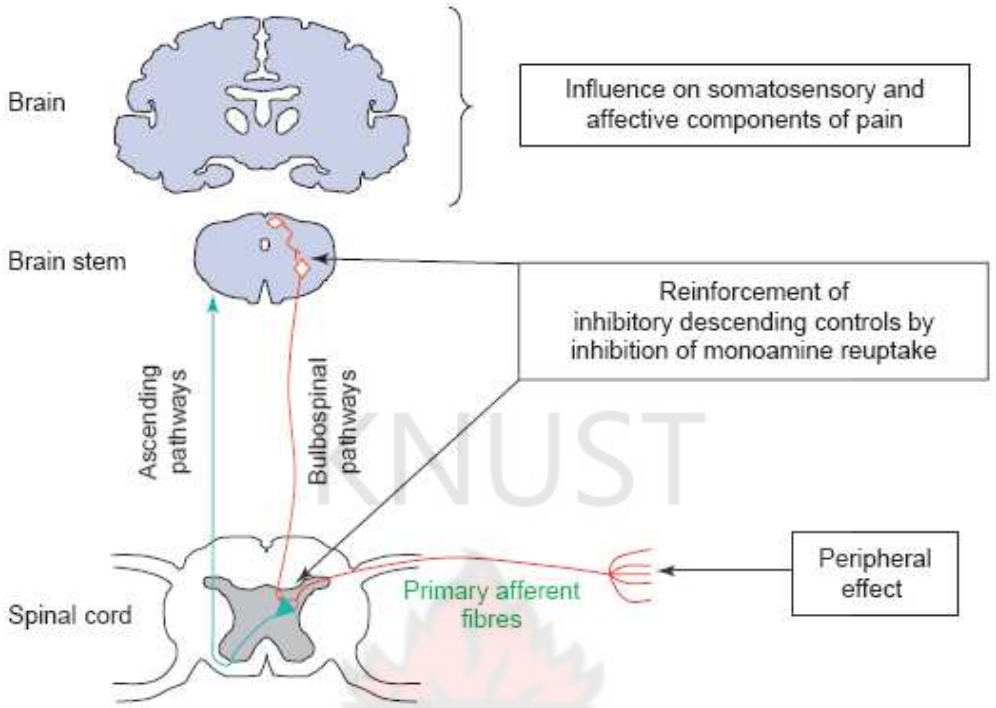

Figure 1.5 Postulated sites of the analgesic action of antidepressants (Adapted from Mico´ *et al.,* 2006)

The challenges of pain management encompass more than just postoperative pain and include other types of acute pain including trauma and burns as well as chronic pain and pain in patients with cancer. Our inability to measure pain effectively is a major problem to progress in pain research and progression in clinical interventions for pain. The principal targets of effective pain control are: to ameliorate nociception, to reduce threshold of pain sensation and to improve quality of life (Chapman, 2005). Chronic pain emerges as a major public health problem as it has a lot of effects on work performance and quality of life (Manca *et al.*, 2008; Smith *et al.*, 2001). There are some unmet needs in pain management more specifically chronic pain. The effective management of pain is not accomplished for many reasons, including fear of adverse events that might be associated with analgesic therapy, legal and regulatory barriers, inability of physicians and other healthcare professionals to accurately assess pain and societal attitudes towards some classes of pain medications (Chapman, 2005; Schim and Stang, 2004b). Consequently, one long-standing focus of drug discovery has been the search for novel analgesics.

1.5 ETHOPHARMACOLOGY

Ethopharmacology can be defined as the study of behavioral and other effects of drugs through the use of ethological concepts. The study of drug effects on natural action patterns, with respect to natural settings and to behavior as a whole represents the basic concepts of ethopharmacology. It applies the methods and concepts of ethology to the analysis of drug-induced changes in behavior, putting primary emphasis on the functional characterization of the behavior itself and, attempting to fit this with molecular notions of drug-action (Krsiak, 1991).

Drug-development programs, particularly those concerned with the development of psychotropic drugs, rely extensively on the use of animal tests. Accordingly, choosing the most appropriate clinical indication for a new drug requires at least some faith in the relevance of animal models for man. At the behavioral level there are many features of human conduct which can be studied in animals and, which

have relevance for the mentally ill population, including depressed and psychotic individuals.

It is commonly believed that stress, anxiety and depression are interrelated phenomena. Stress is typically implicated either in the etiology of depressive and anxiety disorders or as consequence of it (Lloyd, 1980; Sherrill *et al.*, 1997,). In general, anxiety responses of laboratory animals can be measured using behavioral or/and physiological responses to stressful or novel events as indices of emotion. In an evolutionary perspective, it is assumed that animals were phylogenetically shaped by natural selection to display fear or anxiety-mediated responses to specific stimuli, which can thus be utilized for the investigation of anxiolytic agents. For example, naturally aversive situations for mice and rats include being in an unfamiliar open space, exposure to heights or to bright light, and various related factors. All of these features are incorporated in and form the basis of, the exploratory tests for anxiety responses. The traditional animal models of anxiety, such as the open-field or the elevated plus maze are based upon exploration of novel environments which may elicit defensive reactions in that new environments could be potentially dangerous to the individual survivorship (Pellow *et al.*, 1985; Erdogan *et al.*, 2004; Kasture *et al.*, 2002). Crawley and Goodwin, (1980) developed a model based on the natural tendency of rodents to avoid brightly-lit areas of compartmentalized test chamber, i.e. the light/dark exploration test (Hascoet *et al.* 2001). These animal tests of anxiety are based on the `two-factor theory' of the dynamic relationship between exploratory behavior and anxiety typically displaced in unfamiliar environments (Russell, 1973). The fact that they are based on spontaneous behavior has been suggested to have a high degree of ecological validity in that they rely upon unconditional reactions to potentially threatening novel situations. Furthermore, these models of anxiety have been demonstrated to have high sensitivity to anti-anxiety agents (Treit, 1985).

A multiplicity of procedures is devoted to the prediction and evaluation of the antidepressant activity of drugs and to emulate aspects of depression. The `stress

hypothesis' has led to the development of several putative animal models of depression, which are characterized by a common feature: the observed behaviors are triggered by uncontrollable aversive events (i.e. stress). The restraint-induced decrease of locomotor activity, the behavioral despair, the learned helplessness and the chronic mild stress are the most used animal models of depression. The behavioral despair paradigm, i.e. the forced swim test, is currently one of the most frequently used behavioral tests for investigating antidepressant potential (Bannon *et al.*, 1998).

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1.6 AIMS AND OBJECTIVES OF THE STUDY

1.6.1 Aims

Many diseases are now known to have an inflammatory element as part of the pathophysiology (Serhan, 2004). Most of the drugs currently used as anti-inflammatory and analgesic agents include the non-steroidal anti-inflammatory drugs (NSAIDs), opioid analgesics, steroids, disease modifying anti-rheumatic drugs and some centrally-acting drugs including antidepressants and anticonvulsants; which have all proven very effective. Due to the numerous and life-threatening side effects associated with the use of most of these agents (Loewen, 2002, Mirshafiey *et al.*, 2005, Wolfe *et al.*, 1999, Wong *et al.*, 2005), there is still the search for more effective anti-inflammatory and analgesic agents with minimal or no side effects at therapeutic doses and possible therapeutic advantages over the existing ones.

Plants have played a remarkable role in health care since ancient times. Traditional plant-based medicines still exert great deal of importance to people living in developing countries and also serve as source to discovery of new drug candidates for a variety of diseases that threaten human health. Available biomedical evidence suggests that approximately 80% of Africans rely on traditional healthcare practitioners and medicinal plants for their daily healthcare needs (Johnson *et al.*, 2007). African indigenous herbal medicines are widely used throughout the

African continent, despite an apparent lack of scientific evidence for their quality, safety and efficacy (Johnson *et al.*, 2007). Recent pharmaco-chemical exploration of Ghanaian and African medicinal plants in our laboratories and elsewhere has shown that many of these medicinal plants possess therapeutic attributes. Duwiejua and Zeitlin, (1993), reported that several plant–derived products have the capacity of interfering with the predominant pathophysiological processes involved in inflammation and pain. One such therapeutically useful medicinal plant in Ghana and other parts of Africa is *Palisota hirsuta* K. Shum. (Family: Commelinaceae).

There is a lot of information based on community knowledge of existence and application on the various uses of the leaves of *Palisota hirsuta* in analgesia and inflammation among other conditions (Abbiw, 1990; Burkill, 1985; Dokosi, 1998).

This current study therefore aims to provide some pharmacological evidence and basis for the traditional use of the leaves of *Palisota hirsuta* in the management of pain and inflammation and its probable mechanisms of actions together with its central effects. Moreover, this plant has not been subjected to any systematic pharmacological screening so far.

1.6.2 OBJECTIVES

The objective of the present study is to carry out pharmacological evaluation of the leaf extract of *Palisota hirsuta* using animal models.

Specific objectives included evaluating the extract for:

1. Anti-inflammatory activities, both acute and chronic

2. Antioxidant properties *in vitro*

3. Antipyretic activity

4. Analgesic activities

5. Ethopharmacological effects of the extract using anxiety related models and depression related models

6. Acute and sub acute toxicity studies in rats.

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Chapter 2

PLANT COLLECTION, EXTRACTION AND PHYTOCHEMICAL ANALYSIS

2.1 PLANT COLLECTION AND EXTRACTION

2.1.1 Plant collection

The leaves of the plant *Palisota hirsuta* were collected from the Botanic Gardens, KNUST, Kumasi, between January and February, 2007. After the leaves were authenticated by Mr. Amissah, the curator of the garden, a voucher specimen was kept in the Faculty of Pharmacy Herbarium (No. FP 10081). They were then air-dried indoors for a week and pulverized with a hammer-mill.

2.1.2 Preparation of total crude leaf extract

The powder was extracted by cold maceration with 70% (v/v) ethanol over a period of 72 hours. The resulting extract was concentrated at low temperature 60˚C and under low pressure to a syrupy mass in a rotary evaporator. The syrupy mass was then dried to a dark brown semi-solid mass using water bath and kept in a dessicator till it was ready to be used. The final yield was 10.5% (w/w). This is subsequently referred to as PHE or extract.

2.2 PHYTOCHEMICAL ANAYLSIS

The presence of tannins, alkaloids, phytosterols, terpenoids, flavonoids, general test for glycosides (reducing sugars), anthracene glycosides, and saponins were tested by simple qualitative and quantitative methods (Sofowora, 1993, Trease and Evans, 2002).

2.2.1 PHYTOCHEMICAL METHODS

2.2.1.1 Tannins

The powdered leaves of *Palisota hirsuta,* 0.5 g, were boiled with 25 ml of water for 5 minutes. It was then cooled, filtered and the volume adjusted to 25 ml. To 1ml aliquot of the aqueous extract was added 10 ml 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).

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2.2.1.2 Alkaloids

The powdered leaves of *Palisota hirsuta* (0.5 g) were extracted with 30 ml 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).

2.2.1.3 Phytosterols (Lieberman's test)

The powdered leaves of *Palisota hirsuta* were 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 steroids (Sofowora, 1993).

2.2.1.4 Terpenoids (Salkowski test)

The powdered leaves of *P. hirsuta* were 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).

2.2.1.5 Flavonoids

The method outlined by Soforowa was followed with some modification. The powdered leaves 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 flavononones (Sofowora, 1993).

2.2.1.6 General test for Glycosides (Reducing sugars)

About 200 mg of the powdered plant 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 Fehlings 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).

2.2.1.7 Anthrancene glycosides

To test for anthrancene glycosides, *P. hirsuta* extract 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).

2.2.1.8 Saponins

An amount (0.2g) of the powdered plant extract was shaken with a 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, 2002).

2.2.2 RESULTS

The phytochemical analysis of powdered leaf sample revealed the presence of tannins, flavonoids terpenoids, steroids, reducing sugars and traces of alkaloidal compounds with the most dominant being tannins and flavonoids (Table 2.1).

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Table 2.1Chemical constituent of ethanolic leaves extract of *P.hirsuta*

TESTS	RESULTS
Tannins	
Ferric chloride test	+++
Lead acetate test	+++
Flavonoids	+++
Alkaloids	
Draggendoff's test	+
Saponins	
Frothing test	–
Anthrancene glycosides	–
Steroids	
Lieberman's test	++
Terpenoids test	++
Glycosides general test	+

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

2.2.3 DISCUSSION

On preliminary phytochemical screening, the powdered leaf sample of *Palisota hirsuta* was found to contain tannins, reducing sugars, flavonoids, steroids, alkaloids and terpenoids as previously reported by (Benson *et al.*, 2008).

As has been reported by several authors, the presence of many biologically active phytochemicals such as triterpenes, flavonoids, alkaloids, steroids, tannins and glycosides in various plant extracts may be responsible for their respective pharmacological properties (Agarwal and Rangari, 2003; Liu *et al.*, 1996; Mbagwu *et al.*, 2007; Narendhirakannan *et al.*, 2007; Singh *et al.*, 2002). Phytochemicals from *Alstonia boonei* root barks (Kweifio-Okai *et al.*, 1995) and seeds of *Picralima nitida* (Woode *et al.*, 2006) have been used in the management of rheumatoid arthritis in Ghana and found to be effective anti-arthritic agents. Some flavonoids isolated from plant extracts have also shown antinociceptive activity (Clavin *et al.*, 2007; Kupeli and Yesilada, 2007). Some alkaloidal compounds isolated from various parts of several medicinal plants have been reported to be responsible for some pharmacological properties (Duwiejua *et al.*, 2002; Whitehouse *et al.*, 1994).

2.2.4 CONCLUSION

In conclusion, preliminary phytochemical screening of the powdered leaf sample of *Palisota hirsuta* was found to contain tannins, reducing sugars, flavonoids, steroids and terpenoids with traces of alkaloids.

Chapter 3

ANTI-INFLAMMATORY, ANTI-PYRETIC AND ANTI-OXIDANT EFFECTS

3.1 INTRODUCTION

The inflammatory response has received a great deal of interest in the field of medical research because inflammation underlies almost every disease process (Kapoor *et al*., 2005). The ethanolic leaf extract of *P. hirsuta,* a plant locally used in Ghana for various painful inflammatory conditions was assessed for its effect on both acute and chronic inflammation. The carrageenan-induced edema in the seven day old chick, first introduced by (Roach and Sufka, 2003) was used as an acute inflammatory model whilst the rat adjuvant induced arthritis previously described by Pearson, (1956) was used as chronic inflammatory model. Antioxidants are substances capable of counteracting the damaging effects of oxidation in body tissues (Scheibmeir *et al*., 2005). Oxidants including large amounts of superoxide and hydrogen peroxide are produced during inflammation when polymorphonuclear leukocytes and macrophages are stimulated (Ozkan *et al*., 2007). The anti-oxidant potential of the plant extract was assessed by determining its reducing power capacity. The ability of plant extract to scavenge 2, 2-diphenyl-1-picrylhydrazyl hydrate (DPPH) was also assessed in addition to the ability of the extract to protect against lipid peroxidation. This will serve to provide in part, scientific validation for the use of *P. hirsuta* leaves in the management of various inflammatory conditions traditionally. Since fever has been established to be one of the most prominent systemic manifestations of acute inflammation, the yeast-induced hyperthermia in young rats described by Tomazetti *et al*., (2005) was also employed to investigate the anti-pyretic activity of the plant extract. Moreover, most of the drugs currently used as anti-inflammatory agents also have anti-pyretic effects.

3.2 MATERIALS AND METHODS

3.2.1.1 Animals

Cockerels (*Gallus gallus*, strain Shaver 579, Akropong Farms, Kumasi, Ghana); (50-85 g) were obtained 1-day post-hatch and were housed in stainless steel cages (34×57×18 cm^3) at a population density of 12–13 chicks per cage. Food (Chick Mash, GAFCO, Tema, Ghana) and water were readily available.

Sprague-Dawley rats of both sexes (150–200 g) were purchased from Noguchi Memorial Institute for Medical Research, University of Ghana, Legon, 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^3) with soft wood shavings as bedding, fed with normal commercial pellet diet (GAFCO, Tema), given water *ad libitum* and maintained under laboratory conditions. 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).

3.2.1.2 Drugs

Diclofenac sodium was obtained from Troge, Hamburg, Germany, dexamethasone sodium phosphate from Pharm-Inter, Brussels, Belgium, methotrexate sodium from Dabur Pharma, India, Paracetamol powder from Phyto-Riker, Accra, Ghana, carrageenan sodium salt, obtained from Sigma-Aldrich Inc., St. Louis, MO, USA and commercially available dried baker yeast (*Saccharomyces cerevisiae*) from Saf do Brasil Produtos Alimenticios Ltd, Brazil.

3.2.2 CARRAGEENAN-INDUCED EDEMA IN CHICKS

3.2.2.1 Carrageenan-induced edema

The carrageenan foot edema model of inflammation in the chick previously described by (Roach and Sufka, 2003) and as modified by us (Boakye-Gyasi *et al.*, 2008) was used to evaluate the anti-inflammatory properties of the extract. Dexamethasone and diclofenac were used as reference drugs. Carrageenan (10 µl of a 2% suspension in saline) was injected intraplantar into the right footpads of the chicks. Foot volume was measured before injection and at hourly intervals for 5 hours after injection by water displacement plethysmography as described (Fereidoni *et al.*, 2000). The edema component of inflammation was quantified by measuring the difference in foot volume before carrageenan injection and at the various time points.

Effect of extract and drugs on carrageenan-induced edema

The experiment was performed to study the effect of the drugs when given preemptively (30 min for i.p. route and 1 h for oral route) before the carrageenan challenge. Chicks were randomly selected for the following study groups: control; diclofenac (10, 30 and 100 mg kg^{-1}, i.p.); dexamethasone (0.3, 1.0 and 3.0 mg kg^{-1}, i.p.) and extract (10, 30, 100 and 300 mg kg^{-1}, *p.o.*). Extract was prepared in 2 % tragacanth mucilage. All drugs were freshly prepared. Chicks were tested at 7 days of age. Group sample sizes of five–six were used for the study.

3.2.2.2 Analysis of Data

Raw scores for right foot volumes were individually normalized as percentage of change from their values at time 0, then averaged for each treatment group. The time-course curves for foot volume were subjected to two-way (*treatment* × *time*) repeated measures analysis of variance with Bonferroni's *post hoc t* test. Total foot volume for each treatment was calculated in arbitrary units as the area under the curve (AUC) and to determine the percentage inhibition for each treatment, the following equation was used.

$$\%\ inhibition\ of\ edema = \left(\frac{AUC_{control} - AUC_{treatment}}{AUC_{control}} \right) \times 100$$

Differences in AUCs were analyzed by ANOVA followed by Student-Newman-Keuls' *post hoc* test. ED_{50} (dose responsible for 50% of the maximal effect) for each drug was determined by using an iterative computer least squares method, with the following nonlinear regression (three-parameter logistic) equation

$$Y = \frac{a + (b - a)}{\left(1 + 10^{(LogED_{50} - X)}\right)}$$

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Where, *X* is the logarithm of dose and *Y* is the response. *Y* starts at *a* (the bottom) and goes to *b* (the top) with a sigmoid shape.

The fitted midpoints (ED_{50}s) of the curves were compared statistically using *F* test (Miller, 2003; Motulsky *et al.*, 2003). GraphPad Prism for Windows version 4.03 (GraphPad Software, San Diego, CA, USA) was used for all statistical analyses and ED_{50} determinations. $P < 0.05$ was considered statistically significant.

Data was presented as the effect of drugs on the time course and the total edema response for 5 h using GraphPad Prism for Windows version 4.02 (GraphPad Software, San Diego, CA, USA).

3.2.2.3 RESULTS

Administration of 10 µl of 2% carrageenan induced moderate inflammation resulting in foot edema in the 7 day old chicks peaking at 2-3 h as described by (Roach and Sufka, 2003).

Figure 3.1 shows the time courses and the total edema response for the effects of PHE, dexamethasone and diclofenac in carrageenan-induced edema. Two-way ANOVA (treatment x time) revealed a significant effect of drug treatment for PHE ($F_{40,100}$= 5.78, P=0.0029), diclofenac ($F_{3,80}$= 5.46, P=0.0089), and dexamethasone ($F_{3,80}$=

1.62, *P*=0.2241). Total edema produced by each treatment is expressed in arbitrary units as AUC of the time-course curves.

PHE (10-300 mg kg^{-1}) dose dependently and significantly reduced the total foot edema with maximal effect of 54.71±11.04% for PHE administered prophylactically (Figure 3.1a and b). Similarly the NSAID diclofenac (10-100 mg kg^{-1}) dose dependently reduced the edema with a maximal effect of 58.78±17.15% (Figure 3.1c and d). Dexamethasone (0.3-3 mg kg^{-1}), a steroidal anti-inflammatory agent, on the other hand inhibited the carrageenan-induced edema completely (Figure 3.1 e and f).

Figure 3.2 shows the dose-response curves of the effects of the drugs under test. PHE was found to be approximately 4.15× less potent than diclofenac ($F_{1,33}$= 5.82, *P*=0.0022) and 348.89× less potent than dexamethasone ($F_{1,33}$= 108.90, P<0.0001).

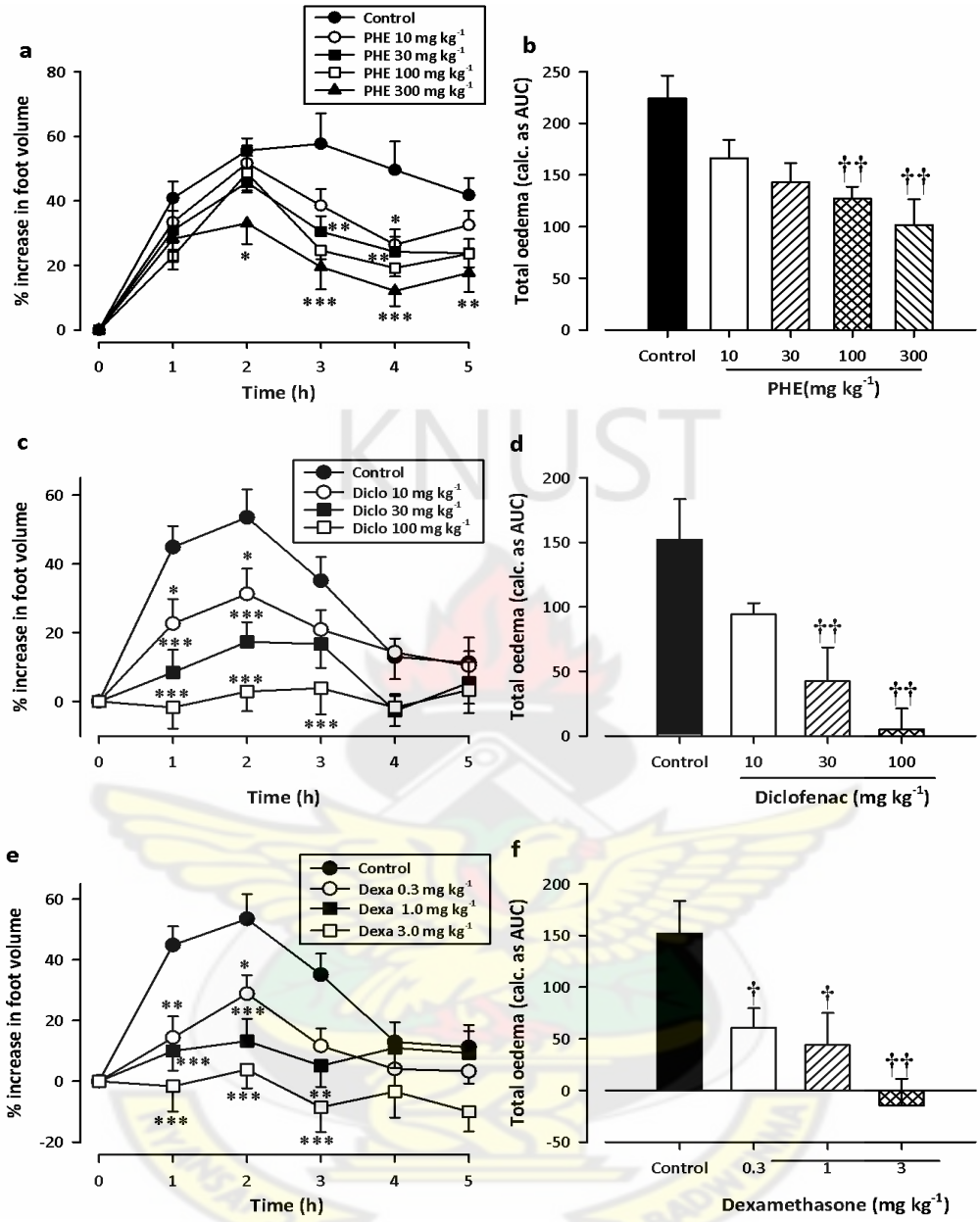

Figure 3.1 Effect of PHE (10-300 mg kg^{-1}; *p.o.*), diclofenac (10-100 mg kg^{-1}; i.p.) and dexamethasone (0.3-3 mg kg^{-1}; i.p.) on time course curve (a, c and e respectively) and the total edema response (b, d and f respectively) in carrageenan-induced paw edema in chicks. Values are means ± S.E.M. (n = 5). ***$P < 0.001$; ** $P < 0.01$; *$P < 0.05$ compared to vehicle-treated group (Two-way ANOVA followed by Bonferroni's *post hoc* test). $^{\dagger\dagger\dagger}P < 0.001$; $^{\dagger\dagger}P < 0.01$; $^{\dagger}P < 0.05$ compared to vehicle-treated group (One-way ANOVA followed by Newman-Keul's *post hoc* test.

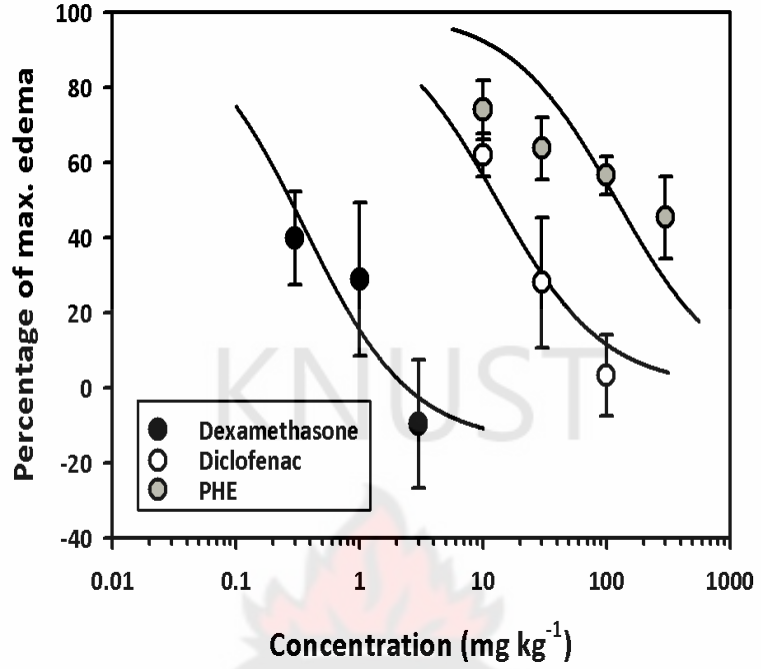

Figure 3.2 Dose response curves for dexamethasone (0.3-3.0 mg kg^{-1} i.p), diclofenac (10-100 mg kg^{-1} i.p) and PHE (10-300 mg kg^{-1} *p.o*) on carrageenan induced foot edema in the chick.

Table 3.1 ED_{50} for *P. hirsuta*, diclofenac and dexamethasone in carrageenan induced foot edema in chicks.

Drugs	ED_{50} (mg kg^{-1})
P. hirsute	125.60±36.80
Diclofenac	30.27±11.10
Dexamethasone	0.36±0.45

3.2.2.4 DISCUSSION

Carrageenan-induced acute footpad edema in laboratory animals (Winter *et al.*, 1962) has been widely used to screen new anti-inflammatory drugs and remains an acceptable preliminary screening test for anti-inflammatory activity (Niemegeers *et al.*, 1975; Singh *et al.*, 2000). It is commonly used to evaluate non-steroidal anti-inflammatory drugs (NSAID) (Di Rosa and Willoughby, 1971). In this study, chicks were used instead of the commonly used rodents. Carrageenan-induced edema has been validated in the chicks by (Roach and Sufka, 2003), and is much more economical than rodent models. Furthermore, chicks are easier to handle. Studies have demonstrated that intraplantar injection of carrageenan in the 7-day-old chick elicits a measurable, reliable and relatively short-lasting state of edema, that is differentially attenuated by the systemic administration of typical anti-inflammatory 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 dose-dependent inhibition of carrageenan-induced foot edema by the extract in this model of acute inflammation depicts the anti-inflammatory potential of the extract in acute inflammation.

According to (Vinegar *et al.*, 1987) the development of the carrageenan-induced paw edema derives from the release of cytoplasmic enzymes and serotonin from mast cells and the increase of prostaglandin in the inflammatory area. In particular, the initial phase of inflammation (0-2hr) has been attributed to the release of histamine and kinins, followed by a late phase (2.5-6 h) mainly sustained by prostaglandin release (Di Rosa, 1972) and more recently have been attributed to the induction of cyclooxygenase-2 in the tissue (Muniappan and Sundararaj, 2003).

Although the actual mechanism of action of PHE is not known, it is possible that, the anti-inflammatory activity exhibited by the extract 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.

The extract was 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).

3.2.2.5 CONCLUSION

In conclusion, this study has demonstrated that the ethanolic leaf extract of *Palisota hirsuta* has anti-inflammatory activity in the chick model of acute inflammation and hence may be potentially useful in the management of inflammatory conditions in humans, a validation of its traditional use as anti-inflammatory agent in Ghana.

3.2.3 ADJUVANT – INDUCED ARTHRITIS

3.2.3.1 Induction of Arthritis

Adjuvant arthritis was induced as previously described by Pearson, (1956) and as modified by Woode *et al.*, (2008). Right hind paw of rats were injected intraplantar with 0.1 ml of Complete Freund's Adjuvant (CFA). The CFA was prepared using a suspension of 5 mg ml^{-1} of heat killed *Mycobacterium tuberculosis* [strains C, DT and PN (mixed) obtained from the Ministry of Agriculture, Fisheries and Food, U.K] in paraffin oil. Arthritic control group received only intraplantar injection of CFA, whilst non-arthritic control/IFA group received only intraplantar injection of 0.1 ml Incomplete Freund's Adjuvant (IFA) (sterile paraffin oil). The drug test groups were pre-treated (30 min for i. p. route and 1 h for oral route) with the test drugs prior to CFA innoculation. Group sample sizes of 6-8 were utilized throughout the study.

Rats were selected for one of the following study groups: dexamethasone (0.3, 1.0, and 3 mg kg^{-1}), metothrexate (0.1, 0.3 and 1 mg kg^{-1}), and *P. hirsuta* extract (30, 100, and 300 mg kg^{-1}).

Foot volume was measured by water displacement plethysmography (Fereidoni *et al.*, 2000) for both the ipsilateral (injected hind paw) and the contralateral paw (non-injected hind paw) before intraplantar injection of CFA (day 0) and every other day up to the 28^{th} day. The edema component of inflammation was quantified by measuring the difference in foot volume between day 0 and the various time points.

The extract was suspended in 2 % tragacanth mucilage and given orally whilst the reference drugs were dissolved in normal saline and given intraperitoneally. Test drugs were prepared such that not more than 1 ml of extract and not more than 0.5 ml of reference drug was administered. All drugs were freshly prepared on each day of drug administration.

Drug challenge: Three sets of experiments were performed. First, to look at the effect of the drugs on established arthritis (curative protocol). Secondly, to study the effect of drugs when given before inducing arthritis (prophylactic protocol) and lastly to study the effect of combining dexamethasone or methotrexate with extract (combination therapy).

In the curative protocol, drugs were administered on day 9 with the onset of polyarthritis.

The animals were grouped randomly into the following study groups in the curative protocol:

Group I Arthritic control/CFA (intraplantar injection of 0.1 ml CFA)

Group II Non-arthritic control/IFA (intraplantar injection of 0.1 ml of IFA)

Groups III-V Treated with dexamethasone (0.3, 1.0, 3 mg kg^{-1} i.p.) from day 9 and administered every other day.

Group VI-VIII Treated with methotrexate (0.1, 0.3, 1 mg kg^{-1} i.p.) from day 9 and administered every 4 days.

Group IX-XI Treated with *P. hirsuta* extract (30,100, 300 mg kg^{-1} *p.o.*) from day 9 and administered every day.

In the prophylactic protocol, initial weights of rats were taken on day 0 after grouping and subsequently every 4 days. Also the initial foot volumes were measured on day 0 and subsequently every other day. Drugs were administered on day 0 and CFA was injected intraplantar 24 h later.

Animals were grouped as follows for the second set:

Group I Arthritic control/CFA (intraplantar injection of 0.1 ml CFA)

Group II Non-arthritic control/IFA (intraplantar injection of 0.1 ml of IFA)

Group III Pretreated with dexamethasone 1.0 mg kg^{-1} i.p. from day 0

Group IV Pretreated with methotrexate 0.3 mg kg^{-1} i.p. from day 0

Group V Pretreated with *P. hirsuta* extract 100 mg kg^{-1} *p.o.* from day 0

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For the combination therapy, the standard drugs and extract were combined and the rats were grouped as follows:

Group VI Pretreated with dexamethasone 1 mg kg^{-1}+*P. hirsuta* 100mgkg^{-1}

Group VII Pretreated with methotrexate 0.3 mg kg^{-1} +*P. hirsuta* 100 mg kg^{-1}

Group VIII Pretreated with dexamethasone 1 mg kg^{-1} + methotrexate 0.3 mg kg^{-1}

3.2.3.2 Analysis of Data

Raw scores for ipsilateral and contralateral paw volumes were individually normalized as percentage of change from their values at day 0 and then averaged for each treatment group.

Data was presented as the effect of drugs on the time course and the total edema response of adjuvant-induced arthritis for the 28 days period and treated as described for the acute inflammation in section 3.2.2.3.

3.2.3.3 Arthritis Score

The arthritis score was evaluated blindly by the same person in all the rats on day 29. The severity of arthritis of each paw was scored as described by Kinne *et al.*, (1995) according to the extent of erythema and edema of the periarticular tissues, using a scale of 0–4. The arthritis score of each rat on day 0 was determined to be 0.

The hind paw volume and arthritis score were used as the measurement parameters of inflammation.

Photographs: Photographs of the affected hind limbs were taken on day 29 using a digital camera (Model DCR-DVD 705E, Sony Corp., Tokyo, Japan).

Radiography: Radiographs of the hind limbs were taken for all the animals on day 29. The animals were anaesthetized by intraperitoneal injection with chloroform. Radiographs were taken with an X-ray apparatus (Softex, Tokyo, Japan) and industrial X-ray film (Fuji Photo Film, Tokyo, Japan). The X-ray apparatus was operated at 30-kV peak and 10-s exposure with a 45-cm tube-to-film distance for lateral projections. The severity of bone and joint destruction was scored blindly by the same person for each hind limb, according to the extent of osteoporosis, osteophytes, joint spaces, and joint structure, as described by Pohlers *et al.*, (2007). The severity of bone destruction of each paw was scored using a scale of 0–4. The radiological score for normal control rats was determined to be 0.

3.2.3.4 RESULTS

Phases of the adjuvant arthritis investigated in this study were assigned acute and polyarthritic/chronic phases corresponding to day 0-10 and days 10-28 post adjuvant inoculation respectively. Two-way ANOVA *(treatment x time)* revealed a significant ($F_{3,144}$= 3.65, P= 0.0355) effect of drug treatment. Total edema produced by each treatment is expressed in arbitrary units as AUC of the time-course curves.

All arthritic control animals showed acute inflammatory edema at the ipsilateral (injected paw) around days 4-6 followed by subsequent chronic polyarthritic phase which begins around day 10-12 as previously described by Weichman, (1989). The progress of inflammatory edema in the contralateral (non-injected) paw was evident on day 12 indicative of a systemic spread of the inflammation. Throughout the 28-day experiment, there was no significant change in the paw volume of the non-inflammed control groups that were injected with IFA.

PHE (30-300 mg kg^{-1}) modified the time course curve significantly ($F_{3,16}$= 3.59, P= 0.0149) and reduced the acute edema in the ipsilatral paw with the highest dose used (300 mg kg^{-1}) significantly ($F_{3,16}$= 4.13, P= 0.0240) reducing the edema with a percentage inhibition of 13.02±8.77% (Figure 3.3a). PHE 300 mg kg^{-1} also significantly ($F_{3,16}$= 3.84, P= 0.0302) prevented the spread of the edema from the ipsilateral to the contralateral paw indicating inhibition of systemic spread (Figure 3.3b). Dexamethasone (0.3-3 mg kg^{-1}) a steroidal anti-inflammatory agent greatly and significantly ($F_{3,16}$= 96.91, P< 0.0001) inhibited in a dose-dependent manner polyarthritis edema with a maximum inhibition of 91.59±2.06% (Figure 3.3c). Dexamethasone also completely prevented the spread of the arthritis ($F_{3,16}$= 36.74, P<0.0001) from the ipsilateral to the contralateral paws of the treated animals (Figure 3.3d). Methotrexate (0.1-1.0 mg kg^{-1}) also dose-dependently reduced the edema in the ipsilateral paw but this effect was not so significant ($F_{3,16}$= 13.76, P= 0.0001). Furthermore, methotrexate completely prevented the spread of the arthritis with a high level of significance ($F_{3,16}$= 21.57, P< 0.0001) as shown in Figure 3.3f.

Figure 3.4 shows the dose-response curves of the effects of the drugs under test. PHE was found to be approximately 75.46× less potent than methotrexate ($F_{1,28}$= 61.55, P<0.0001) and 281.27× less potent than dexamethasone ($F_{1,28}$= 5.28, P=0.0299). Dexamethasone was also found to be 3.73× more potent than methotrexate ($F_{1,28}$= 9.73, P=0.0042) (Table 3.3).

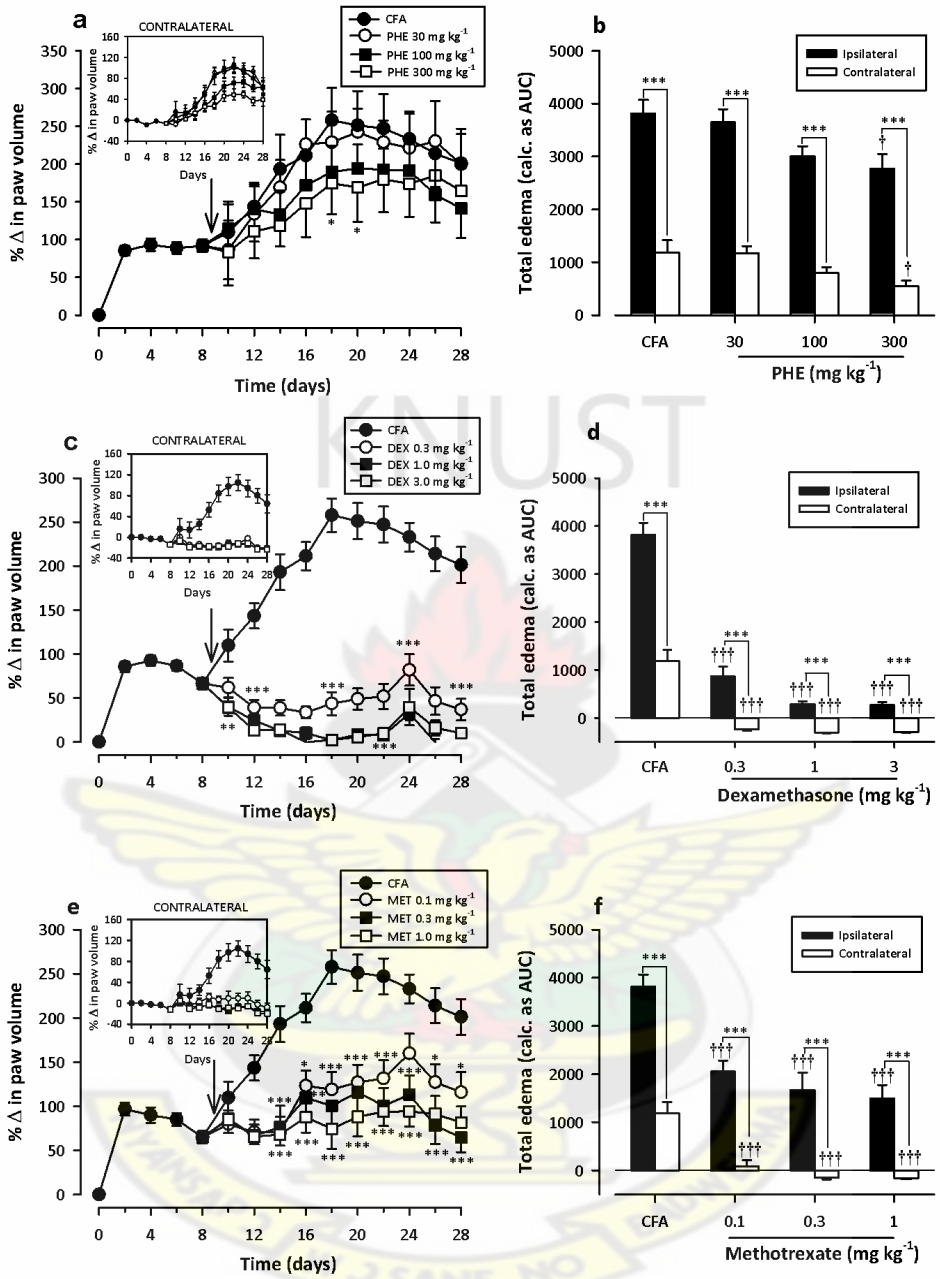

Figure 3.3 Effect of PHE (30-300 mg kg^{-1}; *p.o.*), dexamethasone (0.3-3 mg kg^{-1}; i.p.) and methotrexate (0.1-1 mg kg^{-1}; i.p.) on time course curve (a, c and e respectively) and the total edema response (b, d and f respectively) in adjuvant induced arthritis in rats. Values are means ± S.E.M. (n = 5). ***$P < 0.001$; ** $P < 0.01$; *$P < 0.05$ compared to vehicle-treated group (Two-way ANOVA followed by Bonferroni's *post hoc* test). $^{\dagger\dagger\dagger}P < 0.001$; $^{\dagger\dagger}P < 0.01$; $^{\dagger}P < 0.05$ compared to vehicle-treated group (One-way ANOVA followed by Newman-Keul's *post hoc* test.

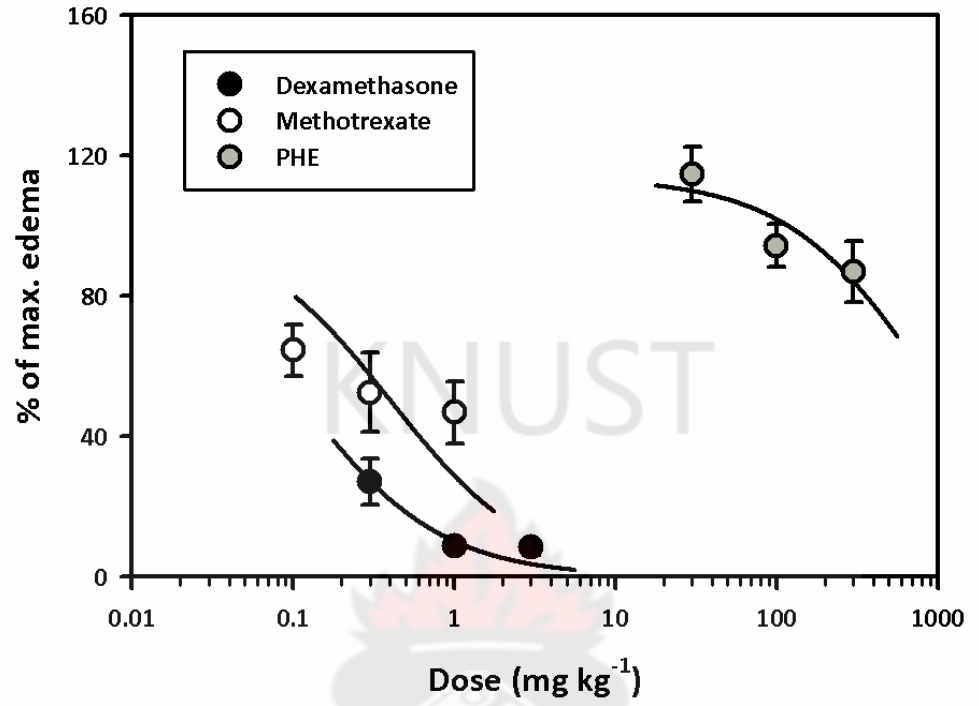

Figure 3.4 Dose response curves for dexamethasone (0.3-3.0 mg kg^{-1} i.p), methotrexate (0.1-1 mg kg^{-1} i.p) and PHE (30-300 mg kg^{-1} *p.o*) on adjuvant induced arthritis in rats.

Table 3.2 ED_{50} values for Adjuvant-induced arthritis

Drugs	ED_{50} (mg kg^{-1})
P. hirsuta	35.59±15.73
Methotrexate	0.41±0.14
Dexamethasone	0.11±0.02

Figure 3.5 shows the effect of PHE (100 mg kg^{-1}), dexamethasone (1 mg kg^{-1}) and methotrexate (0.3 mg kg^{-1}) alone in prophylactic protocol and in various combinations with each other. Two-way ANOVA *(treatment x time)* revealed a significant ($F_{3,224}$= 13.06, P= 0.0001) effect of drug treatment. Total edema produced by each treatment is expressed in arbitrary as AUC of the time-course curves.

PHE when given alone and when combined with methotrexate did not show any significant effect in both the acute (P >0.05) and polyarthritis (P >0.05) phases. PHE in combination with dexamethasone however showed a significant inhibition of arthritis in both the acute ($F_{3,16}$= 8.20, P= 0.0016) and the polyarthritis ($F_{3,16}$= 10.28, P= 0.0005) phases. Dexamethasone in combination with methotrexate gave the greatest inhibition of both phases with an extreme level of significance (phase 1 $F_{3,16}$= 4.27, P= 0.0214; phase 2 $F_{3,16}$= 14.38, P< 0.0001).

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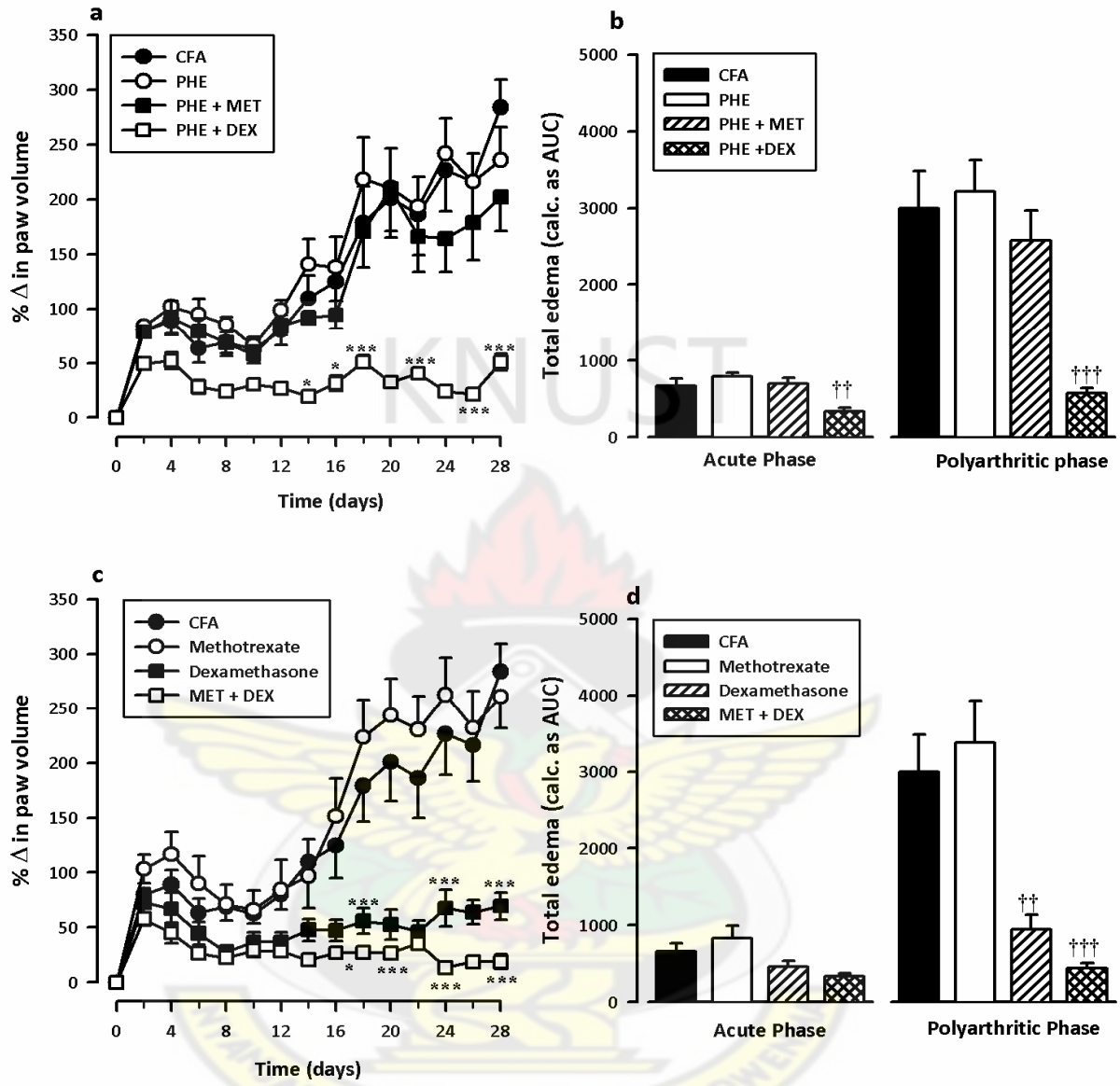

Figure 3.5 Time course effects of *P. hirsuta* 100 mg kg^{-1}, *P. hirsuta* 100 mg kg^{-1}+methotrexate 0.3 mg kg^{-1} & *P. hirsuta* 100 mg kg $^{-1}$+dexamethasone 1.0 mg kg^{-1} (a); methotrexate 0.3 mg kg^{-1} , dexamethasone1.0 mg kg^{-1} & methotrexate 0.3 mg kg^{-1} +dexamethasone 1.0 mg kg^{-1} (b) on CFA induced increase in the ipsilateral paw volume and (b& d) the AUC (total edema) for 28 days in the acute and polyarthritic phase. Each point in (a & c) and column in (b& d) represents the mean ± S.E.M. (n=5). *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ compared to vehicle-treated group (Two-way ANOVA followed by Bonferroni's *post hoc* test). $^{\dagger\dagger\dagger}P<0.001$; $^{\dagger}P<0.05$ compared to vehicle-treated group (One-way ANOVA followed by Neuman-Keul's *post hoc* test.

From the arthritic score for the photographs (Table 3.3 & 3.4), the CFA group showed enormous erythema and swelling in both the ipsilateral and contralateral paws (Plate 3.1). PHE and methotrexate treated animals however exhibited slight erythema and swelling whilst the dexamethasone treated and the IFA groups showed no sign of erythema or swelling (Plates 3.2 – 3.6).

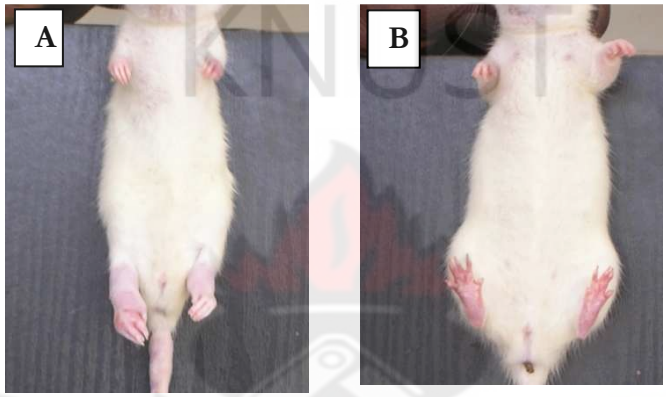

Plate 3.1 Photographs of rat pre treated with CFA/arthritic control (A) and IFA/ non arthritic (B) control in the curative protocol.

Plate 3.2 Photographs of rats treated with *P. hirsuta* extract (30, 100 and 300 mg kg^{-1}) (A, B & C) respectively in the curative protocol.

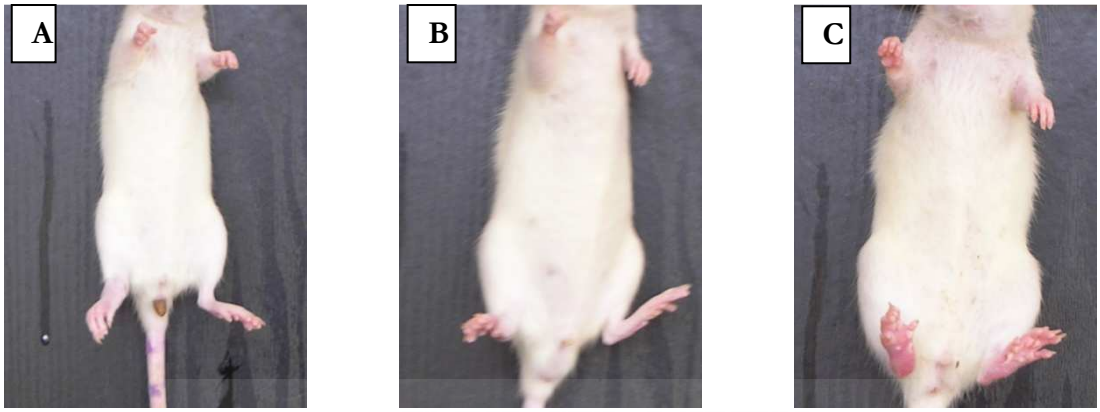

Plate 3.3 Photographs of rats treated with dexamethasone (0.3, 1.0 and 3.0 mg kg^{-1}) (A, B & C) respectively in the curative protocol

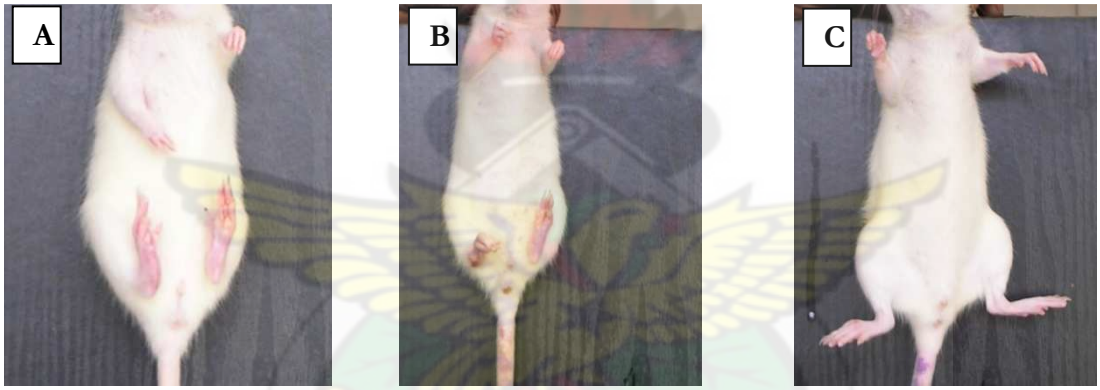

Plate 3.4 Photographs of rats treated with methotrexate (0.1, 0.3 and 1.0 mg kg^{-1}) (A, B & C) respectively in the curative protocol.

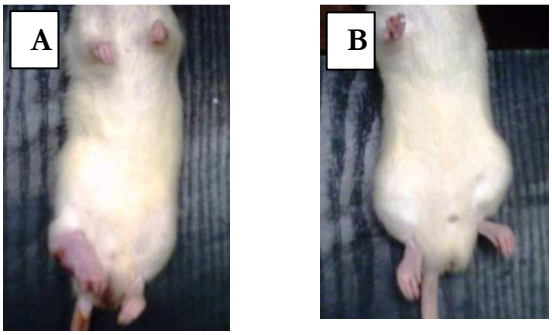

Plate 3.5 Photographs of rat treated with CFA/arthritic control (A) and IFA/ non arthritic (B) control in the prophylactic protocol.

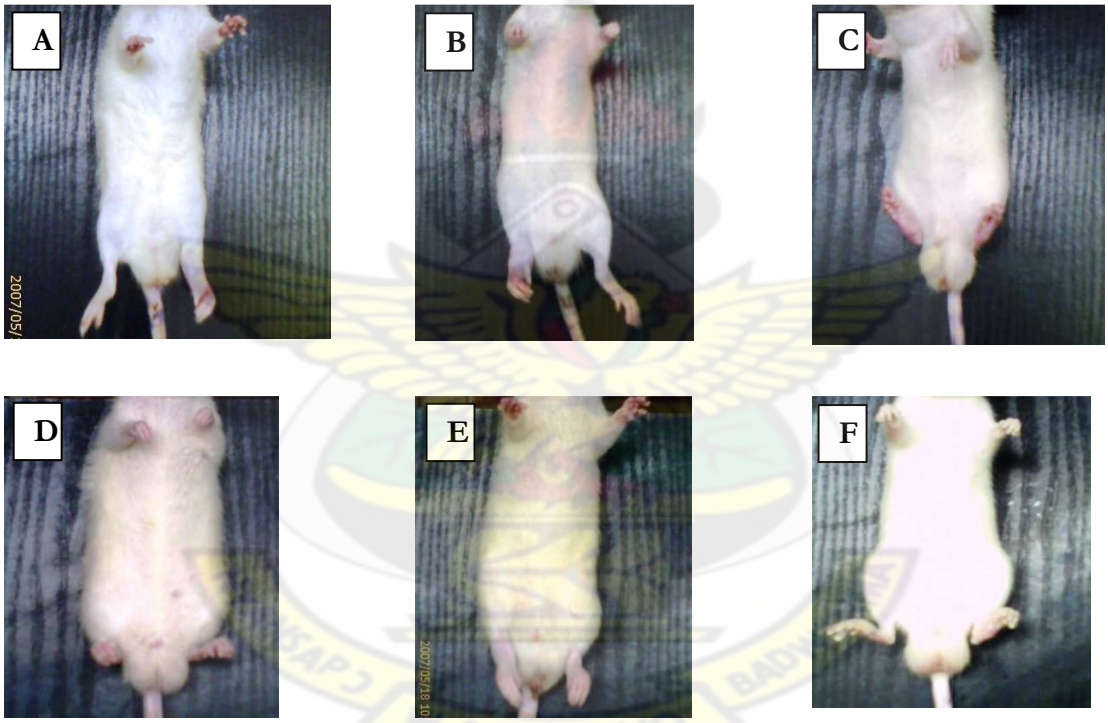

Plate 3.6 Photographs of rats pre- treated with *P. hirsuta* (100 mg kg^{-1}), dexamethasone (1 mg kg^{-1}) and methotrexate (0.3 mg kg^{-1}) alone (A, B &C) in the prophylactic protocol and in combination therapy, *P. hirsuta*+dexamethasone, *P. hirsuta*+methotrexate and dexamethasone+methotrexate (D, E & F) respectively.

Measurement of paw or joint swelling only gives an indication of edematous changes in these regions; however, the most obvious damage takes place in the tibiotarsals joint itself. Hence radiographs of the hind paws for each group were taken as shown in Plates 3.7 – 3.13. These were later scored for the extent of bone damage at the joints as shown in Tables 3.3 and 3.4. CFA/Arthritic control group gave the highest score demonstrating severe bone enlargement with active osteophytosis in the bone metaphysis, reduced bone density, focal areas of excessive bone resorption, and no visible joint spaces, whilst the bones were intact in the IFA/non arthritic control which recorded the lowest score.

PHE, dexamethasone and metothrexate all suppressed the pathological changes seen in adjuvant induced-arthritis with dexamethasone and methotrexate exhibiting dose-dependancy as shown radiological scores (Tables 3.3 & 3.4)

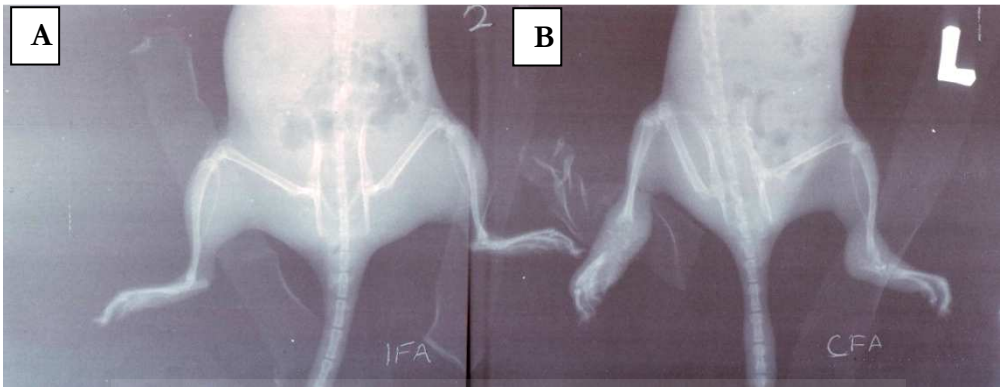

Plate 3.7 Radiographs of rat treated with IFA/ non arthritic control (A) and CFA/arthritic control (B) in the curative protocol.

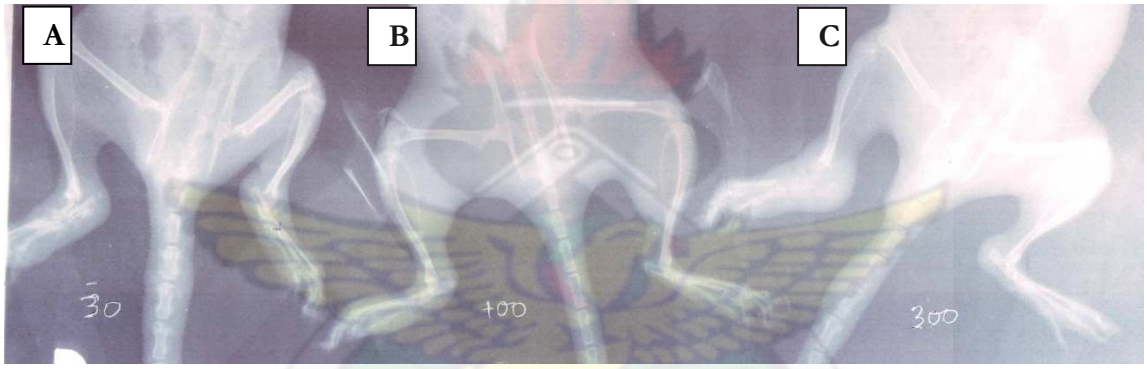

Plate 3.8 Radiographs of rats treated with *P. hirsuta* extract (30, 100 and 300 mg kg^{-1}) (A, B & C) respectively in the curative protocol.

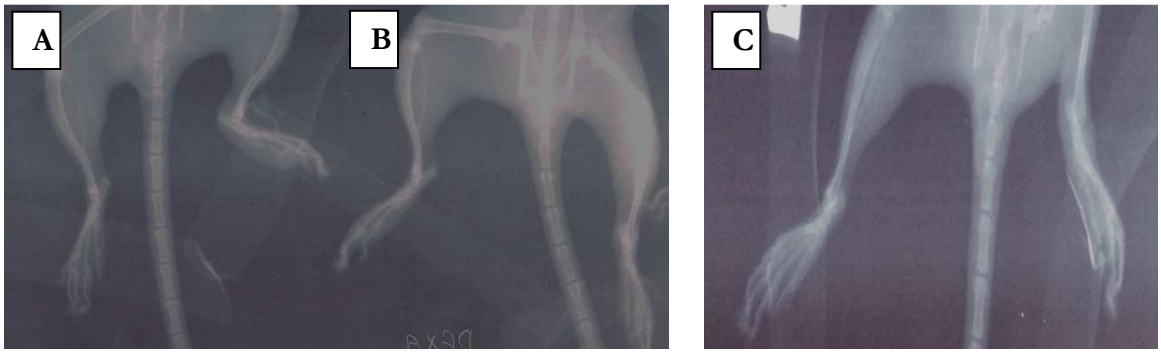

Plate 3.9 Radiographs of rats treated with dexamethasone (0.3, 1.0 and 3.0 mg kg^{-1}) (A, B & C) respectively in the curative protocol

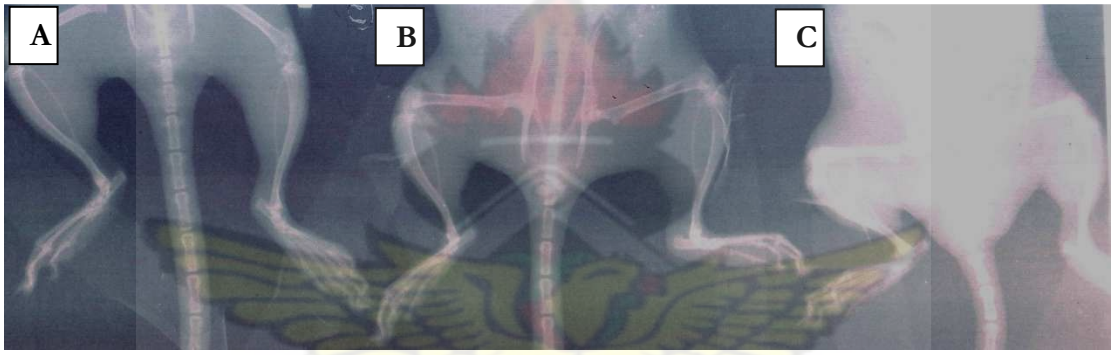

Plate 3.10 Radiographs of rats treated with methotrexate (0.1, 0.3 and 1.0 mg kg^{-1}) (A, B & C) respectively in the curative protocol

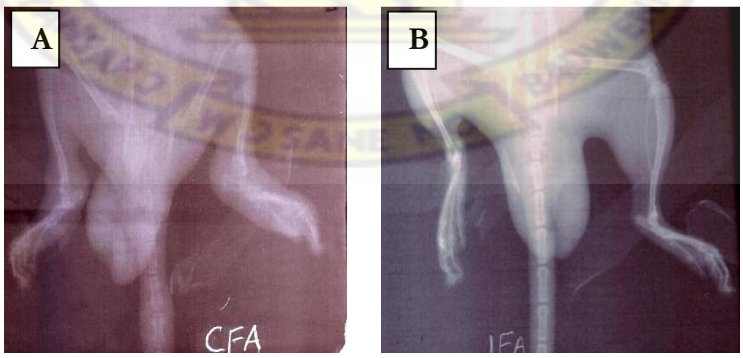

Plate 3.11 Radiographs of rat treated with CFA/arthritic control (A) and IFA/ non arthritic (B) control in the prophylactic protocol.

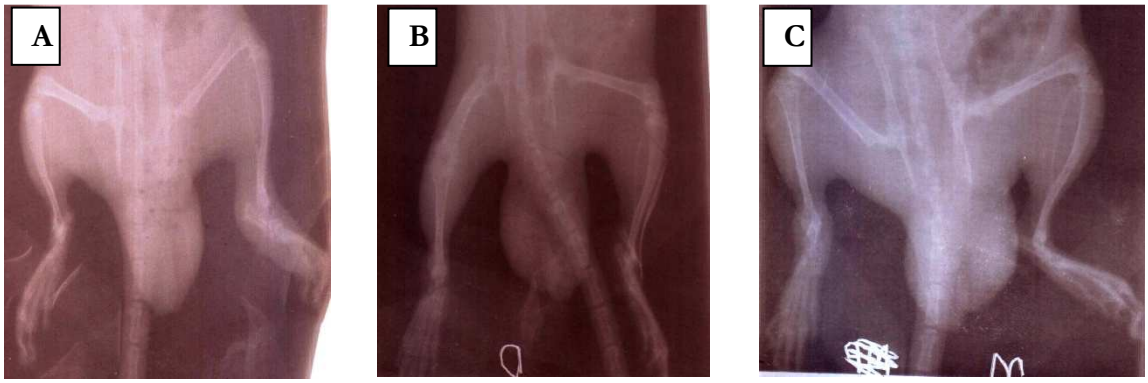

Plate 3.12 Radiographs of rats pre-treated with *P. hirsuta* (100 mg kg^{-1}), dexamethasone (1 mg kg^{-1}) and methotrexate (0.3 mg kg^{-1}) alone (A, B &C) respectively in the prophylactic protocol.

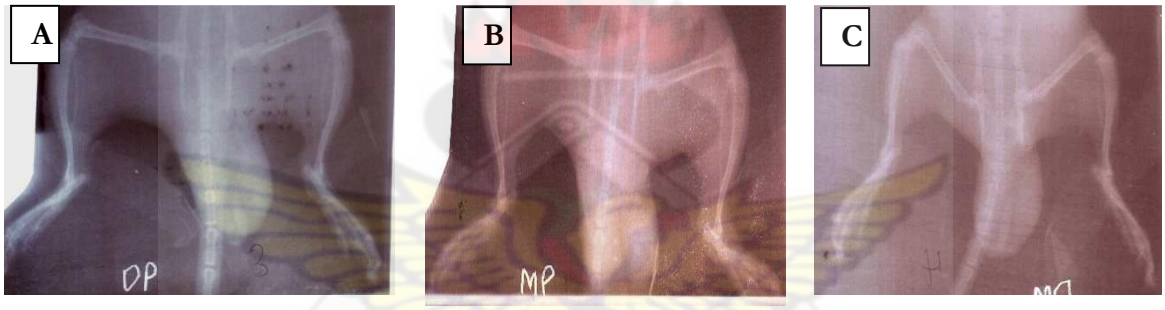

Plate 3.13 Radiographs of rats pre-treated with a combination of, *P. hirsuta*+dexamethasone, *P.hirsuta*+methotrexate and dexamethasone+methotrexate (A, B & C) respectively, in the prophylactic protocol.

Table 3.3 Arthritic scores for representative animals for groups treated curatively with PHE, methotrexate or dexamethasone.

GROUP	Pictures		X-Ray	
	ipsilateral	contralateral	ipsilateral	contralateral
IFA	1	0	0	0
CFA	4	3	4	3
P. hirsuta extract				
30 mg kg^{-1}	2	1	1	1
100 mg kg^{-1}	3	1	3	2
300 mg kg^{-1}	4	3	4	3
Methotrexate				
0.1 mg kg^{-1}	2	1	0	0
0.3 mg kg^{-1}	2	1	0	0
1.0 mg kg^{-1}	1	1	0	0
Dexamethasone				
0.30 mg kg^{-1}	1	0	0	0
1.0 mg kg^{-1}	0	0	0	0
3.0 mg kg^{-1}	0	0	0	0

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Table 3.4 Arthritic scores for representative animals for groups treated with PHE, methotrexate, dexamethasone or vehicle prophylactically and in combination therapy.

Groups	Pictures		X-Ray	
	ipsilateral	contralateral	ipsilateral	contralateral
IFA	1	0	0	0
CFA	4	4	4	3
P.hirsuta 100 mg kg^{-1}	4	2	3	0
Methotrexate 0.3 mg kg^{-1}	3	1	1	0
Dexamethasone 1 mg kg^{-1}	1	0	0	0
MET.0.3 mg kg^{-1}+DEX. 1 mg kg^{-1}	1	0	0	0
MET.0.3 mg kg^{-1}+PHE100 mg kg^{-1}	1	1	0	0
DEX. 1 mg kg^{-1}+PHE. 100 mg kg^{1}	1	1	0	0

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3.2.3.5 DISCUSSION

Rat adjuvant-induced arthritis is a commonly used animal model for preclinical studies of non-steroidal anti-inflammatory drugs and disease-modifying anti-rheumatic drugs and that, it is suggested as the most convenient model for studying drugs affecting human arthritis (Pearson, 1956; Whitehouse, 2007) and has often been used to study the mechanisms of action and preventive effects of a number of disease-modifying anti-rheumatic drugs (Hoffmann *et al.*, 1997).

Prognosis of rat adjuvant-induced arthritis can be divided into three phases just like human rheumatoid arthritis. These phases start with the induction phase without evidence of synovitis, followed by early synovitis, and finally late synovitis with progressive joint destruction (Hoffmann *et al.*, 1997). A good anti-rheumatic agent should be able to block one or more of these phases.

In this study, *P. hirsuta* extract was able to suppress the joint inflammation and synovitis. It also proved very effective in preventing the systemic spread and ultimately reducing the destruction of joints as seen in the scores for the pictures and the radiographs. Radiographs are necessary to determine true remission of disease and for accurate evaluation of disease status (Kitamura *et al.*, 2007). Reduced bone formation and increased resorption are the causes of bone loss in adjuvant-induced arthritis in rats (Aota *et al.*, 1996; Findlay and Haynes, 2005; Makinen *et al.*, 2007). The x-ray scores clearly show increased bone loss in arthritic groups and decreased bone loss in drug treated groups. It has been suggested that, new therapeutic strategies for chronic forms of arthritis have to aim at both, suppression of inflammation and bone protection and as such, joint protection plus suppression of synovitis are known to be the ultimate goals of a better rheumatoid arthritis treatment (Atzeni and Sarzi-Puttini, 2007; Hoffmann *et al.*, 1997; Sharma *et al.*, 2004) which is indicative that PHE can have a role to play in the management of human rheumatoid arthritis.

Corticosteroids are effective against adjuvant arthritis whether administered prophylactically or therapeutically (Walz *et al*., 1971). Dexamethasone, a steroidal anti-inflammatory drug and a first line drug which quickly reduces symptoms of inflammation in rheumatoid arthritis, dramatically suppressed inflammation and wiped out the spread of arthritis. Dexamathasone is known to inhibit the release of pro-inflammatory cytokines (tissue necrosis factor-• and interleukin-1•), which are known to play a central role in the propagation of the disease process in rheumatoid arthritis thus being able to arrest the edema produced (Issekutz and Issekutz, 1991). Dexamathasone in this study was able to suppress completely the joint inflammation and synovitis as well as effectively preventing the systemic spread and ultimately reducing the destruction of joints. Surprisingly, for reasons not very clear, dexamethasone caused a reduction in foot volume which was however not statistically different from zero.

Methotrexate, a disease modifying anti rheumatic drug was used for comparison because it is a commonly prescribed "front-line" treatment for rheumatoid arthritis (Swierkot and Szechinski, 2006). Low dose methotrexate is the most widely used anti-rheumatic drug and it is the "gold standard" against which other systemic medications are compared (Ochaion *et al.*, 2006). Methotrexate is better tolerated with fewer side effects than other DMARDs, it promotes disease remission and prevent progressive joint destruction that can result from uncontrolled inflammation; it helps maintain bone mass by increasing bone formation and decreasing bone resorption (Segawa *et al.*, 1997). It is also an immunosuppressant (Cronstein, 2005; Tian and Cronstein, 2007). Methotrexate is often preferred by rheumatologists because if it does not control arthritis on its own then it works well in combination with many other drugs (Hisadome *et al.*, 2004). Methotrexate inhibited AIA and completely wiped out the spread of arthritis. However, its effect was not as drastic as dexamethasone because it is slow acting but when given early enough before the onset of polyarthritis as was the case in this experiment, it is able

to prevent long term irreversible damaging effects of chronic inflammation to the joints (Puolakka *et al.*, 2005).

As a number of disease-modifying anti-rheumatic drugs in monotherapy often have unexpected side effects, combined treatment at lower doses may be necessary in order to expand the margin between efficacy and toxicity (Hisadome *et al.*, 2004; Makinen *et al.*, 2007). Based on this premise, the effect of combined lower doses of PHE and methotrexate or dexamethasone on the progression of hind paw inflammation and joint destruction in rats was studied. PHE in combination with dexamethasone had strong inhibitory effect on arthritis in rats; showing a synergistic suppression of both the increase in hind paw volume and also joint destruction thus producing a better remission of adjuvant-induced arthritis than PHE or dexamethasone alone. It is therefore possible that PHE and dexamethasone suppress different stages of the inflammatory process in arthritic rats thus their combined effect given a better remission. The effect of PHE was however not potentiated by methotrexate which is indicative that both drugs may be suppressing a similar stage of the inflammatory process in arthritic rats. As expected, dexamethasone combined with methotrexate synergistically suppressed arthritic progression which was extremely significant (Brown *et al.*, 2006).

In respect to joint destruction, there is very little evidence on the role of most of the anti-rheumatic drugs currently being used, such as gold, sulfasalazine, and methotrexate, when used alone in the modification of long term disease outcome (Zhao *et al.*, 2006). Combination therapy in rheumatoid arthritis is acknowledged to give a better remission of disease than monotherapy (Capell *et al.*, 2007; Mottonen *et al.*, 2006). It is therefore necessary to develop new agents from natural sources, which when used in combination with other anti rheumatic drugs will be less toxic and at the same time, be affordable and effective for preventing joint destruction, as well as synovial inflammation, in RA ,thus increasing efficacy of the treatment for patients with RA (Ronday *et al.*, 1998).

3.2.3.6 CONCLUSION

PHE is a very promising candidate for monotherapy and/or combination therapy in the effective treatment of rheumatoid arthritis.

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3.2.4 ANTI-PYRETIC EFFECTS

3.2.4.1 Induction of brewer's yeast pyrexia

Hyperthermia was induced in rats as previously described by Tomazetti *et al.,* (2005). Animals were fasted overnight and for the entire duration of the experiment but given water *ad libitum*. Initial rectal temperatures (TR) were recorded before induction of pyrexia with a lubricated digital thermometer inserted about 3 cm into the rectum of each rat. To induce pyrexia, rats were injected with a pyrogenic dose of baker's yeast (0.135 mg kg^{-1} *i.p*). TR changes were recorded every hour up to 4 h. Animals that showed an increase of not less than 0.5 •C in rectal temperature were selected for the experiment.

Effect of extract on brewer's yeast-induced pyrexia

Animals were divided randomly into seven groups of five animals each. Three groups received the ethanolic extract (30, 100, 300 mg kg^{-1}, *p.o.*) whilst three other group were given paracetamol (10, 30, 100 mg kg^{-1}, *p.o.*) which served as the reference drug. Control group received 0.5 ml saline solution. Rectal temperatures were determined before and at hourly intervals up to 4 h after extracts/drugs administration.

3.2.4.2 Data Analysis

Raw scores for basal and changes in rectal temperature were individually normalized as percentage of change from their values at time 0, and then averaged for each treatment group. The time-course curves for changes in rectal temperature was subjected to two-way (*treatment* × *time*) repeated measures analysis of variance with Bonferroni's *post hoc* test. Total change in rectal temperature for each treatment was calculated in arbitrary unit as the area under the curve (AUC). Data was subsequently treated as described for the acute inflammation in section 3.2.2.2.

3.2.4.3 RESULTS

Rectal temperatures before yeast injection ranged from 36.2 to 37.3 °C with an overall mean ± s.e.m of 36.59±0.03 °C (n=42). Intraperitonial injection of yeast

increased significantly (t=16.8; paired t-test), the rectal temperature to between 36.8 to 38.3 °C with a mean of 37.52±0.06. Mean differences in the pre- and post-injection temperatures was 0.93 °C with a 95% confidence interval of 0.82-1.05. PHE (30, 100 and 300 mg kg^{-1}) administered orally, dose-dependently and significantly ($F_{4,120}$=37.99; P<0.0001) reduced the increased rectal temperature induced by intraperitonial injection of the yeast as shown in Figure 4.11. Furthermore, PHE produced a significant ($F_{4,\ 15}$=32.70; P<0.0001) and dose-dependent decrease in total pyrexia represented as AUCs in Figure 4.11b. Similarly, a two-way ANOVA (*treatment group* × *time*) of time course curves revealed a significant treatment effect for paracetamol ($F_{4,120}$=88.84; P<0.0001).(Figure 4.11c). Also, paracetamol significantly ($F_{4,15}$=78.87; P<0.0001) and dose-dependently decreased the area under the curves of the time course curves compared to that of vehicle-treated group (Figure 4.11d). On analysis of data by non-linear regression, the IC_{50}s obtained were 265.10±63.73 and 18.05±4.08 mg kg^{-1} respectively for PHE and paracetamol. Thus the extract was ≈ 15 times less potent than the standard, paracetamol.

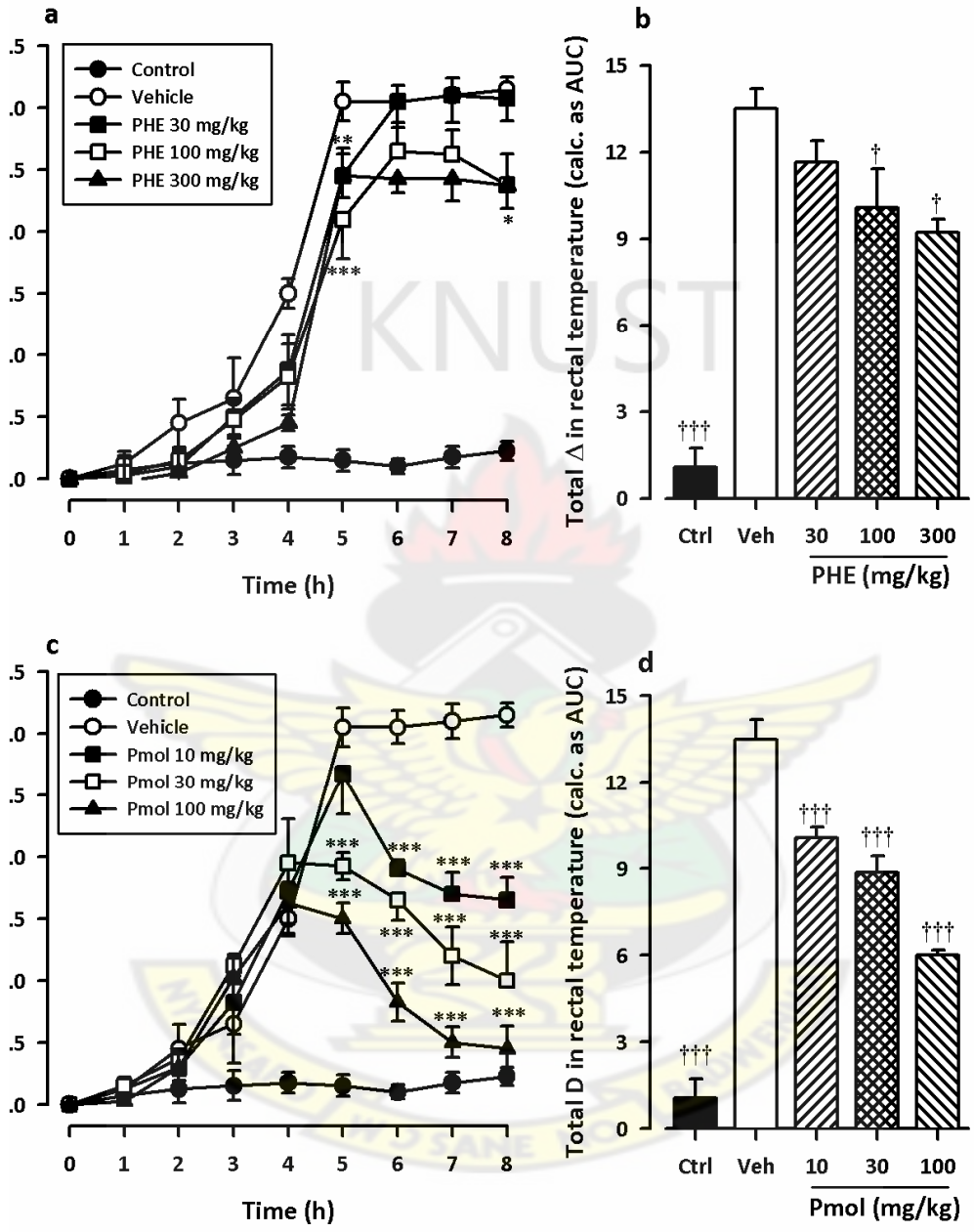

Figure 3.6 (a and c)Time course effect of PHE (10-300 mg kg^{-1} *p.o*) and Paracetamol (10-100 mg kg^{-1} *p.o*) on baker yeast-induced fever in young rats and (b and d)the AUC (total edema) for variation of rectal temperature along time, compared to 0.9% NaCl or vehicle. Each point in a and c represents the 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 followed by Bonferroni's *post hoc*); each column in (b and d) represent the mean ± S.E.M. $^{\dagger}P \leq 0.05$, $^{\dagger\dagger}P \leq 0.01$, $^{\dagger\dagger\dagger}P \leq 0.001$ (one-way ANOVA followed by Newman-Keuls *post hoc* test)

3.2.4.4 DISCUSSION

The extract exhibited antipyretic activity in yeast-induced pyrexia in rats. Fever may be a result of infection or one of the sequelae of tissue damage, inflammation, graft rejection, or other disease states (Ryan *et al.*, 2003). Baker's yeast (a lipopolysaccharide which is a cell wall component of gram negative bacteria) is an exogenous pyrogen which binds to an immunological protein called lipopolysaccharide-binding protein (LBP). It is currently accepted that prostaglandin E_2 (PGE_2) is the final fever mediator in the brain, specifically in the preoptic area of the anterior hypothalamus (Li *et al.*, 2008). It slows the rate of firing of warm sensitive neurons and results in increased body temperature. The set-point temperature of the body will remain elevated until PGE_2 is no longer present (Ryan *et al.*, 2003; Santos and Rao, 1998). Antipyretic activity is commonly mentioned as a characteristic of drugs or compounds which have an inhibitory effect on prostaglandin-biosynthesis and an indispensable role of prostaglandins in the febrile response has been demonstrated (Panthong *et al.*, 2003; Romanovsky *et al.*, 2005), thus it may be plausible to conclude that the extract inhibits the synthesis of prostaglandins. However, it must be noted that several biochemical events occur leading ultimately to the synthesis of PGE_2. Fever is believed to result from a finely tuned, complex event that involves both the peripheral immune system and the brain, through which a series of inflammatory and metabolic processes are regulated (Inoue *et al.*, 2008; Roth *et al.*, 2006). It is established that there are two pathways leading to the transcription and induction of cyclooxygenase (COX)-2, the rate limiting enzyme for prostaglandin (PGE_2) synthesis (Inoue *et al*, 2008). Both pathways are activated by cytokines e.g. IL-1•, IL-6 and tumor necrosis factor (TNF) which trigger central mechanisms that act via the transcription factors such nuclear factor (NF)•B and signal transducer and activator of transcription (STAT-3) (Inoue *et al*, 2008). It may therefore be worthwhile to investigate the exact point in the biochemical events where the extract exerts it antipyretic effect.

3.2.4.5 CONCLUSION

The ethanolic leaf extract of *Palisota hirsuta* exerts anti-pyretic activity probably through the inhibition of prostaglandins synthesis.

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3.2.5 ANTI-OXIDANT ACTIVITY

3.2.5.1 Reducing Power

The reducing power of the total crude extract (0.1, 0.3, 1, 3 mg ml^{-1}, methanol) was determined by its ability to reduce Fe^{3+} to Fe^{2+} (Oyaizu, 1986) as previously described by Amarowicz *et al.*, (2005) with modifications using *n*-propyl gallate (0.001-0.03 mg ml^{-1}, in methanol) as standard.

Principle:

The method depends upon the ability of a test compound to reduce Fe^{3+} to Fe^{2+}. The resultant Fe^{2+} then reacts with ferricyanide ion to form a Prussian blue complex with maximum absorbance at 700 nm.

$$Fe^{3+} \rightarrow Fe^{2+}$$

$$K_3Fe(CN)_{6(aq)} + Fe^{2+}{}_{(aq)} \rightarrow KFe\,[Fe(CN)_6] + 2\,K^{+}{}_{(aq)}$$

Equation 3.1 Equations for the reduction of Fe^{3+} to Fe^{2+}

The greater the reducing power, the greater the intensity of blue complex and the higher the absorbance.

Experimental design:

The drug/extract (1 ml) was mixed with 2.5-ml of 0.2M sodium phosphate buffer (pH 6.6) and 2.5 ml 1% potassium ferricyanide solution [$K_3Fe(CN)_{6(aq)}$] in a test tube. The mixture was incubated at 50˚C for 20 min. Following this 1.5 ml of 10% trichloroacetic acid solution (TCA) was added to the incubated mixture, and centrifuged at 3000 rpm for 10 minutes. Four replicates were used.

The supernatant (2.5 ml) was then mixed with 2.5 ml distilled water and 0.5 ml of 0.1% ferric chloride solution ($FeCl_{3(aq)}$) in a test tube. The absorbance was then measured at 700 nm using spectrophotometer (Model CE 7200, Cecil Instrument Limited, Milton Technical Centre, England).

Distilled water (1 ml) was added to 2.5ml sodium phosphate buffer and 2.5 ml potassium ferricyanide [$K_3Fe(CN)_6$] in a test tube. This mixture was then treated through the same processes as the test drugs and used as blank.

Four replicates were used. Data was presented as concentration-absorbance curves and the EC_{50} (concentration that gives 50% of maximal response) computed using GraphPad Prism for Windows version 4.02 (GraphPad Software, San Diego, CA, USA).

3.2.5.2 DPPH Scavenging Assay

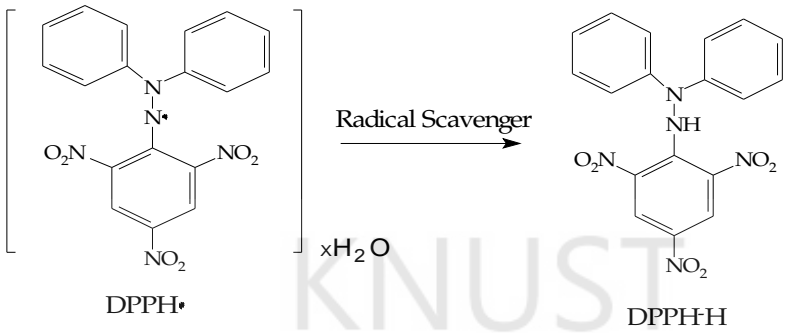
The experiment was carried out as previously described by Govindarajan *et al.*, (2003) with a few modifications.

Principle:

DPPH, 2, 2-diphenyl-1-picrylhydrazyl hydrate is a stable radical with characteristic violet colour (and maximum absorption at 517 nm) accepts an electron or hydrogen in the presence of a suitable free radical scavenger (reducing agent) to form reduced 2, 2-diphenyl-1-picrylhydrazyl, which is yellow in colour (Equation 3.2). The residual DPPH is then determined at 517 nm in a spectrophotometer. The absorbance decreases with increasing free radical scavenging ability.

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

$$\%\ \text{DPPH Scavenging effect} = \frac{\text{ABSROBANCE}_{\text{control}} - \text{ABSORBANCE}_{\text{test}}}{\text{ABSORBANCE}_{\text{control}}} \times 100$$



Equation 3.2 DPPH molecular structure and its reduced form

The absorbance decreases with increasing free radical scavenging ability.

Experimental design:

The extract (0.1, 0.3, 1, 3 mg ml^{-1} in methanol) was compared to *n*-propyl gallate (0.00083-0.025 mg ml^{-1} in methanol) as standard free radical scavenger.

The test drug (1 ml) was added to 3 ml methanolic solution of DPPH (20 mg l^{-1}) in a test tube. The reaction mixture was kept at 25˚C for 1 h in an orbital shaker incubator (BoroLabs, Aldermaston Berkshire). The absorbance of the residual DPPH was determined at 517nm in a spectrophotometer (Model CE 7200 spectrophotometer, Cecil Instrument Limited, Milton Technical Centre, England). One ml of methanol (99.8%) (1 ml) was added to 3 ml DPPH solution, incubated at 25˚C for 1 h and used as control. Methanol (99.8%) was used as blank.

Four replicates were used. Data was presented as % DPPH scavenging effect against concentration and the EC_{50} determined. All data was analysed using GraphPad Prism for Windows version 4.02 (GraphPad Software, San Diego, CA, USA). Levels of significance were determined by analysis of variance (ANOVA).

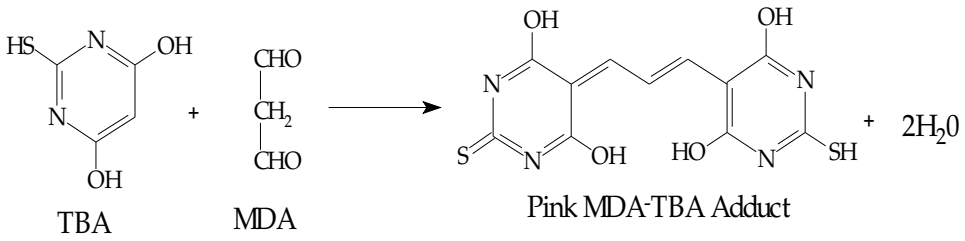
3.2.5.3 Lipid Peroxidation Assay

The extent of lipid peroxidation in rat brain homogenate was determined using thiobarbituric acid (TBA), as previously described (Auddy *et al.*, 2003; Ohkawa *et al.*, 1979), with modifications.

Principle:

The oxidation of polyunsaturated fatty acid during oxidative burst results in oxidized derivatives whose level can be assayed as a characteristic index of oxidation. Amongst a great variety of aldehydes that are produced during lipid peroxidation, malondialdehyde (MDA) is the oxidized derivative most widely used as indicator of free radical damage. In the assay, MDA reacts with TBA (thiobarbituric acid) giving a pink colored TBA-MDA adduct (Equation 3.3) that is spectrophotometrically measured at 535 nm to determine the degree of lipid peroxidation.

The assay involves the initiation of lipid peroxidation in rat brain homogenate (as a source of PUFA) by the Haber-Weiss and Fenton reactions (Equation 3.4), in which the combination of ascorbic acid and Fe^{3+} leads to a cascade of reactions, converting Fe^{3+} to Fe^{2+} with the subsequent generation of highly charged hydroxyl radical. The hydroxyl radical formed attacks the polyunsaturated fatty acids (PUFA) leading to the formation of MDA amongst other oxidative products. The reaction of TBA (thiobarbituric acid) with MDA (malondialdehyde) give a pink colored TBA-MDA adduct that can be assayed spectrophotometrically at 532nm



Equation 3.3 The reaction of TBA (thiobarbituric acid) with MDA (malondialdehyde) give a pink colored TBA-MDA adduct.

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$$Fe^{3+} + \text{Ascorbic acid} \rightarrow Fe^{2+} + \text{Ascorbic acid}\bullet$$

$$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2\bullet^- \quad \left.\right\} \text{Haber-Weiss}$$
$$2O_2\bullet^- + 2H^+ \rightarrow H_2O_2 + O_2$$

$$Fe^{3+} + O_2\bullet^- \rightarrow Fe^{2+} + O_2 \quad \left.\right\} \text{Fenton reaction}$$
$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH\bullet$$

Equation 3.4 The Haber-Weiss and Fenton reactions.

Experimental design

Brain homogenate: Male Sprague-Drawley rats weighing (200-250 g) were sacrificed by decapitation and whole brain was dissected out and homogenized (100 mg ml^{-1}) in ice-cold 0.1M phosphate buffer (pH 7.4) using Ultra-Turrax T 25 homogenizer (IKA Labortehnic, Staufen, Germany).

Initiation of lipid peroxidation: Brain homogenate (2.5 ml) was mixed with 1 ml phosphate buffer and 0.5 ml of the test drug. Lipid peroxidation was then initiated by the addition of 0.5 ml of 0.1 mM ascorbic acid and 0.5 ml 5 mM $FeCl_3$. The mixture was then incubated in an orbital shaker incubator (BoroLabs, Aldermaston Berkshire) at 37˚C for 1 h.

Assay of Thiobarbituric Acid Reactive Substances (TBARS)

After 1 h of incubation, 0.1ml of the reaction mixture was taken into a test tube containing 1.5 ml of 10% trichloroacetic acid (TCA), and allowed to stand for 10 min. After 10 min, tubes were centrifuged at 5000 *g* for 10 min. The supernatant was separated and mixed in a tube containing 1.5 ml of 0.67% thiobarbituric acid (TBA) in 20% acetic acid. The mixture was heated in a hot water bath at 85˚C for 1 h to complete the reaction and allowed to cool. The intensity of the pink-colored complex formed was measured at 535 nm in a spectrophotometer (Model CE 7200, Cecil Instrument Limited, Milton Technical Centre, England). The absorbance decreases with increasing ability to inhibit lipid peroxidation.

Phosphate buffer was used as blank throughout the experiment.

The extract (0.01-0.1 mg ml^{-1} in methanol) was compared to *n*-propyl gallate (0.01-0.10 mg ml^{-1} in methanol).

Each test was carried out using three replicates.

To calculate the percentage inhibition of lipid peroxidation, the following controls were prepared:

Homogenate alone (H): (2.5-ml homogenate + 2.5-ml phosphate buffer)

Full Reaction Mixture (FRM): (2.5-ml homogenate + 1-ml phosphate buffer + 0.5 methanol + 0.5 ml ascorbic acid + 0.5-ml $FeCl_3$(aq)

Drug alone/Extract alone: (0.5-ml of the selected dose of the drug/extract + 3.5-ml phosphosphate buffer).

The percentage inhibition of lipid peroxidation was then calculated from the following equation:

$$\text{\% Inhibition of lipid peroxidation} = \frac{(\text{FRM - H}) - (\text{DRUG/EXTRACT TEST - (DRUG/EXTRACT ALONE - H)})}{(\text{FRM - H})} \times 100$$

Where

FRM (Full Reaction Mixture) determines the degree of lipid peroxidation in the absence of antioxidant.

H (Homogenate alone) determines the underlying lipid peroxidation before the initiation of lipid peroxidation.

Data was presented as % inhibition of lipid peroxidation against concentration and the EC_{50} (concentration that produces 50% of the maximal effect of drug) for each drug determined from concentration-response curves using GraphPad Prism for Windows version 4.02 (GraphPad Software, San Diego, CA, USA).

3.2.5.4 RESULTS

Reducing Power

The extract (0.001- 0.03 mg ml^{-1}) and the standard antioxidant *n*-propyl gallate (0.1-3 mg ml^{-1}) concentration dependently reduced Fe^{3+} to Fe^{2+} resulting in concentration dependent increase in absorbance (Figure3.10 b). From the EC_{50} (in mg ml^{-1}; Table 3.5) obtained for the extract (133.70 ± 7.59) and *n*-propyl gallate (3.77 ±0.07), the extract was found to be about 35 fold less potent than *n*-propyl gallate.

DPPH Scavenging Assay

Both the standard, *n*-propyl gallate (0.00083 - 0.025 mg ml^{-1}) and the extract (0.1- 3 mg ml^{-1}) exhibited concentration dependent free radical scavenging activity (Figure 3.10a). The rank order of potency (defined by EC_{50} in mg ml^{-1}; Table 3.5) was found

to be: *n*-propyl gallate ($8.02 \pm 0.01 \times 10^{-4}$) > extract ($1.77 \pm 0.40 \times 10^{-1}$). The extract was however found to be about 220 fold less potent than *n*-propyl gallate.

Lipid Peroxidation

Several concentrations of *n*-propyl gallate (0.01- 0.1 mg ml^{-1}) and the extract (0.1-1.0 mg ml^{-1}) were tested for inhibitory action on ascorbatate/Fe^{3+} induced lipid peroxidation. All drugs showed concentration dependent ability to inhibit lipid peroxidation (Figure 3.10c). The rank order of potency (defined by EC_{50} in mg ml^{-1}; Table 3.5) was found to be: *n*-propyl gallate ($1.31 \pm 3.00 \times 10^{-2}$) > extract ($4.29 \pm 0.99 \times 10^{-1}$). The extract was found to be about 32 fold less potent than *n*-propyl gallate a physiological antioxidant used as standard.

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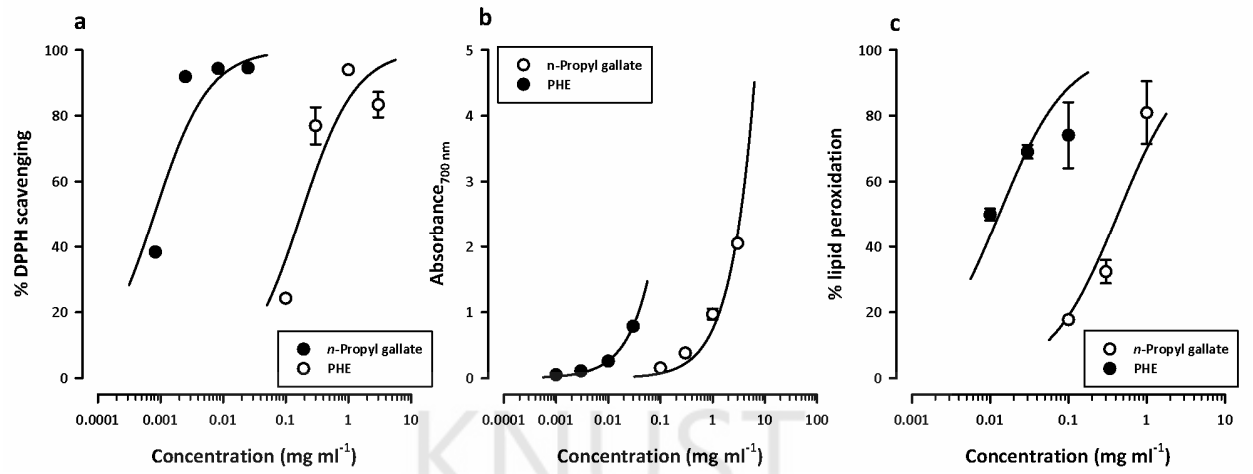

Figure 3.7 Free radical scavenging ability of PHE (0.1-3 mg ml^{-1}) compared to n-propyl gallate (0.00083-0.025 mg ml^{-1}) in the DPPH radical assay, reducing power of PHE (0.1-3 mg ml^{-1}) compared to n-propyl gallate (0.001-0.03 mg ml^{-1}) and percentage inhibition of lipid peroxidation by PHE (0.1-1 mg ml^{-1}) compared to *n*-propyl gallate (0.01-0.1 mg ml^{-1}). Each point represents the mean ± S.E.M

Table 3.5 ED_{50} Antioxidant Properties

Drug	**ED_{50} (mg ml^{-1})**		
	Reducing Power	*DPPH Scavenging*	*Lipid Peroxidation*
P.hirsuta extract	133.70±7.59***	$1.77\pm0.40\times10^{-1}$***	$4.29\pm0.99\times10^{-1}$***
n-Propyl gallate	3.77±0.07	$8.02\pm0.01\times10^{-4}$	$1.31\pm3.00\times10^{-2}$

*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ compared to reference antioxidant (One-way ANOVA followed by Neuman-Keul's *post hoc* test.

3.2.5.5 DISCUSSION

In the present study the antioxidant activity of the extract was evaluated using three different assays: reducing power test, DPPH scavenging activity and the of lipid peroxidation assay.

Polyphenols are electron-rich compounds and capable of entering into efficient electron-donation reactions with oxidizing agents (Kang *et al.*, 2005). Various plant phenols have been found to interfere with the oxidation of different biomolecules important for life (Amarowicz *et al.*, 2005). Prior to this, a routine test using ferric chloride was done to determine the presence of phenols in the extract, which gave a positive result indicating that the extract contains some amount of plant phenols. The potent reductive capabilities exhibited by the extract through the reduction of Fe^{3+} to Fe^{2+} in the reducing power test demonstrates the potential of the leaves extract to donate electrons to free radicals or reactive oxygen species as is exhibited by chain-breaking antioxidants like *n*-propyl gallate and α-tocopherol (Vitamin E) (Scheibmeir *et al.*, 2005). The process gives rise to low energy products that are unable to propagate free radical formation any further (Cui *et al.*, 2004). The extract caused a concentration dependent de-colorization of DPPH solution. This is an expression of the extracts 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 extract and the standard drug effectively inhibited lipid peroxidation, a proof of their potent antioxidant properties. There are many possible mechanisms involved in the anti-lipid peroxidation property. The effect may be mediated through their scavenging effect on the super oxide anion radical ($O_2^{\bullet-}$) and the hydroxyl radical ($OH^{\bullet}$) formed during the Haber-Weiss reaction and Fenton reaction receptively.

3.2.5.6 CONCLUSION

P. hirsuta leaf extract showed potent antioxidant properties by exhibiting potent reductive capabilities, causing a dose dependent de-colorization of DPPH solution and inhibiting lipid peroxidation.

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Chapter 4

ANTI-NOCICEPTIVE EFFECT

4.1 INTRODUCTION

Pain is a common situation and it is one of the most frequent presenting symptoms of different pathologies. The whole plant of *P. hirsuta* and various parts are used extensively in West African medicine for various pain related conditions (Burkill, 1985; Dokosi, 1998). Based on the traditional use of the plant as an anti-nociceptive agent, this study was carried out in various experimental animal models of pain to substantiate this traditional claim. Moreover, pain is one of the cardinal signs of inflammation and most drugs used as anti-inflammatory agents double as analgesics. The anti-nociceptive effect of the plant extract was assessed using animal models that predict both peripheral and centrally mediated pain. Some receptors and neurotransmitters involved in the pain mediation were also investigated using different antagonist and agonist to help predict the possible mechanism of action of the extract.

4.2 ANTI-NOCICEPTIVE EFFECT

4.2.1 METHODS

4.2.1.1 Animals

Sprague-Dawley rats (150–200 g) and ICR mice (20-25 g) of both sexes were used and cared for as previously described in section 3.2.

4.2.1.2 Drugs

Diclofenac sodium was purchased from Troge, Hamburg, Germany, morphine hydrochloride from Phyto-Riker, Accra, Ghana, carrageenan sodium salt, naloxone hydrochloride and N^G-Nitro-L-arginine methyl ester (L-NAME) were also obtained from Sigma-Aldrich Inc., St. Louis, MO, USA, formalin, acetic acid and theophylline were also purchased from British Drug House, Poole, England whilst glibenclamide, (Daonil®) was from Sanofi-Aventis, Guildford, UK.

4.2.1.3 Tail immersion test

The tail immersion test was carried out according to the method described by Janssen *et al.*, (1963) and modified by Savegnago *et al.*, (2007). Tail withdrawal latency, defined by the time (in seconds) to withdraw the tail from hot water maintained at 50.0± 1.0 °C, was measured using a stopwatch. A cut-off time of 10 s was set to avoid tissue damage. Increase in tail withdrawal latency was defined as anti-nociception and calculated as % maximum possible effect (MPE). The maximum possible anti-nociceptive effect was reached when the animals did not show a tail withdrawal reaction within the cut-off time of 10 s. % MPE was calculated according to the formula: $[(T_1-T_0)/ (T_2-T_0)] \times 100$, where T_0 and T_1 are the latencies obtained before and after drug treatment, and T_2 is the cut-off time. Animals were tested before and at 30, 60, 90, 120, 150 and 180 min after administration of PHE (30-300 mg kg^{-1}, *p.o.*), morphine (1-10 mg kg^{-1}, i.p.) or diclofenac (10-100 mg kg^{-1}, i.p.). A single habituation test was used before baseline test to minimize learning effects.

4.2.1.4 Carrageenan-Induced Mechanical Hyperalgesia

Mechanical nociceptive thresholds were measured in the rat paw pressure test (Randall and Selitto, 1957) and as modified by Villetti *et al.*, (2003) and Stohr *et al.*, (2006) using an analgesimeter (Model No. 15776, Ugo Basile, Comerio, Varese, Italy) which is based on the Randall–Selitto test (Randall and Sellito, 1957). The analgesimeter was used to apply a linearly-increasing pressure, by means of a blunt perspex cone, to the dorsal region of the right hind paw until the rat withdrew the paw. Rats received two training seasons before the day of testing. Pressure was gradually applied to the right hind paw and paw withdrawal thresholds (PWTs) were assessed as the pressure (grams) required to elicit paw withdrawal. A cut-off point of 250 g was used to prevent any tissue damage to the paw. A change in hyperalgesic state was calculated as a percentage of the maximum possible effect (% MPE). On the test day, a baseline measurement was taken before animals were administered carrageenan (100 µl of a 20 mg ml^{-1}

solution) into the right hind paw. PWTs were determined again 2.5 h after carrageenan to establish that mechanical hyperalgesia had developed. PHE (30-300 mg kg^{-1}, *p.o*), morphine (1-10 mg kg^{-1}, i.p) or diclofenac (10-100 mg kg^{-1}, i.p) were then administered 3 h post-carrageenan, and PWTs were taken again at 3.5, 4, 4.5, 5, 5.5 and 6 h post-carrageenan.

4.2.1.5 Acetic acid-induced abdominal constriction

ICR mice (20-30 g) were used according to the method described by Amresh *et al.*, (2007) with slight modifications. The total number of writhing following intraperitoneal administration of 10 ml kg^{-1} of 0.6% acetic acid was recorded over a period of 20 min, starting 10 min after the acetic acid injection. The response induced by i.p. injection of acetic acid consists of a contraction of the abdominal muscle, together with a stretching of the hind limbs. Animals received PHE (30-300 mg kg^{-1}, *p.o.*), diclofenac (10-100 mg kg^{-1}, i.p.) or morphine (1-10 mg kg^{-1}, i.p.) 30 min before the acetic acid administration. Control animals received vehicle.

4.2.1.6 Formalin- induced nociception

The formalin test first described by Dubuisson and Dennis, (1977) was carried out as described by Malmberg and Yaksh, (1995), with a few 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 1h 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 behavior of the animal was then captured (60 min) 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 defined conservatively as 0-10 minutes and the second phase 10-60 minutes post formalin injection (Wilson *et al*., 2002). Nociceptive behavior was quantified 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. Mice were randomly selected for one 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

The extract was prepared in 2 % tragacanth mucilage. Drug solutions and suspensions were prepared such that not more than 1-ml of extract was given orally and not more than 0.5 ml of the standard drugs were injected intraperitoneally. All drugs were freshly prepared.

4.2.1.7 Mechanism of action of PHE in the formalin test

To investigate the possible mechanisms by which PHE inhibits formalin-induced nociception, mice were pretreated with different drugs. The doses of antagonist, agonist and other drugs were selected on the basis of previous literature data and in pilot experiments in our laboratory. The formalin test was chosen for this purpose because of the specificity and sensitivity in nociception transmission that this model provides. (Le Bars *et al.*, 2001)

Involvement of opioid system: To assess the role of the opioid system, mice were pretreated intraperitoneally (i.p.) with naloxone (2 mg kg^{-1}, a nonselective opioid

receptor antagonist). After 15 min the animals received PHE (100 mg kg^{-1}, *p.o.*), morphine (3 mg kg^{-1}, i.p.) or vehicle (10 ml kg^{-1}, *p.o.*). The nociceptive response to the formalin intraplantar injection was recorded 60 min after administration of PHE or vehicle and 30 min after administration of morphine.

Involvement of adenosinergic system: To investigate the role played by the adenosinergic systems in the anti-nociception caused by PHE, mice were pretreated with theophylline (5 mg kg^{-1}, i.p., a nonselective adenosine receptor antagonist). After 15 min the mice received PHE (100 mg kg^{-1}, *p.o.*), morphine (3 mg kg^{-1}, i.p.) or vehicle (10 ml kg^{-1}, *p.o.*). The nociceptive response to the formalin intraplantar injection was recorded 60 min after administration of PHE or vehicle and 30 min after morphine administration.

Involvement of ATP sensitive K^+ channels: To explore the possible contribution of K^+ channel in the anti-nociceptive effect of PHE, mice were pretreated with glibenclamide (an ATP-sensitive K^+ channel inhibitor, 8 mg kg^{-1}, *p.o.*), or vehicle and after 30 min they received PHE (100 mg kg^{-1}, *p.o.*), morphine (3 mg kg^{-1}, i.p.) or vehicle. The nociceptive responses to formalin were recorded 60 min after administration of PHE or vehicle and 30 min after morphine administration.

***Involvement of the nitric oxide system*:** To verify the possible involvement of nitric oxide/cyclic GMP pathway in the anti-nociceptive action caused by PHE, mice were pretreated with N^G-L-nitro-arginine methyl ester (L-NAME, a NO synthase inhibitor; 10 mg kg^{-1}, i.p.) or saline (0.9% NaCl, i.p.) 30 min before PHE (100 mg kg^{-1}, *p.o.*), morphine (3 mg kg^{-1}, i.p.) or vehicle administration. The nociceptive responses to formalin were recorded 60 min after administration of PHE or vehicle and 30 min after morphine administration.

4.2.1.8 Tolerance Studies

The mouse paw formalin test was used to ascertain whether, after chronic treatment, tolerance develops to the anti-nociceptive activity of PHE and morphine. Mice were divided randomly into five groups (n =5) and treated once

daily for 8 days as follows: three groups with saline i.p., one group with PHE 200 mg kg^{-1}, *p.o.*, and one group with morphine 6 mg kg^{-1}, i.p. On day 9, these groups were treated in the following manner: one saline-pretreated group was treated with saline i.p.; two saline-pretreated groups were treated either with PHE 100 mg kg^{-1}, *p.o*. or with morphine 3 mg kg^{-1}, i.p.; the group pretreated with PHE 200 mg kg^{-1} was treated with PHE 100 mg kg^{-1}, *p.o.*, and the group pretreated with morphine 6 mg kg^{-1} was treated with morphine 3 mg kg^{-1}, i.p. PHE and morphine were administered 60 and 30 min before formalin injection, respectively. In a separate study, PHE was administered to animals chronically treated with morphine to establish whether morphine-induced tolerance cross-generalizes with PHE. In the second study, two groups of animals (n =5) were treated once daily for 8 days with morphine 6 mg kg^{-1}, i.p. Three other groups of animals (n =5) received chronic dosing of saline i.p. also for 8 days. On day 9, animals treated with chronic morphine received either morphine (3 mg kg^{-1}, i.p., 30 min before formalin) or PHE (100 mg kg^{-1}, *p.o.*, 60 min before formalin, respectively), whereas three saline-treated groups received either a similar administration of saline, morphine (3 mg kg^{-1}, i.p.), or PHE (100 mg kg^{-1}, *p.o.*).

4.2.1.9 Data Analysis

In all experiments, a sample size of five animals (n=5) were used. Raw data was calculated as the percentage change in maximum possible effect (%MPE).The time-course curves were subjected to two-way (*treatment* × *time*) repeated measures analysis of variance (ANOVA) with Bonferroni's *post hoc test*. Total nociceptive score for each treatment was calculated in arbitrary units as the area under the curve (AUC). Percentage inhibition for each treatment and ED_{50} values were calculated and treated as described for the acute inflammation in section 3.2.2.2.

4.2.2 RESULTS

4.2.2.1 Tail-immersion test

All the test drugs caused an increase in the tail withdrawal latency. PHE (30–300 mg kg^{-1}, *p.o.*) (Fig. 4.1a) caused a significant and dose dependent increase in the withdrawal latencies of the tail as depicited in the time-course curve ($F_{3,16}$= 5.44, P=0.009). As shown in Fig. 4.1b, PHE (300 mg kg^{-1}, p.o.) increased the withdrawal latency by 43.83±11.62%. Similarly, diclofenac (10–100 mg kg^{-1}, i.p.) (Fig. 4.1c) produced a significant anti-nociceptive activity by dose-dependently increasing the tail withdrawal latencies of animals pretreated with the drug ($F_{3,16}$= 10.81, P<0.0004) with the highest dose of 100 mg kg^{-1} causing a percentage increase of 73.75±14.99% as shown in (Fig. 4.1d). Animals pretreated with morphine (1–10 mg kg^{-1}, i.p.) also showed a great increase ($F_{3,16}$= 15.76, P<0.0001) in their tail withdrawal latencies as observed in the time-course curve in a dose-related manner (Fig. 4.1e) with the highest dose of 10 mg kg^{-1} causing total anti-nociceptive effect with %MPE of 103.83± 20.46% within the cut-off time of 10s. (Fig. 4.1f). Comparison of ED_{50}s obtained by non-linear regression (Fig. 4.2) revealed that the extract [ED_{50}: 52.12±19.86 mg kg^{-1}] was 26× less potent than morphine [ED_{50}: 1.99±0.76 mg kg^{-1}] and 3× less potent than diclofenac [ED_{50}: 13.74±5.24 mg kg^{-1}].

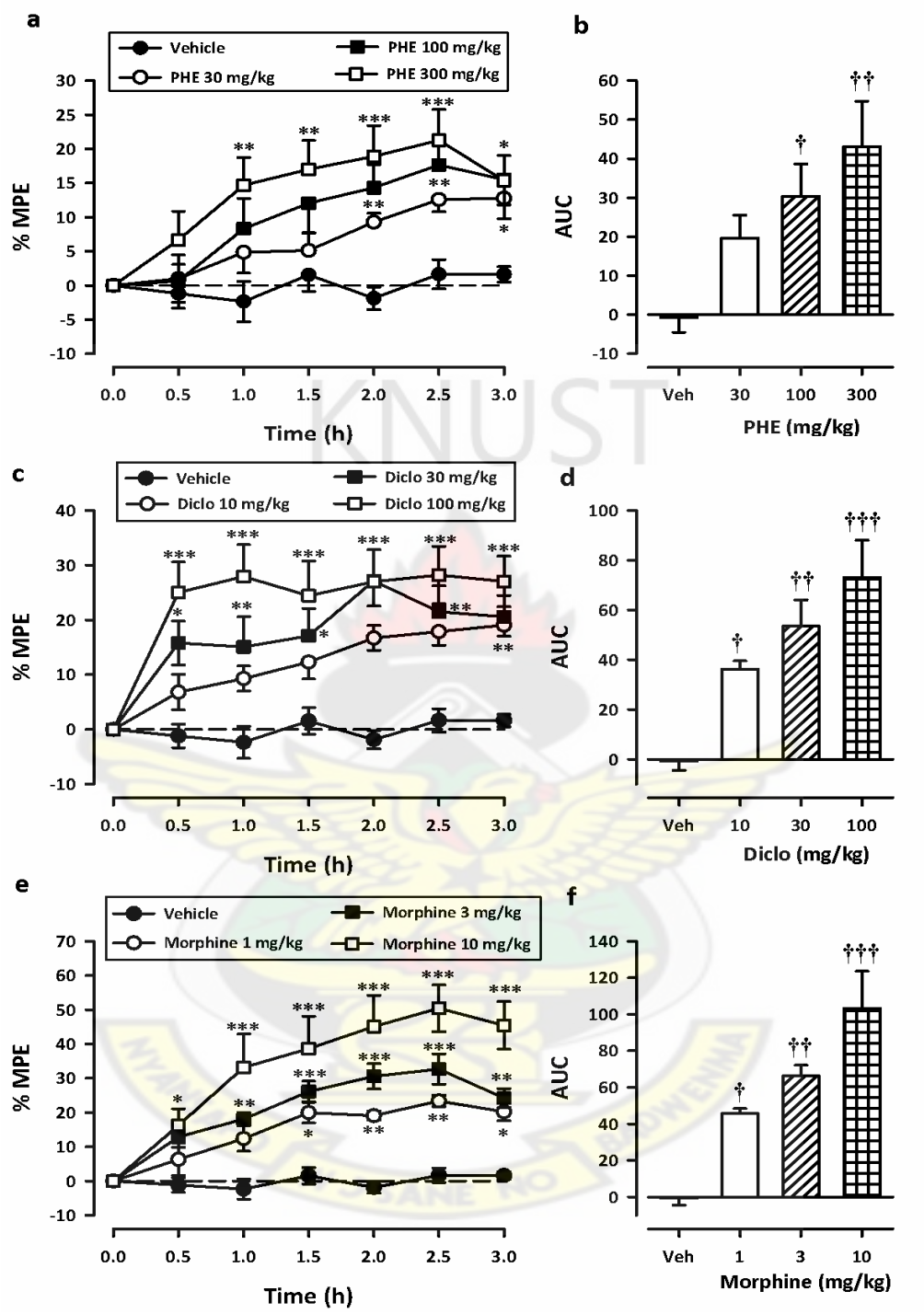

Figure 4.1 Effect of PHE (30-300 mg kg^{-1} *p.o*), diclofenac (10-100 mg kg^{-1} *i.p*) and morphine (1-10 mg kg^{-1} *i.p*) on time course curve of tail immersion test (a, c and e) and the AUC (b, d and f). Data was presented as mean ± S.E.M. (n = 5); ***$P < 0.001$; ** $P < 0.01$; *$P < 0.05$ compared to vehicle-treated group (Two-way ANOVA followed by Bonferroni's *post hoc* test). $^{\dagger\dagger\dagger}P<0.0001$ $^{\dagger}P<0.05$ compared to vehicle-treated group (One-way ANOVA followed by Neuman-Keul's *post hoc* test).

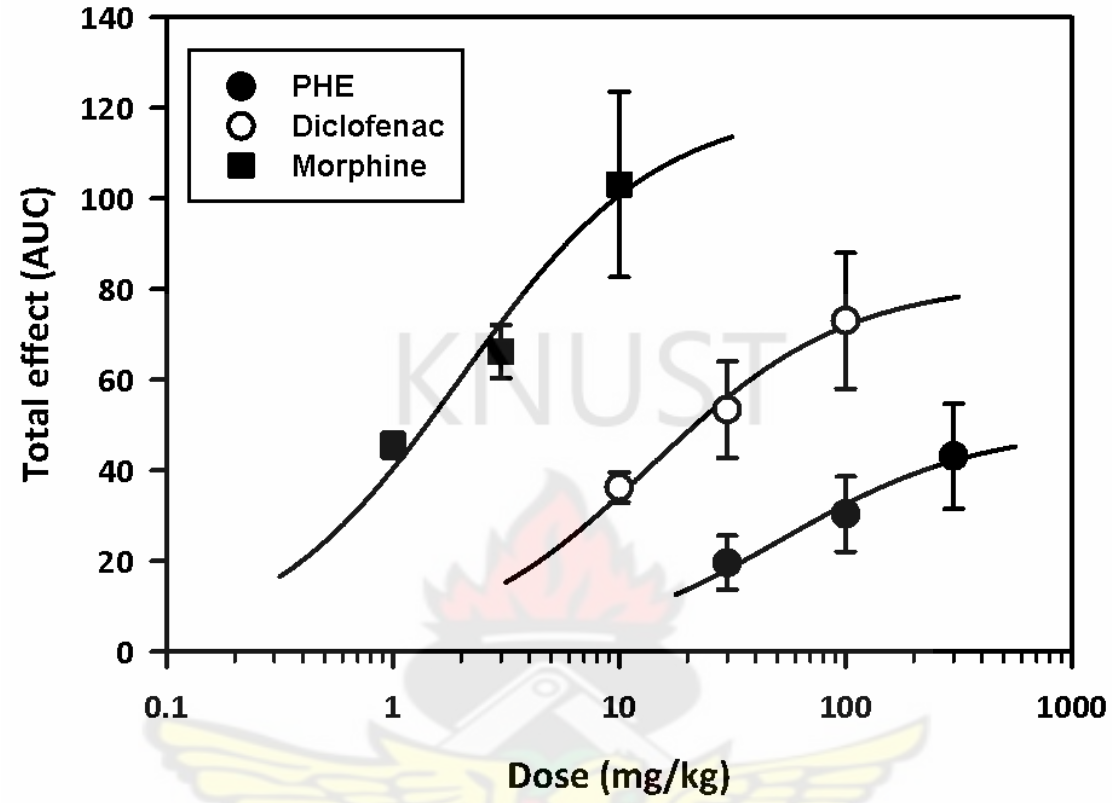

Figure 4.2 Dose response curves for PHE (30-300 mg kg^{-1} i.p), diclofenac (10-100 mg kg^{-1} i.p) and morphine (1-10 mg kg^{-1} *p.o*) on tail immersion test in rats.

4.2.2.2 Carrageenan-Induced Mechanical Hyperalgesia using Randall Sellito test

On the day of experiment, animals showed baseline withdrawal thresholds of about 60 to 150 g. Two and half hours (2.5 h) after carrageenan injection, the ipsilateral paw exhibited marked mechanical hyperalgesia in all experiments which was maintained in vehicle-treated animals throughout experiment. A change in hyperalgesic state was calculated as a percentage of the maximum possible effect. PHE (30–300 mg kg^{-1}, *p.o.*) administered 3 h after carrageenan produced a significant and dose-dependent reversal of mechanical hyperalgesia ($F_{3,16}$= 25.03, P<0.0001)(Fig. 4.3a). The highest dose of PHE completely reversed the inflammatory-induced mechanical hyperalgesia with %MPE of 154.79±15.84% as shown in Fig. 4.3b. The i.p. administration of diclofenac (10–100 mg kg^{-1}) significantly ($F_{3,16}$= 17.77, P<0.0001) and dose dependently relieved the mechanical hyperalgesia as decipited in (Fig. 4.3c). The highest dose of diclofenac also completely reversed the inflammatory-induced mechanical hyperalgesia with a %MPE of 153.09±16.52% (Fig. 4.3d). Morphine (1-10 mg kg^{-1}) after i.p. administration similarly antagonized mechanical hyperalgesia significantly ($F_{3,16}$= 18.31, P<0.0001) in a dose-dependent manner as shown in Fig.4.3e; with the highest dose completely and the highest reversing the hyperalgesia with %MPE of 178.19±19.83% as shown in the AUC curve (Fig. 4.3f).

When ED_{50}s obtained by non-linear regression were compared (Fig. 4.4), the extract [ED_{50}: 141.58±53.95 mg kg^{-1}] was found to be 56× less potent than morphine [ED_{50}: 2.55±0.97 mg kg^{-1}] and 9× less potent than diclofenac [ED_{50}: 14.69±5.60 mg kg^{-1}].

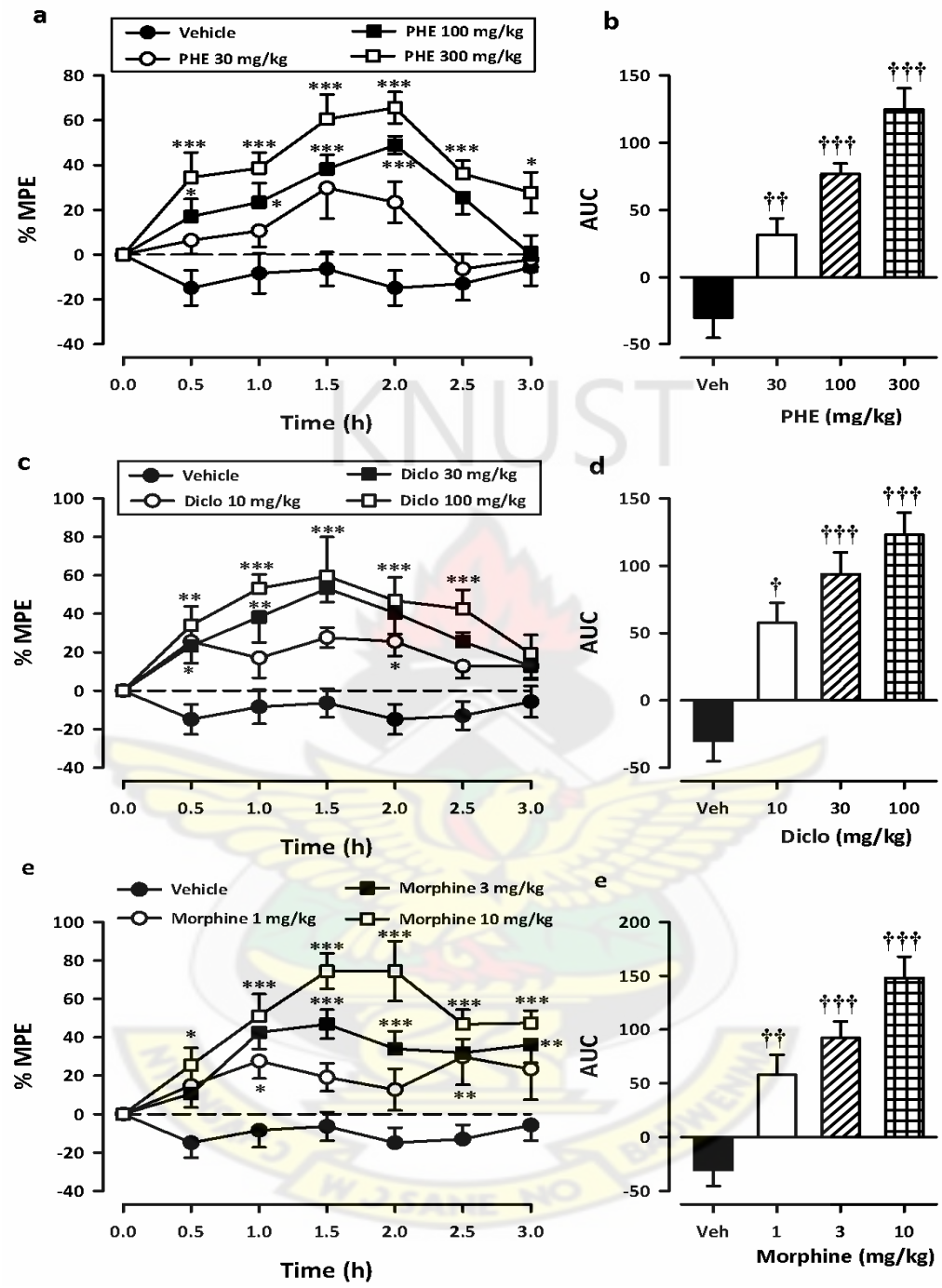

Figure 4.3 Effect of PHE (30-300 mg kg^{-1} *p.o*), diclofenac (10-100 mg kg^{-1} i.p) and morphine (1-10 mg kg^{-1} i.p) on time course curve in carrageenan-induced mechanical hyperalgesia in the rat using the Randall sellito model (a, c and e) and the AUC (b, d and f). Data was presented as mean ± S.E.M. (n = 5); ***$P < 0.001$; ** $P < 0.01$; *$P < 0.05$ compared to vehicle-treated group (Two-way ANOVA followed by Bonferroni's post hoc test). $^{\dagger\dagger\dagger}P<0.0001$ $^{\dagger}P<0.05$ compared to vehicle-treated group (One-way ANOVA followed by Neuman-Keul's post hoc test).

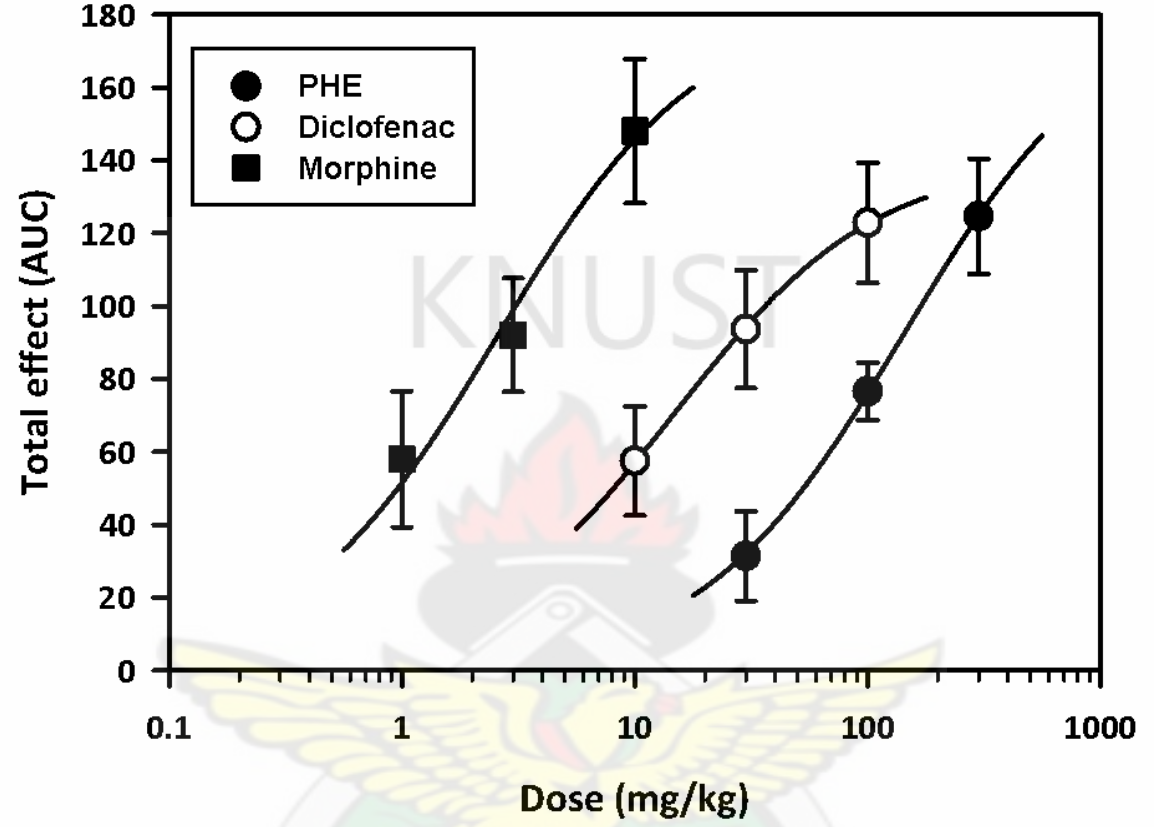

Figure 4.4 Dose response curves for PHE (30-300 mg kg^{-1} i.p), diclofenac (10-100 mg kg^{-1} i.p) and morphine (1-10 mg kg^{-1} *p.o*) on carrageenan-induced mechanical hyperalgesia in rats in the Randall sellito model

4.2.2.3 Acetic acid-induced writhing assay

Figure 4.5a represents the total number of writhes induced by acetic acid, during 20 min of observation, beginning 10 min after the i.p. injection. PHE (30-300 mg kg^{-1}, *p.o.*, 60 min before) significantly ($F_{3,16}$= 5.87, P=0.0067) reduced the number of writhing induced by acetic acid in mice (Fig. 4.5a) with the highest dose used reducing the total number of writhes by 70.85±8.73%. Morphine (1-10 mg kg^{-1}, i.p., 30 min before) and diclofenac (10–100 mg kg^{-1}, i.p., 30 min before) both significantly ($F_{3,16}$= 18.06, P<0.0001) and ($F_{3,16}$= 13.93, P=0.0001) respectively, also reduced the number of writhing induced by the acetic acid. The highest dose of morphine (10 mg kg^{-1}) used caused a percentage inhibition of 91.96±2.91% whilst the highest dose of diclofenac (100 mg kg^{-1}) gave a percentage inhibition of 83.92±10.35%.

Comparison of ED_{50}s obtained by non-linear regression (Fig. 4.5b) revealed that the extract [ED_{50}: 80.20±0.58 mg kg^{-1}] was 127× less potent than morphine [ED_{50}: 0.63±0.52 mg kg^{-1}] and 14× less potent than diclofenac [ED_{50}: 5.91±0.56 mg kg^{-1}].

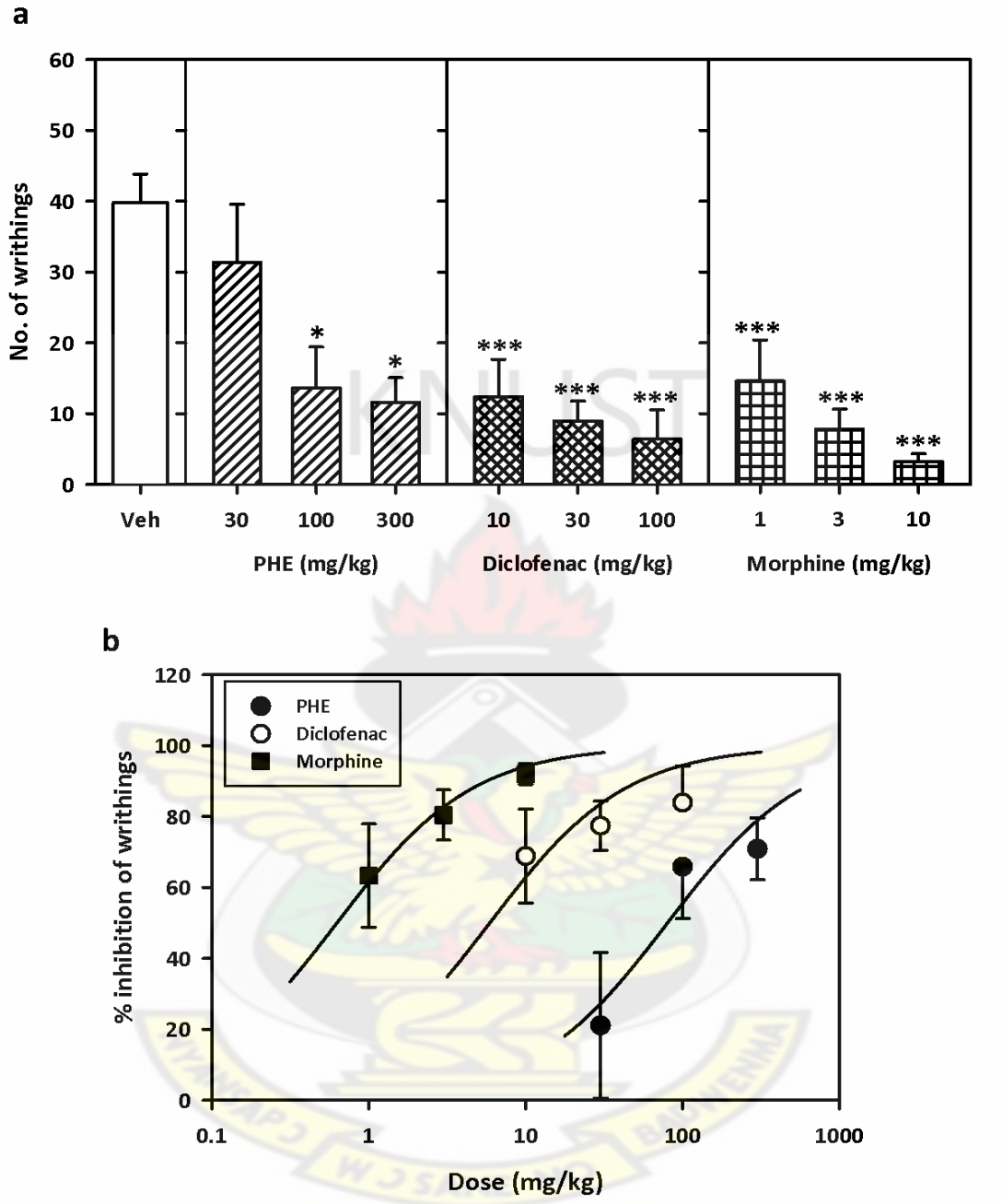

Figure 4.5 Effect of PHE (30 – 300 mg kg^{-1}), diclofenac (10 – 100 mg kg^{-1}) and morphine (1 – 10 mg kg^{-1}) on the total number of writhes induced by acetic acid in mice Data was presented as mean ± S.E.M. (n = 5); ***$P < 0.001$; ** $P < 0.01$; *$P < 0.05$ compared to vehicle-treated group (One-way ANOVA followed by Neuman-Keul's *post hoc* test). (b) Dose response curves for PHE (30-300 mg kg^{-1} i.p.), diclofenac (10-100 mg kg^{-1} i.p.) and morphine (1-10 mg kg^{-1} *p.o.*) on acetic acid induced writhing in mice.

4.2.2.4 Formalin-induced nociception

Formalin administration produced a typical pattern of flinching and licking behavior. The first phase started immediately after administration of formalin and then diminished gradually in about 10 min. The second phase started at about 15 min and lasted until 1 h. Treatment of mice with PHE (10–300 mg kg^{-1}, *p.o.*, 60 min before) (Fig. 4.6a and b) produced a marked and dose-related inhibition of both phases of formalin-induced nociception first phase ($F_{4,20}$= 17.35, P<0.0001) second phase ($F_{4,20}$= 22.729, P<0.0001) with the highest dose causing a maximal inhibition of 83.46±6.67% and 94.56±4.12% of the licking time in the early and late phase, respectively(Fig. 4.6a and b). Similarly, morphine (1-10 mg kg^{-1}, i.p.) produced marked inhibition of both the neurogenic ($F_{3,16}$= 18.61, P<0.0001) and inflammatory($F_{3,16}$= 18.39, P<0.0001) pain phases (Fig. 4.6c and d). Morphine, reduced the duration of formalin evoked nociceptive behavior by a maximum percentage of 90.36±4.68% in the early phase and 96.04±5.50% in the late phase of the formalin test (Fig. 4.6c and d). Comparison of ED_{50}s obtained by non-linear regression (Fig. 4.7) revealed that the extract was 4× more potent ($F_{1,38}$= 32.89, P<0.0001) in the second phase [ED_{50}: 5.36±2.04 mg kg^{-1}] than the first [ED_{50}: 21.88±8.34 mg kg^{-1}]. Likewise, morphine was three fold more potent ($F_{1,28}$= 19.97, P=0.0001) in the second phase [ED_{50}: 0.85±0.33 mg kg^{-1}] compared to the first phase [ED_{50}: 2.72±1.04 mg kg^{-1}]

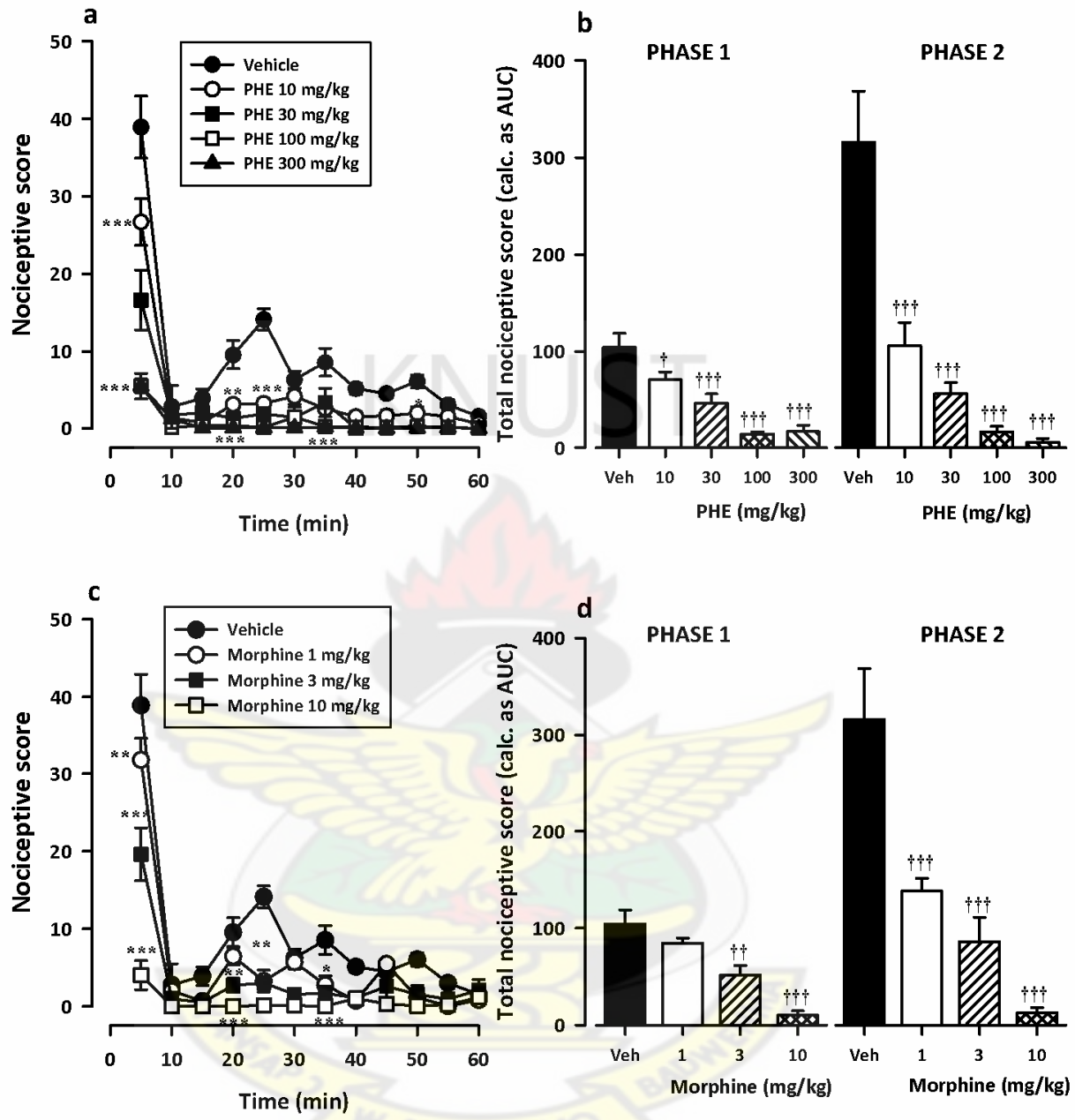

Figure 4.6(a) Effect of PHE (10-300 mg kg^{-1} *p.o*) and (c) Morphine (1-10 mg kg^{-1} i.p) on the time course of formalin induced pain in mice. Nociceptive/pain scores are shown in 5 min blocks up to 60 min post formalin injection. Each point represents Mean ± S.E.M (n = 5). $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ compared to respective controls (two-way repeated measures ANOVA followed by Bonferroni's *post hoc* test); (b and d) the AUC (total response) for phase 1 and phase 2. Each column in b and d represent the mean ± S.E.M. $^{\dagger}P \leq 0.05$, $^{\dagger\dagger}P \leq 0.01$, $^{\dagger\dagger\dagger}P \leq 0.001$ (one-way ANOVA followed by Newman-Keuls *post hoc* test)

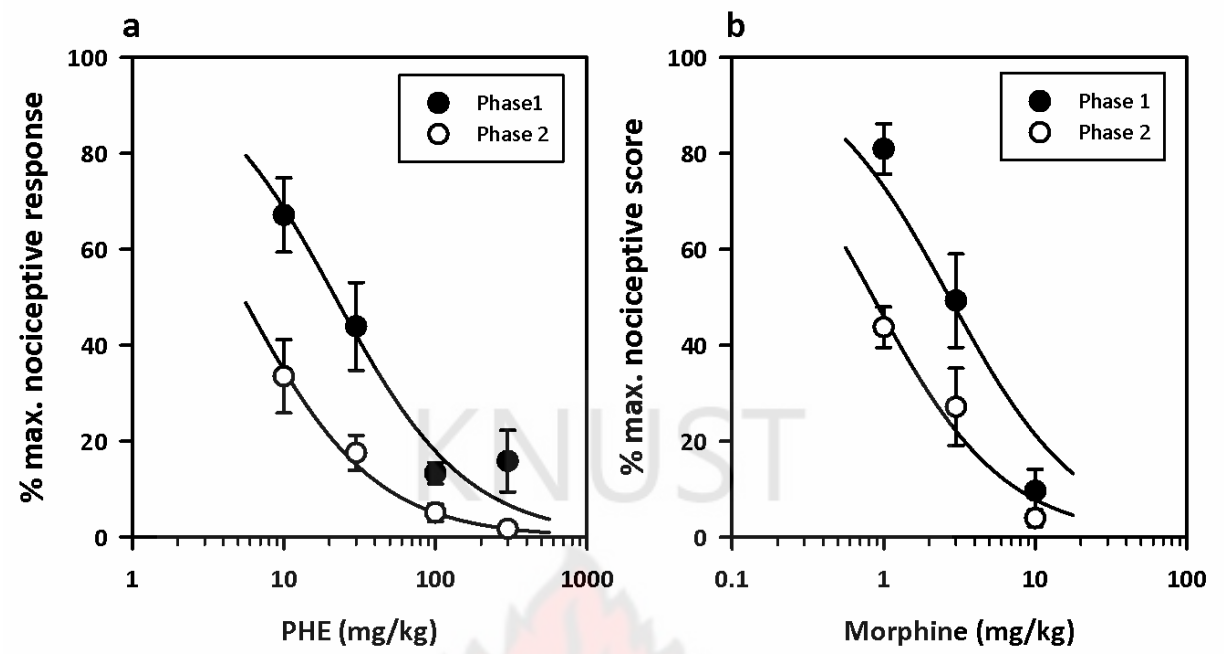

Figure 4.7 Dose response curves of PHE (a) and morphine (b) on the total nociceptive score for the first phase and the second phase of the formalin test in mice. Each point represents mean ± S.E.M (n = 5)

4.2.2.5 Analysis of mechanism of action of PHE

The results presented in Fig. 4.8 show that, the pretreatment of mice with naloxone (2 mg kg^{-1}, i.p., a non-selective opioid receptor antagonist), administered 30 min beforehand, completely and significantly reversed the anti-nociception of both PHE (100 mg kg^{-1}, p.o.) and morphine (3 mg kg^{-1}, i.p.). The adenosine antagonist theophylline (5 mg kg^{-1}) however did not have any significant effect ($p<0.05$) on the anti-nociceptive effects of both morphine (3 mg kg^{-1}, i.p.) and PHE (100 mg kg^{-1}, p.o.) in the formalin test.

Systemic pretreatment of mice with L-NAME (a NO synthase inhibitor; 10 mg kg^{-1}, i.p.) significantly and completely prevented the anti-nociceptive effect induced by the oral administration of PHE (100 mg kg^{-1},*p.o.*) and morphine (3 mg kg^{-1}, i.p.) (Fig. 4.9 a and b) in the formalin test.

Pretreatment with an ATP-sensitive K^+ channel inhibitor, glibenclamide (8 mg kg^{-1}, i.p.), also prevented the anti-nociception produced by PHE (100 mg kg^{-1}, *p.o.*) as well as morphine (3 mg kg^{-1}, i.p.) in the formalin test (Fig.4.9 a and b).

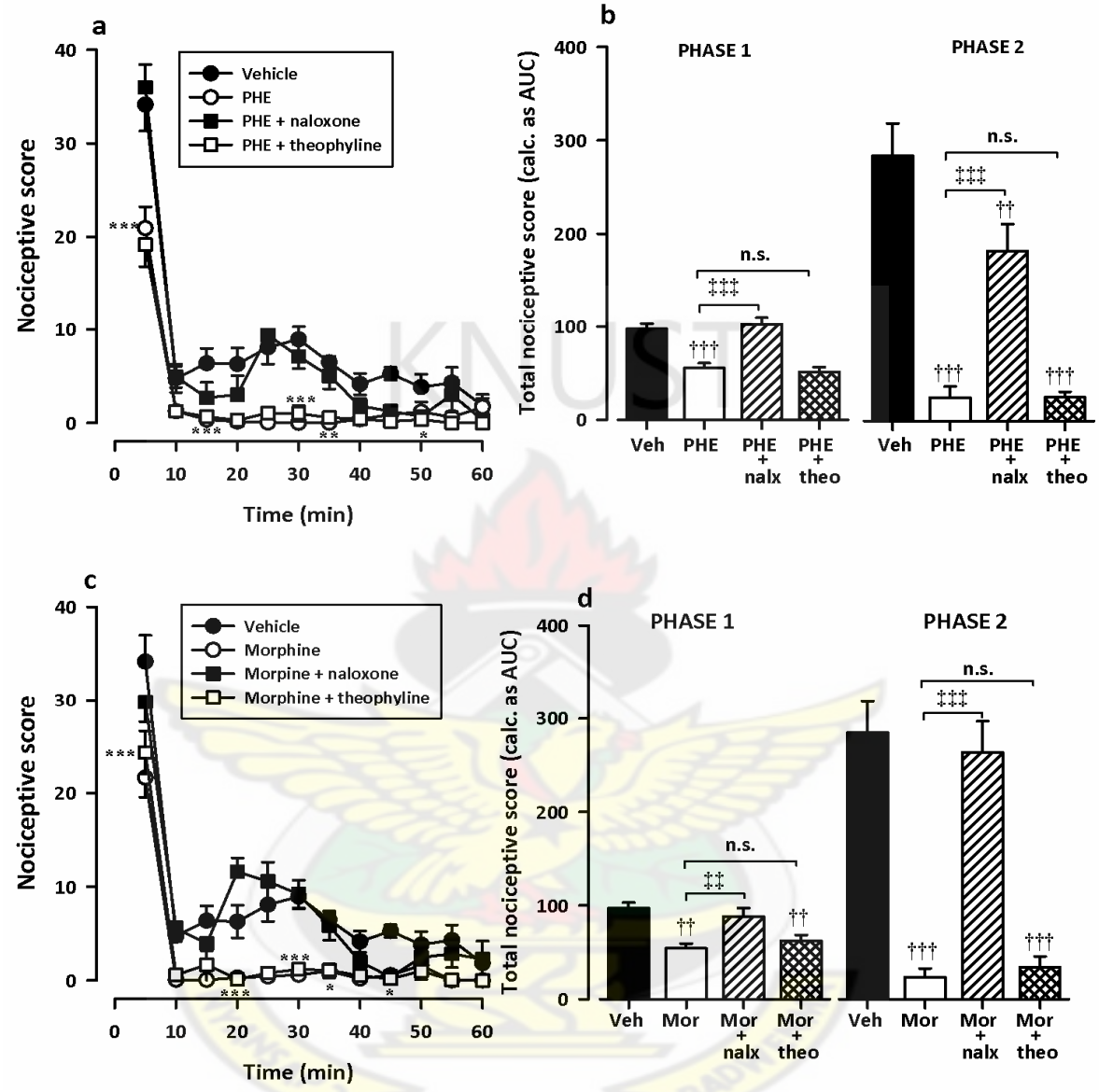

Figure 4.8 Effect of intraperitonial injection of naloxone and theophylline on the antinociceptive effect of PHE (10-300 mg kg^{-1} *p.o*) and Morphine (1-10 mg kg^{-1} i.p*)* on the total nociceptive score of formalin-induced licking test in mice with (a and c) showing the time course effect and (c and d) representing the AUC for phase 1 and phase 2 of formalin-induced pain. Each column represent the mean ± S.E.M. $^{\dagger}P \leq 0.05$, $^{\dagger\dagger}P \leq 0.01$, $^{\dagger\dagger\dagger}P \leq 0.001$, $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, $^{\ddagger}P \leq 0.05$, $^{\ddagger\ddagger}P \leq 0.01$, $^{\ddagger\ddagger\ddagger}P \leq 0.001$compared to respective controls (one-way ANOVA followed by Newman-Keuls *post hoc* test)

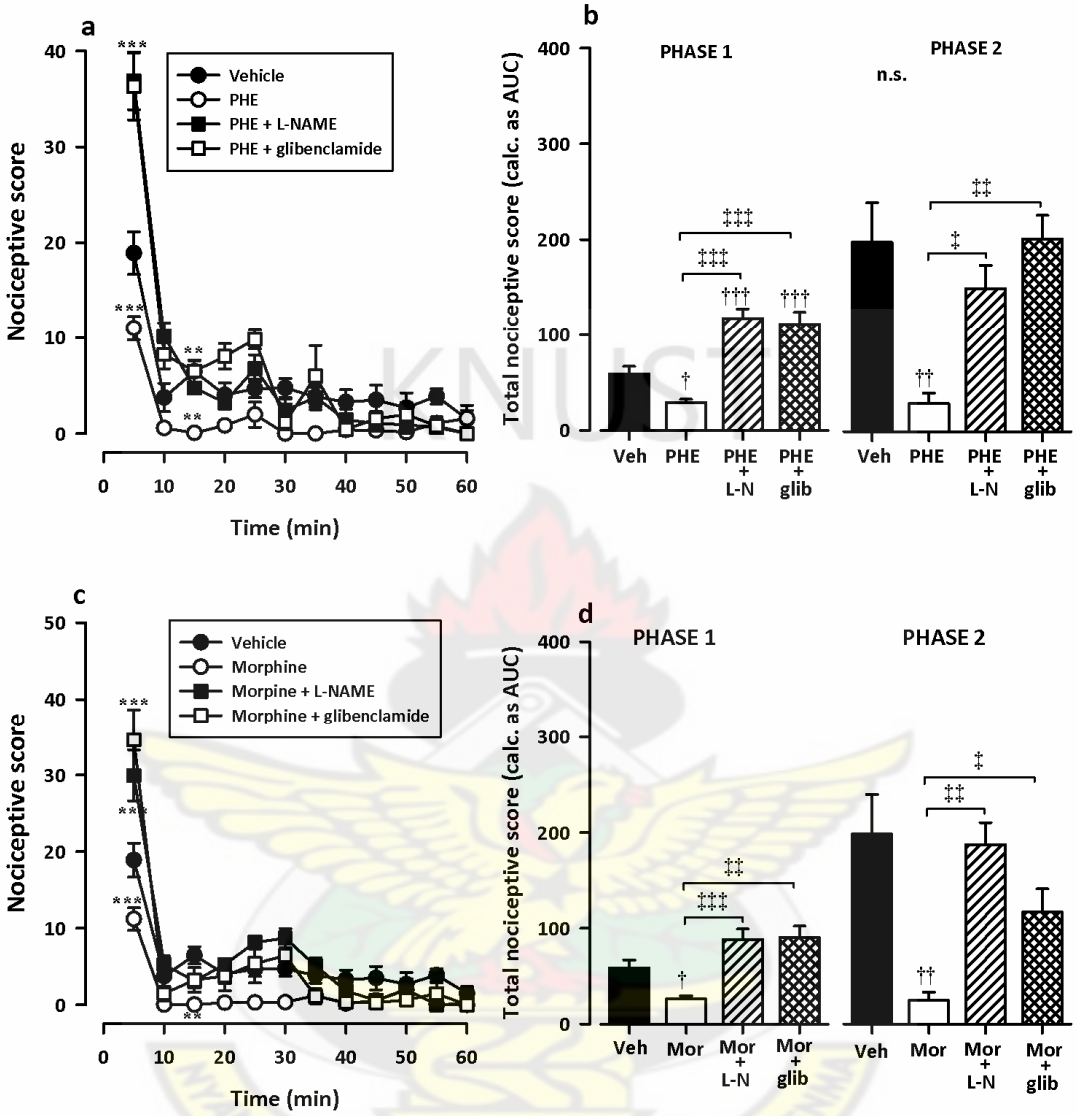

Figure 4.9 Effect of intraperitonial injection of L-NAME and glibenclamide on the antinociceptive effect of PHE (10-300 mg kg^{-1} *p.o*) and Morphine (1-10 mg kg^{-1} i.p*)* on the total nociceptive score of formalin-induced licking test in mice with (a and c) showing the time course effect and (b and d) representing the AUC for phase 1 and phase 2 of formalin-induced pain. Each column represent the mean ± S.E.M. $^{\dagger}P \leq 0.05$, $^{\dagger\dagger}P \leq 0.01$, $^{\dagger\dagger\dagger}P \leq 0.001$, $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, $^{\ddagger}P \leq 0.05$, $^{\ddagger\ddagger}P \leq 0.01$, $^{\ddagger\ddagger\ddagger}P \leq 0.001$ compared to respective controls (one-way ANOVA followed by Newman-Keuls *post hoc* test

4.2.2.6 Tolerance Studies

Morphine (3 mg kg^{-1}, i.p.) significantly ($F_{5,24}$= 10.01, P<0.0001 phase 1, $F_{5,24}$= 10.01, P<0.0001 phase 2) attenuated basal nociceptive response in both phases of formalin test in chronic vehicle-treated animals. However, the same dose of morphine administered at day 9 in animals chronically treated with 6 mg kg^{-1}, i.p. morphine failed to show such effect indicating development of tolerance (Fig. 4.10). In contrast, 100 mg kg^{-1}, i.p. PHE showed a comparable activity in mice given chronic treatment of either 200 mg kg^{-1}, i.p. PHE or vehicle, indicating lack of tolerance development (Fig. 4.10). Moreover, 100 mg kg^{-1}, i.p. PHE still demonstrated anti-nociceptive activity in mice chronically treated with morphine, indicating that no cross-tolerance exists with morphine (Fig. 4.10).

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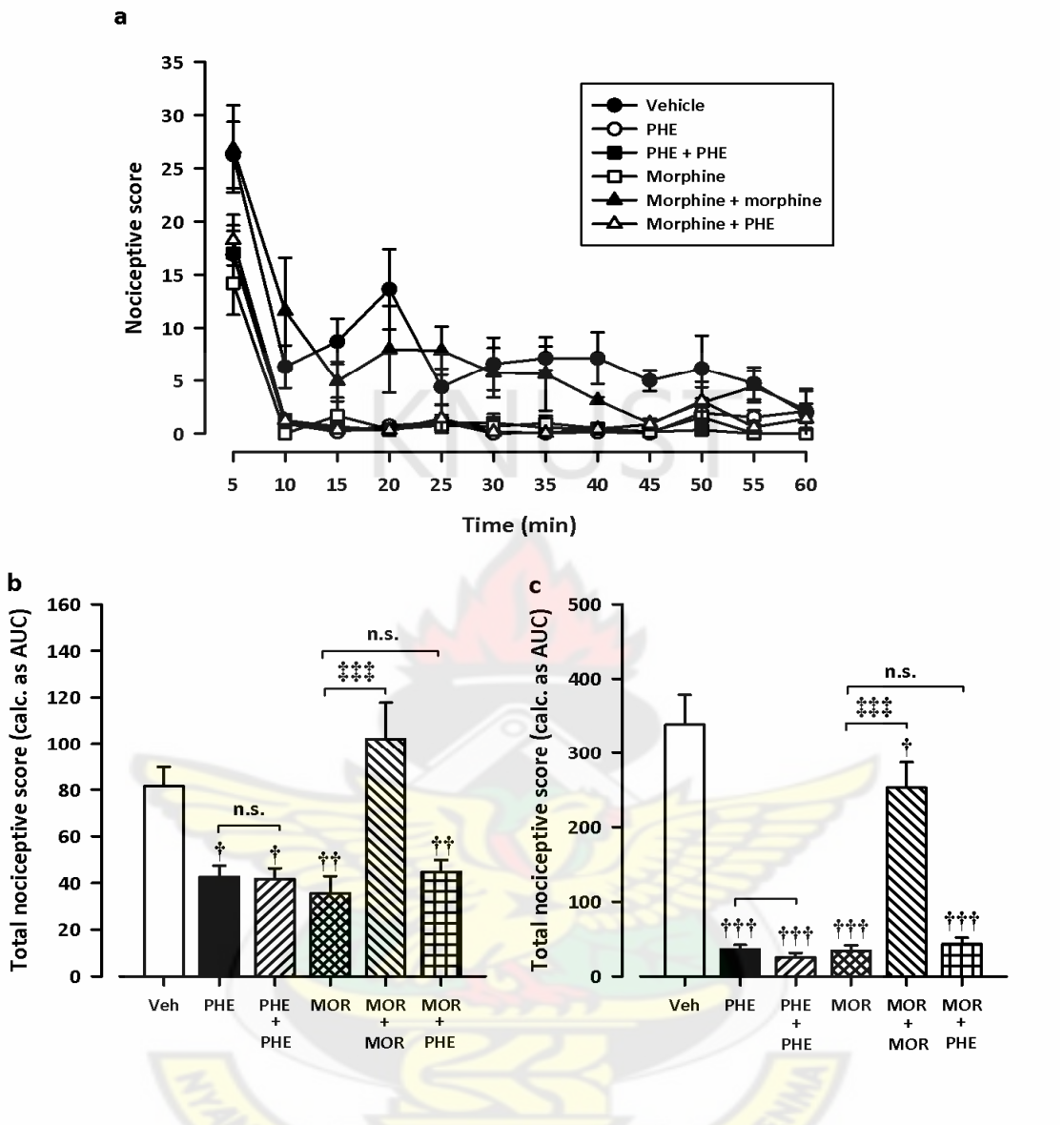

Figure 4.10 Effect of PHE (100 mg kg^{-1} *p.o.*) and morphine (3 mg kg^{-1} i.p.) challenge on mice chronically treated with saline, PHE (200 mg kg^{-1} *p.o.*) or morphine (6 mg kg^{-1} i.p.) for 9 days on the total nociceptive score of formalin-induced licking test in mice with (a) showing the time course effect and (b and c) representing the AUC for phase 1 and phase 2 of formalin-induced pain respectively. Each column represent the mean ± S.E.M. $^{\dagger}P \leq 0.05$, $^{\dagger\dagger}P \leq 0.01$, $^{\dagger\dagger\dagger}P \leq 0.001$, $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, $^{\ddagger}P \leq 0.05$, $^{\ddagger\ddagger}P \leq 0.01$, $^{\ddagger\ddagger\ddagger}P \leq 0.001$compared to respective controls (one-way ANOVA followed by Newman-Keuls *post hoc* test)

4.2.3 DISCUSSION

This study has demonstrated that oral administration of an ethanolic extract of the leaves of *Palisota hirsuta* caused potent anti-nociception in the tail immersion, acetic acid-induced writhing, carrageenan-induced mechanical hyperalgesia and the formalin-induced paw licking tests in rodents. This anti-nociceptive effect was reversed by the systemic administration of the non-selective opioid antagonist, naloxone, the NO synthase inhibitor, L-NAME and an ATP-sensitive K^+ channel inhibitor, glibenclamide. The non-selective adenosine receptor antagonist, theophylline however did not alter the anti-nociceptive effect of the extract.

Several behavioral nociceptive tests which differ with respect to stimulus quality, intensity and duration, were employed in evaluating the analgesic effect of PHE in order to obtain a holistic picture of the analgesic properties of the extract. The models were selected such that both centrally and peripherally mediated effects were investigated. At the doses tested, the ethanolic extract showed anti-nociceptive activity in all the nociceptive models thus indicating that the extract had both centrally- and peripherally-mediated activities (Vongtau *et al.*, 2004).

The writhing response of mice to an intraperitoneal injection of noxious chemical is used to screen for both peripherally and centrally acting analgesic activity. Acetic acid causes pain by releasing endogenous substances and other substances that excite pain nerve endings. The abdominal constriction is related to the sensitization of nociceptive receptors to prostaglandins (Bose *et al.*, 2007). Diclofenac and other NSAIDs can inhibit the number of writhes in this model by inhibiting cyclooxygenase in peripheral tissues, thus interfering with the mechanism of transduction in primary afferent nociceptors by blocking the effect or the synthesis and/or release of inflammatory mediators (Panthong *et al.*, 2007). It is therefore plausible to suggest that the extract may be acting via mechanisms similar to NSAIDs. However, further experiments may be needed to consolidate this view.

The extract together with morphine and diclofenac also had a significant effect in the tail-immersion test even though this model is known to be more sensitive to centrally acting analgesics (Santos *et al.*, 2005). Such analgesic agents elevate pain threshold of animals towards heat and pressure and as such some amount of central activity (spinal and supra spinal mechanisms), can be conferred on the extract since it exhibited significant activity in this pain model (Jain *et al.*, 2001).

The Randall–Sellito paw pressure test which detects the time to withdrawal of an inflamed hind paw from noxious stimuli revealed hyperalgesia in all carrageenan-treated animals. The extract together with morphine and diclofenac at all doses tested, exhibited significant analgesic activity in this pain model which is often used to distinguish between central and peripheral analgesic actions. Inflammation is known to lower the thresholds of various mechanoreceptors and mechanotransduction pathways (Park *et al.*, 2008). Stimulus applied in this model of nociception, pressing of a blunt tip into inflamed hind paws, is likely to activate slowly-adapting mechanoreceptors with decreased thresholds, which are predominantly C-fibers located in cutaneous and subcutaneous structures that would have required greater stimulus intensities for activation (Birder and Perl, 1994; Lewin and Moshourab, 2004).

Results obtained in the formalin test showed that both PHE extract and morphine significantly reduced the time spent in licking the injured paw. In this 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 by formalin, results from a combination of peripheral input and spinal cord sensitization (Tjølsen *et al*., 1992). The intraplantar injection of formalin, releases EAAs, PGE_2, NO and kinins in the spinal cord (Tjølsen *et al.*, 1992). Taking this into account, the anti-nociception of *Palisota hirsuta* could be dependent on either peripheral or central sites of action. Centrally acting drugs, such as opioids, inhibit both phases of pain by equally inhibiting the effect produced by

prostaglandins released at this level in 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. It has been demonstrated (Tjølsen *et al.*, 1992) 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 PHE as exhibited in the formalin test suggest an involvement at both central and peripheral levels, which further implies that the extract possesses not only anti-nociceptive but also anti-inflammatory activity.

This study also looked at the possible mechanism of action of the extract. The formalin test was selected for this study, since it is more specific and it is possible to identify two distinct phases of nociception (Basile *et al.*, 2007; Yin *et al.*, 2003) and also since it reflects different pathological processes and it allows the elucidation of the possible mechanism involved in analgesia (Tjølsen *et al.*, 1992). The anti-nociceptive effect of PHE was determined in the presence of naloxone, theophylline, L-NAME or glibenclamide. Naloxone, a non selective opioid antagonist, reversed the anti-nociceptive effect of both morphine and the ethanolic leaves extract of PHE in both phases of the formalin test. This finding clearly suggests that activation of opioid receptors and/or an increment of endogenous opioids, either centrally or peripherally, might be involved in the anti-nociceptive effect of PHE (Bjorkman, 1995). The anti-nociceptive effect of PHE as well as that of morphine were also blocked by the nitric oxide synthase inhibitor L-NAME, suggesting that the anti-nociceptive action of PHE like morphine involves the activation of the nitric oxide-cyclic GMP pathway at peripheral and/or central levels (Duarte and Ferreira, 1992; Granados-Soto *et al.*, 1995; Granados-Soto *et al.*, 1997; Islas-Cadena *et al.*, 1999; Nozaki-Taguchi and Yamamoto, 1998; Tonussi and Ferreira, 1994). It has been clearly established that, downstream, NO is a signaling molecule released in response to central analgesics specifically morphine (Cadet *et*

al., 2004). Hence, the release of NO or its production is an important step for the anti-nociceptive action of *P. hirsuta* and may contribute for the plant effects against formalin-induced nociception.

Glibenclamide, an ATP-sensitive K^+ channel blocker also blocked the analgesic activity of both PHE and morphine. It is well established that glibenclamide specifically blocks ATP sensitive K^+ channels, with no effect on Ca^{2+-} or voltage dependent K^+ channels (Amoroso *et al.*, 1990; Edwards and Weston, 1993). Therefore, this data suggest that opening of ATP-sensitive K^+ channels plays a role in the analgesic action of PHE. It is likely that PHE has a mechanism of action similar to diclofenac, metamizol, ketorolac, sodium nitroprusside and morphine all of which activate the nitric oxide-cyclic GMP-K^+ channel pathway (Alves *et al.*, 2004; Carrier *et al.*, 1997; Lazaro-Ibanez *et al.*, 2001; Ortiz *et al.*, 2002; Rodrigues and Duarte, 2000; Soares *et al.*, 2000). It is likely that, compounds that open K^+ channels by direct activation like PHE may gain importance as effective pain relievers since these have been shown to be very effective in models of acute and chronic pain (Ocana *et al.*, 2004).

Theophylline, an adenosine receptor antagonist however was not able to modify PHE-induced anti-nociception giving an indication that, the adenosinergic pathway is not involved in the anti-nociceptive effects of PHE. Morphine anti-nociception was also not block by theophylline as reported by other workers (Mantegazza *et al.*, 1984; Ribeiro *et al.*, 2003; Sawynok and Liu, 2003). This may be accounted for by various reasons including the type of species used (Malec and Michalska, 1990), the doses of theophylline used (Sawynok *et al.*, 1998) as well as pharmacokinetic factors (Misra *et al.*, 1985).

At this point, it is important to mention that one of the current trends in nociception studies is the search for opioid analgesics acting at opioid receptors outside the central nervous system, with the prospect of avoiding centrally-mediated side effects such as tolerance and dependence (Stein *et al.*, 2000). The

results reported here suggest that, unlike morphine, PHE does not induce tolerance to its anti-nociceptive effect after chronic administration in the formalin test. Eight days of 6 mg kg^{-1} morphine administration produced significant tolerance in mice treated at day 9 with 3 mg kg^{-1} morphine. In contrast, chronic treatment with 200 mg kg^{-1} PHE did not modify the day 9 anti-nociceptive activity of 100 mg kg^{-1} PHE. The absence of tolerance with PHE treatment cannot be attributed to the dose being low, because PHE was chronically administered at the dose maximally active in both phases of the formalin-induced pain. Based on this, it can be speculated that, PHE might have a greater effect on the opioid receptors at the periphery since tolerance is known to be induced centrally. The present study further demonstrates that at doses tested, morphine tolerance does not cross-generalize to PHE; that is cases where there tolerance to morphine has develop, PHE could be used.

4.2.4 CONCLUSION

Overall, the results demonstrate that the central and peripheral anti-nociceptive effects exhibited by PHE might partially or wholly be due to the stimulation of peripheral and/or central opioid receptors through the activation of the nitric oxide-cyclic GMP- ATP-sensitive K^+ (NO/cGMP/K^+ATP)-channel pathway. These findings therefore may contribute to the design of pharmacological strategies directed towards a better management of some painful conditions.

Chapter 5

ETHOPHARMACOLOGICAL EXPERIMENTS

5.1 INTRODUCTION

In West African and Ghanaian traditional medicine, an infusion is prepared from the leaves of *Palisota hirsuta* K. Schum. (Family: Commelinaceae) and administered orally for treating various painful conditions (Burkill, 1985; Dokosi, 1998). The central analgesic property of this plant has been established in this study. Moreover, leaves decoction of this plant is also taken orally for CNS diseases (Abbiw, 1990). Also, considering the increasing evidence supporting the role of centrally acting drugs like anxiolytics, anti-depressants and anti-convulsants in the management of some types of pain like neuropathic pain which does not respond to conventional analgesics (Arnold *et al.*, 2005; Maizels and McCarberg, 2005), the central effects of this extract was investigated. Moreover, despite the popular use of the plant, it was not possible to find pharmacological data confirming the CNS activity of this plant. Experimental paradigms such as elevated plus-maze, open field, light–dark box, tail suspension and forced swimming tests are widely used for identifying possible candidates for new treatment obtained from natural sources for anxiety and depression. The objective of this study therefore, was to evaluate the effect of the hydroalcoholic extract from *Palisota hirsuta* leaves on anxiety and depression in mice and further attempt to investigate its probable its mechanism of action.

5.2 MATERIALS AND METHODS

5.2.1.1 Animals

Male ICR mice were purchased from Noguchi Memorial Institute for Medical Research, University of Ghana, Accra and used and cared for as previously described in section 3.2. All behavioral experiments were carried out under dim

light and therefore to acclimatize the animals to the test conditions, they were brought to the laboratory and exposed to dim light at the stipulated time of testing daily for 6 days before the experiments.

5.2.1.2 Drugs

Fluoxetine hydrochloride (Prozac®), imipramime hydrochloride, α-methyldopa (Aldomet®) and reserpine were purchased from Eli Lilly and Co., Basingstoke, England, Phyto-Riker Pharmaceuticals, Accra, Ghana, Merck Sharp Dohme, Herts., England and BDH, Poole, England respectively. Diazepam and caffeine hydrochloride were obtained from Sigma Chemicals (St. Louis, MO, USA) and flumazenil, Anexate®, from Roche products Ltd., Herts, England

5.2.2 ANTIDEPRESSANT EFFECT

5.2.2.1 Forced Swimming Test (FST)

The FST was based on that described by Porsolt *et. al.*, (2001) and other workers (Bannon *et al.*, 1998) with slight modifications. Mice were divided into ten groups of six animals each, and received either the extracts (30, 100 or 300 mg kg^{-1}, *p.o.*), the vehicle or the standard reference drugs imipramine (3, 10 or 30 mg kg^{-1}, *p.o.*) or fluoxetine (3, 10 or 30 mg kg^{-1}, *p.o.*). Thirty minutes after administration of the test compound, mice were gently dropped individually into transparent cylindrical polyethylene tanks (25 cm high, 10 cm internal diameter) containing water (25°C to 28°C) up to a level of 20 cm and left there for 6 minute. Four identical polyethylene cylinders were prepared and four animals, separated by opaque screens, were exposed simultaneously and videotaped. Each session was recorded by a video camera suspended approximately 100 cm above the cylinders. After each session, animals were removed from the cylinders, dried with absorbent towels, placed in cages near to a heater until they were completely dried and then returned to their home cages. Water was changed for each mouse and tanks were cleaned in between studies.

5.2.2.2 Tail Suspension test (TST)

The TST was carried out as previously described (Steru *et al*., 1985). Mice were allowed to acclimatize to the room for 3.5–4 h before the test. Groups of six mice were treated with PHE (30, 100 or 300 mg kg^{-1}, *p.o.*), imipramine (3, 10 or 30 mg kg^{-1}, *p.o.*), fluoxetine (3, 10 or 30 mg kg^{-1}, *p.o.*) or vehicle. Thirty minutes after i.p. and 1 h after oral administration of the test compounds, mice were individually suspended by the tail from a horizontal bar (distance from floor = 30 cm) using adhesive tape (distance from tip of tail = 1 cm). Duration of immobility, defined as the absence of all movement except for those required for respiration, was recorded by an observer for 6 min from video recordings of the test as described above for forced swimming test. Mice that climbed up on their tails during the test session were gently pulled down and testing continued. Mice that continued to climb their tails were excluded from the study.

5.2.2.3 Effect of catecholamine depletion on the anti-depressant actions of PHE

To investigate the possible role of noradrenergic system in the actions of PHE, a separate experiment in which catecholamines were depleted by treatment with α-methyldopa (αMD) (400 mg kg^{-1}, i.p.) and/or reserpine (1 mg kg^{-1}, s.c.) was carried out. Because the TST presents some advantages over the FST in allowing an objective measure of immobility and does not induce hypothermia by immersion in water (Ripoll *et al*., 2003), it was chosen for this study. The doses of αMD and reserpine were chosen on the basis of the work by (van Giersbergen *et al*., 1990) and O'Leary *et al*., (2007). To deplete newly synthesized pools of noradrenaline (NE) and dopamine (DA), mice were treated with a single dose of •-MD (400 mg kg^{-1}, i.p.) 3.5 h before behavioral testing. To deplete vesicular pools of NE and DA, mice were treated with a single dose of reserpine (1 mg kg^{-1}, s.c.) 24 h before behavioral testing. In an effort to deplete both the vesicular and cytoplasmic pools of NE and DA, mice were pretreated with a combination of reserpine (1 mg kg^{-1}, s.c., 24 h before behavioral testing) and α-MD (200 mg kg^{-1}, i.p., 3.5 h before

behavioral testing), respectively. All control animals received 0.9% saline on the same schedule as the treated groups.

5.2.2.4 Motor Co-ordination – Rotarod Test

Deficits in motor-coordination and increase in activity could invalidate conclusions drawn from the tail suspension test and the forced swimming test. Therefore, the effect of the various treatments was assessed using the rotarod apparatus. The rotarod apparatus (model 7600, Ugo Basile, Cormerio, Italy) rotated at a speed of 12 rpm. This apparatus consists of a base platform and a rotating rod of 3 cm diameter with a non-skid surface. The rod, 50 cm in length, is divided into five equal sections by six disks. Five mice were tested simultaneously. The mice were placed individually on the rod. Before the start of the experiment, animals were trained to stay on the rotarod for 300 s. Mice that failed to learn the test or did not reach the criterion (300-s endurance) were excluded from the study. On the test day, the length of time each mouse remained on the rod ("endurance time," maximal score 300 s) was measured after administration of the test compounds or vehicle. The animals were acclimatized to the revolving drum and habituated to handling in order to avoid stress during testing. The integrity of motor coordination was assessed as the performance time on the rod, measured from acceleration start until fall from the drum. The mice were acclimatized to acceleration by three training runs.

5.2.2.5 Analysis of Data

An observer scored the behavior from the videotapes with the aid of 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/), for duration of immobility during the last 4 minutes of the 6-minute period. The ED_{50} (dose responsible for 50 % of the maximal effect) and inhibitory effects of drugs were analyzed as previously described in section 3.2.2.2. Levels of significance were determined by analysis of variance

(ANOVA) and Student-Newman-Kewls post test using GraphPad Prism. All values were expresses as mean ± S.E.M. $P < 0.05$ were considered significant.

5.2.2.6 RESULTS

Effect on immobility periods in FST and TST

P. hirsuta extract (30, 100, and 300 mg kg^{-1} *p.o.*), fluoxetine (3, 10 and 30 mg kg^{-1} *p.o.*) and imipramine (3, 10 and 30 mg kg^{-1} *p.o.*) all administered 60 min before the test period, significantly decreased the immobility periods of mice in a dose dependent manner in both the FST and the TST when compared to control group, indicating significant antidepressant activity. Comparing the dose-response curves obtained in the FST (fig. 5.1a), the curve for PHE (ED_{50}114.55±72.69) was found to be significantly different from that of both fluoxetine ($F_{1,32}$= 24.24, P< 0.0001) and imipramine ($F_{1,32}$= 12.43, P= 0.0013). The curves for fluoxetine (ED_{50}6.12±4.87) and imipramine (ED_{50}13.21±11.11) were also found to be significantly ($F_{3,16}$= 10.28, P= 0.0005) different from each other. The order of the test drugs in terms of potency was fluoxetine > Imipramine > PHE.

From the dose-response curves obtained for the TST (fig. 5.1b), all the drugs tested caused a reduction in the periods of immobility compared to the control. The curve for PHE was found to be significantly different from that of fluoxetine ($F_{1,28}$=33.60, P<0.0001) and imipramine ($F_{1,28}$= 34.83, P< 0.0001). However, the curves for imipramine and fluoxetine were not significantly different from each other ($F_{1,26}$= 0.202, P= 0.2019) indicating similar antidepressant effect in the TST.

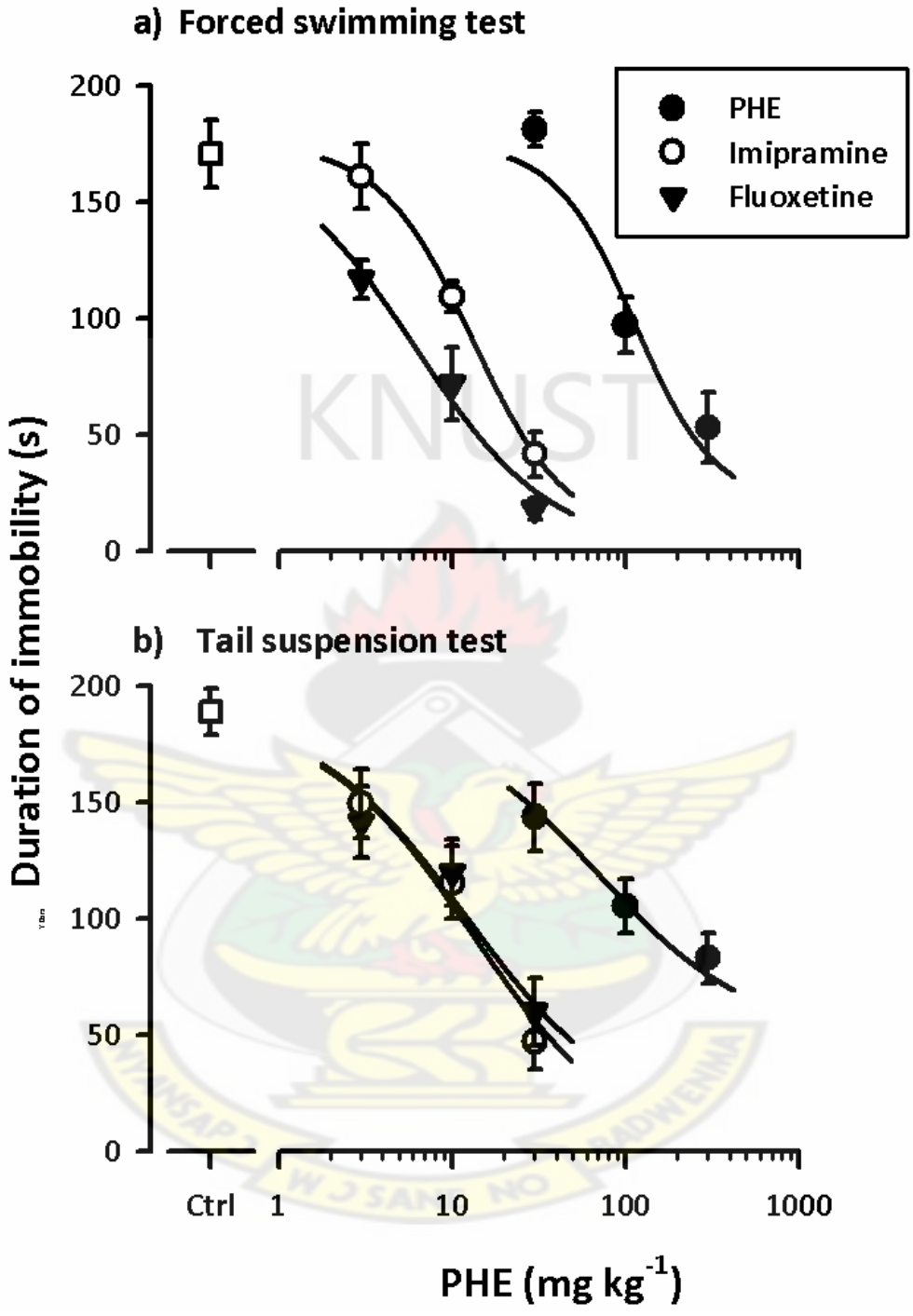

Figure 5.1Dose response curves for *PHE* (10-300 mg kg^{-1} *p.o*), Fluoxetine (3-30 mg kg^{-1} *p.o.*) and Imipramine (3-30 mg kg^{-1} *p.o.*) on immobility periods in (a) FST and (b) TST Each point represents mean ± S.E.M (n = 5).

Table 5.1 ED_{50} for FST and TST

Drug	ED_{50} (mg kg^{-1})	
	FST	TST
Palisota hirsuta extract	114.55±72.69	70.42±0.06
Imipramine	13.21±11.11	12.15±11.03
Fluoxetine	6.12±4.87	12.96±0.01

From the ED_{50} values (in $mgkg^{-1}$; Table 5.1) the potency of the drugs in the TST was in this order: fluoxetine (12.15±11.03) > imipramine (12.96±0.01) > PHE (70.42±0.06). The standard drugs decreased the immobility periods more than the *P. hirsuta* in both the FST and the TST, indicating that these drugs at the dose used in this study has greater efficacy as an antidepressant than the plant extract.

Pretreatment with α-methyldopa

Figure 5.2 shows the effects of •-methyldopa (MeDOPA) pretreatment on the behavioral effects of antidepressants in the TST. PHE ($F_{3,16}$=1.10, P=0.3776) and imipramine ($F_{3,16}$=1.95, P=0.1618) were not able to significantly attenuate or reverse the immobility induced by MeDOPA. Fluoxetine on the other hand, was able to significantly ($F_{3,16}$=12.61, P=0.0002) reverse the immobility in a dose-related manner. Pretreatment with MeDOPA significantly inhibited catecholamine synthesis as revealed by the inability of imipramine to reduce or reverse this immobility without producing significant effect on serotonin content as revealed by the ability of fluoxetine to reverse the immobility induced by MeDOPA.

Pretreatment with reserpine

From the effects of pretreatment with reserpine, fluoxetine (Figure 5.2) was able to significantly ($F_{3,16}$=18.18, P<0.0001) reverse or attenuate the reserpine-induced immobility in a dose dependent manner whereas PHE ($F_{3,16}$=1.66, P=0.2148) and imipramine ($F_{3,16}$=1.66, P=0.2146) (Figure 5.2) were not able to cause any significant change in this effect of reserpine-induced immobility. However, the reversal effect

of the fluoxetine was not as pronounce as it is when the monoamines were depleted with MeDOPA.

Pretreatment with reserpine + α-methyldopa

Figure 5.2 also shows the effect of pretreatment with a combination of reserpine (1 mg kg^{-1}, s.c.) 24 h before the TST and MeDOPA (200 mg kg^{-1}, i.p.) 3.5 h before the TST. This was to deplete both the newly formed stores of catecholamines by MeDOPA and also vesicular storage by reserpine because the behavioral effects of antidepressants could involve catecholamines located in different cellular pools or compartments. Although the behavioral effects of all the tested antidepressant drugs were blocked by this combination [PHE ($F_{3,16}$=1.08, P=0.3873); imipramine ($F_{3,16}$=0.65, P=0.5969)], fluoxetine still had a significant ($F_{3,16}$=5.06, P=0.0118) reduction in the percentage immobility at the dose of 30 mg kg^{-1} after reserpine + MeDOPA pretreatment.

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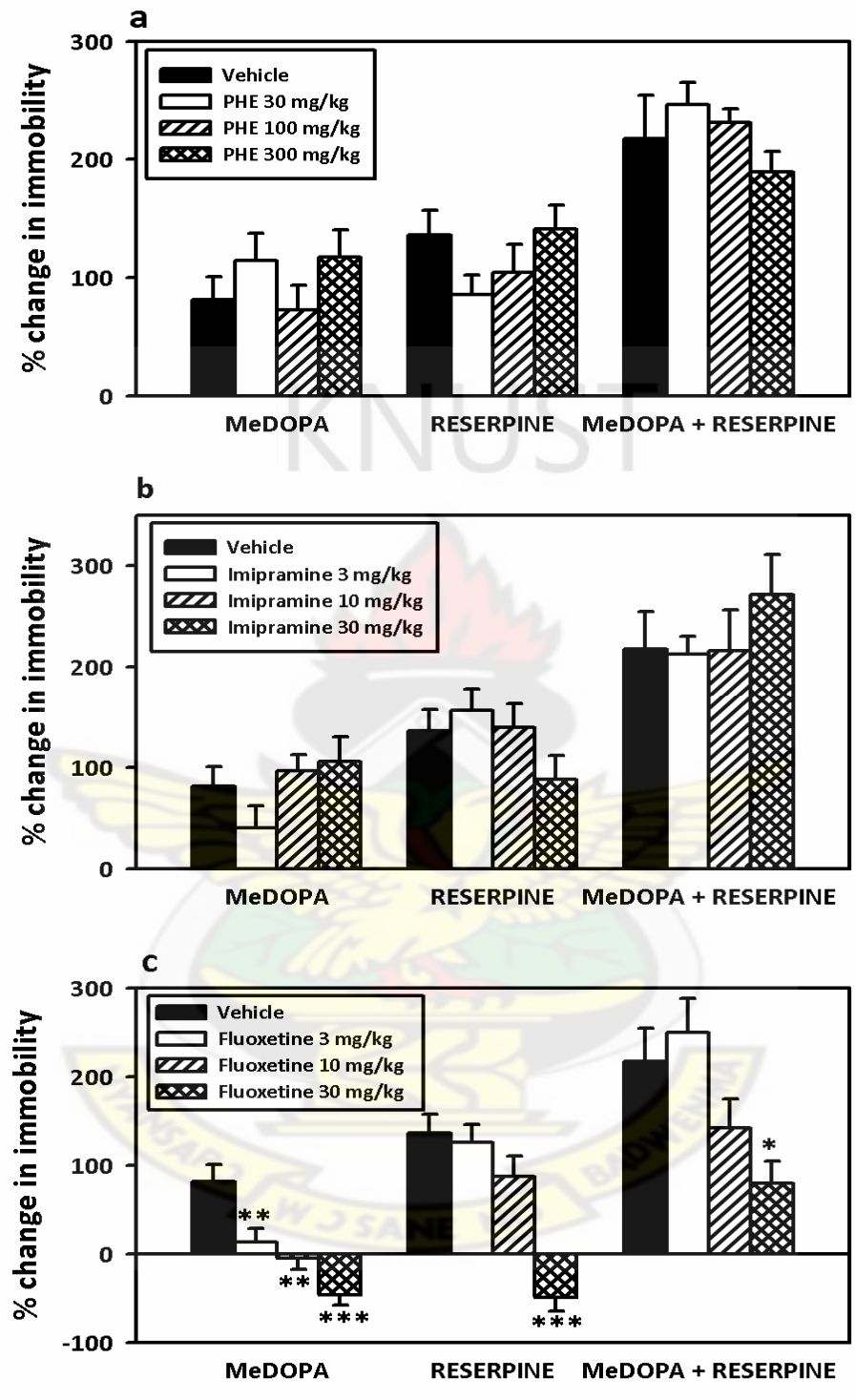

Figure 5.2 Behavioural effect of acute (a) PHE (30, 100 and 300 mg kg^{-1}) (b) Imipramine (3, 10 and 30 mg kg^{-1}) (c) Fluoxetine (3, 10 and 30 mg kg^{-1}) on the TST Data are presented as group means (±SEM) significantly different from vehicle: *P<0.05, **P<0.01, ***P<0.001 by Newman-Keuls test.

Effect on motor coordination in rota-rod

The result indicate that there is no significant difference in the duration of stay on the rotating drum when the PHE treated mice ($F_{3,19}$= 1.668; P=0.2074), imipramine treated mice ($F_{3,19}$= 0.9428; P=0.4396) or fluoxetine treated mice were compared to the control group ($F_{3,20}$= 0.9711; P=0.4256) (Figure 5.3).

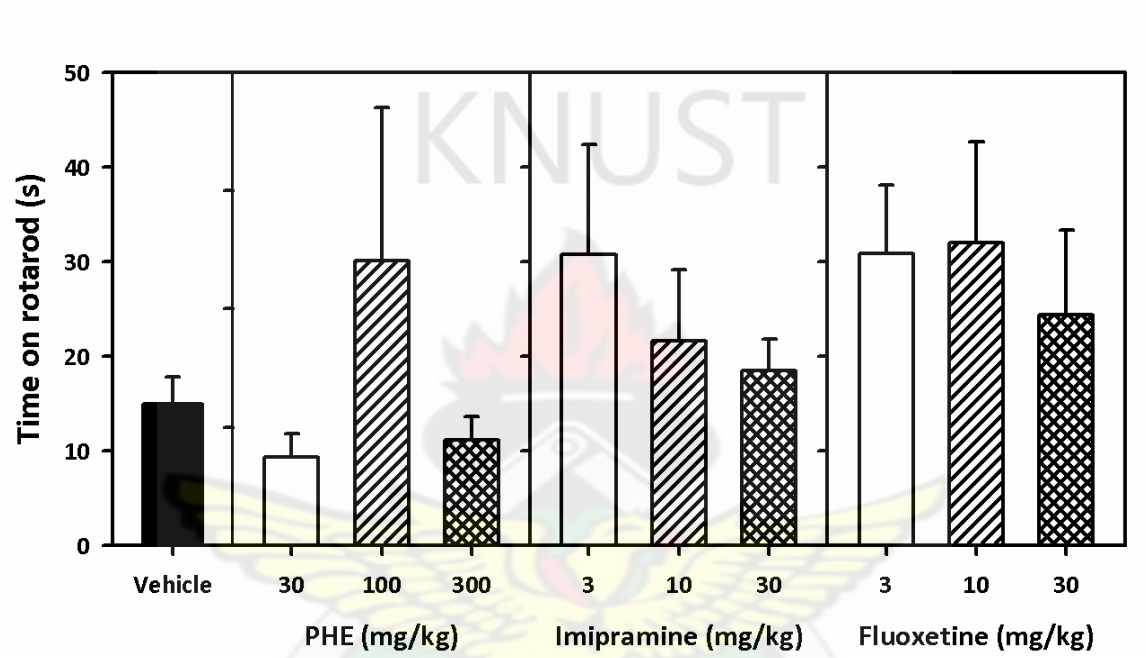

Figure 5.3 Behavioural effect of acute PHE (30, 100 and 300 mg kg^{-1}), Imipramine (3, 10 and 30.0 mg kg^{-1}) and Fluoxetine (3, 10 and 30 mg kg^{-1}), on motor coordination on the rotarod Data are presented as group means ± S.E.M.

5.2.2.7 DISCUSSION

Data presented here indicate that the ethanolic leaf extract of *P. hirsuta* has an antidepressant-like effect in two widely-used animal models of depression. The

forced swimming test has been used in preclinical tests to evaluate "behavioral despair"- a measure of failure to seek escape from an aversive stimulus (Crawley *et al.*, 1997). FST has a high degree of predictive validity as shown by its sensitivity to major classes of antidepressants, tricyclic antidepressants (TCAs), monoamine oxidase inhibitors (MAOIs), atypical antidepressants, selective serotonin reuptake inhibitors (SSRIs) and electroconvulsive therapy (Borsini and Meli, 1988; Dalvi and Lucki, 1999). In the tail suspension test, mice immediately engage in several "agitation- or escape-like" behaviors, followed temporally by increasing bouts of immobility. Like the forced swimming test, immobility is reduced by a broad range of pharmacological and somatic treatments (Cryan *et al.*, 2005; Cryan *et al.*, 2004; Perrault *et al.*, 1992; Teste *et al.*, 1990).

Though statistical analyses did not show any significant difference, the ED_{50s} for PHE and imipramime were lower in the TST thus confirming the superior sensitivity of the TST. However, fluoxetine was more effective in the FST in contrast to a report indicating that TST shows a greater sensitivity to antidepressant effects of 5-HT uptake inhibitors (Steru *et al.*, 1987). Different experimental conditions may explain the difference in observations since factors such as strain and temperature affect such results (Porsolt *et al.*, 2001).

To eliminate the involvement of compromised motor activity and coordination, the rotarod test was performed. PHE at the doses used did not have any effect on motor cordination. An attempt was made to investigate the mechanism of the antidepressant action of PHE. The effect of pretreatment of mice with •-methyldopa and reserpine, are known to alter the monoaminergic systems. Consistent with views from the aminergic theories of depression, an earlier literature has employed monoamine depletion strategies in animals to show that acute and/or adaptive changes in either serotonergic or noradrenergic transmission mediate the attenuation by antidepressants of many depressive-like behaviors in animals. The monoamines, dopamine, serotonin (5-HT), noradrenaline, and adrenaline in the frontal cortex play crucial roles in processes

involved in the control of mood, cognition and motor behavior functions that are compromised in depression (Millan *et al.*, 2000). Etiology of depression has been shown to involve corticolimbic serotonergic and noradrenergic transmissions, and antidepressants act by elevating the monoamine neurotransmission, in particular 5-HT, NE and/or DA in the frontal cortex (Brocco *et al.*, 2002; Brunello *et al.*, 2002; Stone *et al.*, 2003).

•-Methyldopa, an L-aromatic amino acid decarboxylase inhibitor is known to inhibit the biosynthesis of catecholamines and 5-HT (DeMuth and Ackerman, 1983; Schinelli *et al.*, 1993). Furthermore, •-methyldopa may serve as a substrate in catecholaminergic pathway leading to production of the 'false transmitters'; •-methyldopamine and •-methylnoradrenaline (DeMuth and Ackerman, 1983). Moreover, these false transmitters are $•_2$-adrenoceptor agonists (Hey *et al.*, 1988) and thus prevent the release of noradrenaline from nerve endings. It was therefore, hypothesized that pretreatment with •-methyldopa will have more effect on catecholaminergic than the serotoninergic pathways. This is confirmed by this results which showed that the antidepressant effect of imipramime (a tricyclic antidepressant) was abolished by pretreatment by •-methyldopa whilst the SSRI fluoxetine reversed the effects of •-methyldopa. It must however be pointed out that imipramine is a non-selective inhibitor of monoamine transporters; inhibiting both norepinephrine (NET) and serotonin transporter (SERT) (Iversen, 2006). In comparison, pretreatment with •-methyldopa similar to imipramime, though not quantitatively, abolished the antidepressant effects of the extract. The effect of methyldopa on responses to imipramime was intriguing. Imipramine is a non-selective inhibitor of monoamines and thus it was not expected for •-methyldopa to completely abolish its antidepressant effect. This may possibly be explained by the fact that imipramime is normally metabolized *in vivo* to desipramine, a highly potent and selective NE reuptake inhibitor (Iversen, 2006; O'Leary *et al.*, 2007).

Pretreatment with reserpine increased baseline immobility and attenuated the effects of imipramine and PHE in the TST but did not affect that of flouxetine. The

results obtained for imipramime in reserpine-pretreated mice is consistent with the effects of reserpine. Reserpine is an irreversible inhibitor of the vesicular monoamine transporter 2 (VMAT-2) which is located primarily within the CNS and is responsible for transporting monoamines from the cytoplasm into secretory vesicles (Ji *et al.*, 2007; Metzger *et al.*, 2002). Treatment with reserpine therefore leads to depletion of vesicular monoamine stores – both serotonin and noradrenaline (Fukui *et al.*, 2007) suggesting both serotonin and noradrenaline might be important in the antidepressant effects of PHE as well as imipramine. The inability of reserpine pretreatment to affect the actions of fluoxetine seem to suggest that reserpine does not affect vesicular storage of 5-HT to the same extent as that of noradrenaline. At the dose level used in this experiment, reserpine is reported to depleted tissue in the frontal cortex of 5-HT, noradrenaline and dopamine by 78%, 93% and 95% respectively (O'Leary *et al.*, 2007).

From these, it may be inferred that actions of PHE were similar to the TCA, imipramine. However, the involvement of noradrenergic and dopaminergic systems seems to be greater in PHE than imipramine which can be due in part to the several secondary metabolites contained in the extract. Further experiments however, may be necessary therefore to confirm the exact mechanism/s involved in the CNS effects.

5.2.2.8 CONCLUSION

Based on the results from this study, it is clear that the ethanolic extract of *P. hirsuta* leaves has anti-depressant properties in rodent models of depression and may have actions similar to imipramine by affecting levels of noradrenaline and dopamine.

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5.2.3 ANXIOLYTIC EFFECT

5.2.3.1 Open-field test

The test was based on that described previously by other workers (Erdogan *et al.*, 2004; Kasture *et al.*, 2002). Testing was conducted in clear Plexiglas boxes (40 × 40 × 30 cm^3) whose floor was divided into 16 equal squares by black lines. For behavioral analysis, the arena of the open field was designated as (i) corner (one of the four corner squares); (ii) periphery (the squares along the walls); or (iii) center (the four inner squares).The animals were divided into ten groups of six animals each, and received either the extracts (30, 100 or 300 mg kg^{-1}, *p.o.*), the vehicle or the standard reference drugs diazepam (0.1, 0.3 or 1 mg kg^{-1}, i.p.) or caffeine (10, 30 or 100 mg kg^{-1}, *p.o.*). Thirty minutes after i.p. and 1 h after oral administration of the test compound, mice were placed individually in the centre of the open field and allowed to explore freely for 5 minutes. Each session was recorded by a video camera suspended approximately 100 cm above the arena. All animals were regularly handled before individual tests in order to minimize handling-related stress. Videotapes of the arena and the following variables of motor activity were recorded: number of entries as well as the duration of stay in individual zones. Thereafter, behavior in the open field was analyzed for 5 min. Mean values ± S.E.M. were calculated for each and compared to vehicle-treated animals.

Behavioral parameters for all the tests were scored from videotapes with the aid of 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/). Mean values ± S.E.M. were calculated for each and compared to vehicle-treated animals.

To compute total distances travelled by the mice, the software Behavior Collect (http://cas.bellarmine.edu/tietjen/DownLoads/computer_programs_for_data_colle.htm) was used to obtain raw XY data from the videos. These data were then exported into Microsoft® Office Excel 2007 and further analyzed. Distance between two X-Y coordinate pairs was calculated from the formula:

$$\sqrt{[(X_1 - X_2)^2 + (Y_1 - Y_2)^2]}$$

In this experiment and subsequent ones, the apparatus was cleaned between each session with 70% w/v ethanol to preclude possible cueing effects of odors left by previous animals (Phillips, 1982).

5.2.3.2 Light/Dark Test

The light–dark exploration test is typically used to more directly assess anxiety-related responses. This apparatus was based on the initial model described by Crawley, (1981) and as modified by other workers (Belzung and Le Pape, 1994; Belzung *et al.*, 1987). It consists of wooden boxes (45 cm long × 30 cm wide × 30 cm deep), which are divided into two equal compartments by a wooden board with a 7 × 7 cm opening located centrally at the floor level, connecting the compartments. One compartment was painted black and covered with a wooden lid. The other box (not covered) was painted white and lit by a 60-W light bulb set 30 cm above the box. Mice were grouped and treated with drugs as described for the other behavioral tests described above. At the beginning of the experiment, mice were placed individually in the center of the illuminated box, facing the opening away from the dark compartment. Behaviors of the animals were recorded for 5 minutes with a digital camera placed 1 m above the box. Videotapes were scored manually with the aid of the public domain software JWatcher™ Version 1.0 for following parameters: 1) frequency of compartment entries; 2) total time spent by mice in each compartment.

5.2.3.3 Elevated Plus-Maze Test

The method used was as described for rats (Pellow *et al.*, 1985) with some modifications. The elevated plus maze was made from opaque Plexiglas. It consisted of two opposite open arms (15 cm·× 5 cm) without side walls and two enclosed arms (15 cm ×5 cm×·30 cm), extending from a central square platform (5·×5 cm). A rim of Plexiglas (0.5 cm in height) surrounded the perimeter of the

open arms to provide additional grip and thus prevent the mice falling off (Rodgers and Johnson, 1995). The maze was elevated to the height of 80 cm from the floor, and placed in a lit room. The animals were divided into ten groups of six animals each, and received treatments similar to that described for the open field test. Animals were placed individually in the central platform of the EPM for 5 minutes and their behavior recorded on a videotape with a digital camera placed 100 cm above the maze. Behavioral parameters were scored from the videotapes as follows: 1) number of closed and open arm entries—(absolute value and percentage of the total number); 2) time spent in exploring the open and closed arms of the maze — absolute time and percentage of the total time of testing 3) number of head-dips (absolute value and percentage of the total number) — protruding the head over the ledge of either an open (unprotected) or closed (protected) arm and down toward the floor; 4) number of stretch-attend postures (absolute value and percentage of the total number) —the mouse stretches forward and retracts to original position from a closed (protected) or an open (unprotected) arm. An arm entry was counted only when all four limbs of the mouse were within a given arm. Total distances travelled by the mice, was computed as described earlier for the open-field test.

Behavioral parameters for all the tests were scored from videotapes with the aid of the public domain software JWatcher™ Version 1.0

In a separate antagonism experiment, the mice were treated with flumazenil 3 mg kg^{-1}, 15 min before PHE (30, 100, 300 mg kg^{-1}) or diazepam (0.3 mg kg^{-1}) treatment.

5.2.3.4 Analysis of Data

All data are presented as mean ± SEM. To compare differences between groups, one-way ANOVA was performed with Newman-Keuls's test as *post hoc*. Also, two-way analysis of variance (ANOVA) with groups as a between-subject factor and compartment as a within-subject factor followed Bonferroni's as *post hoc* test were performed.

5.2.3.5 RESULTS

Open-field Test

In the open field test, all drug treated mice showed significant differences in both the number of entries into the various fields as well as the time spent in the various zones (figs. 5.4 and 5.5). *P. hirsuta* treated mice exhibited anxiolytic activity similar to diazepam by significantly increasing the percentage number of center entries ($F_{3,16}$=6.08, P=0.0058) (fig. 5.4d) and the percentage time spent in the center of the open field ($F_{3,16}$=7.95, P=0.0018) (fig. 5.5d). There was a significant difference in both the number ($F_{2,36}$=36.54; P<0.0001) (fig. 5.4a) and duration ($F_{2,36}$=201.44; P<0.0001) (fig. 5.5a) of entries into the various zones.

Caffeine treatment did not have much effect on both the percentage number ($F_{3,16}$=1.78, P=0.1918) (fig. 5.4f) and duration of entries ($F_{3,16}$=0.33, P=0.8021) (fig. 5.5f) into the center of the field. There was a significant difference in both the number ($F_{2,36}$=247.60; P<0.0001) (fig. 5.4c) and duration ($F_{2,36}$=370.31; P<0.0001) (fig. 5.5c) of entries into the various zones.

Consistent with the anxiolytic nature of diazepam, it significantly increased both the percentage number of entries ($F_{3,16}$=5.62, P=0.0079) (fig.5.4e) and the percentage time spent in the more exposed center of the arena ($F_{3,16}$=5.37, P=0.0094) (fig. 5.5e). There was a significant difference in both the number ($F_{2,36}$=42.28; P<0.0001) (fig. 5.4b) and duration ($F_{2,36}$=88.37; P<0.0001) (fig. 5.5b) of entries into the various zones.

All treatment groups did not show any significant differences compared to the vehicle treated group in the total distance travelled in the arena [PHE ($F_{3,16}$=0.17, P=0.9136) caffeine ($F_{3,16}$=3.27, P=0.0487) diazepam ($F_{3,16}$=0.92, P=0.4522) (fig.5.6)]. However, comparing the 3D line plots generated from the time and XY data, the PHE and diazepam treated animals made much more visits to the center of the arena indicating decrease in thigmotactic behavior compared to the vehicle treated animals. Exposure to caffeine however made the mice highly thigmotactic,

spending most of the time along the walls of the open field arena (Fig. 5.6 lower panel).

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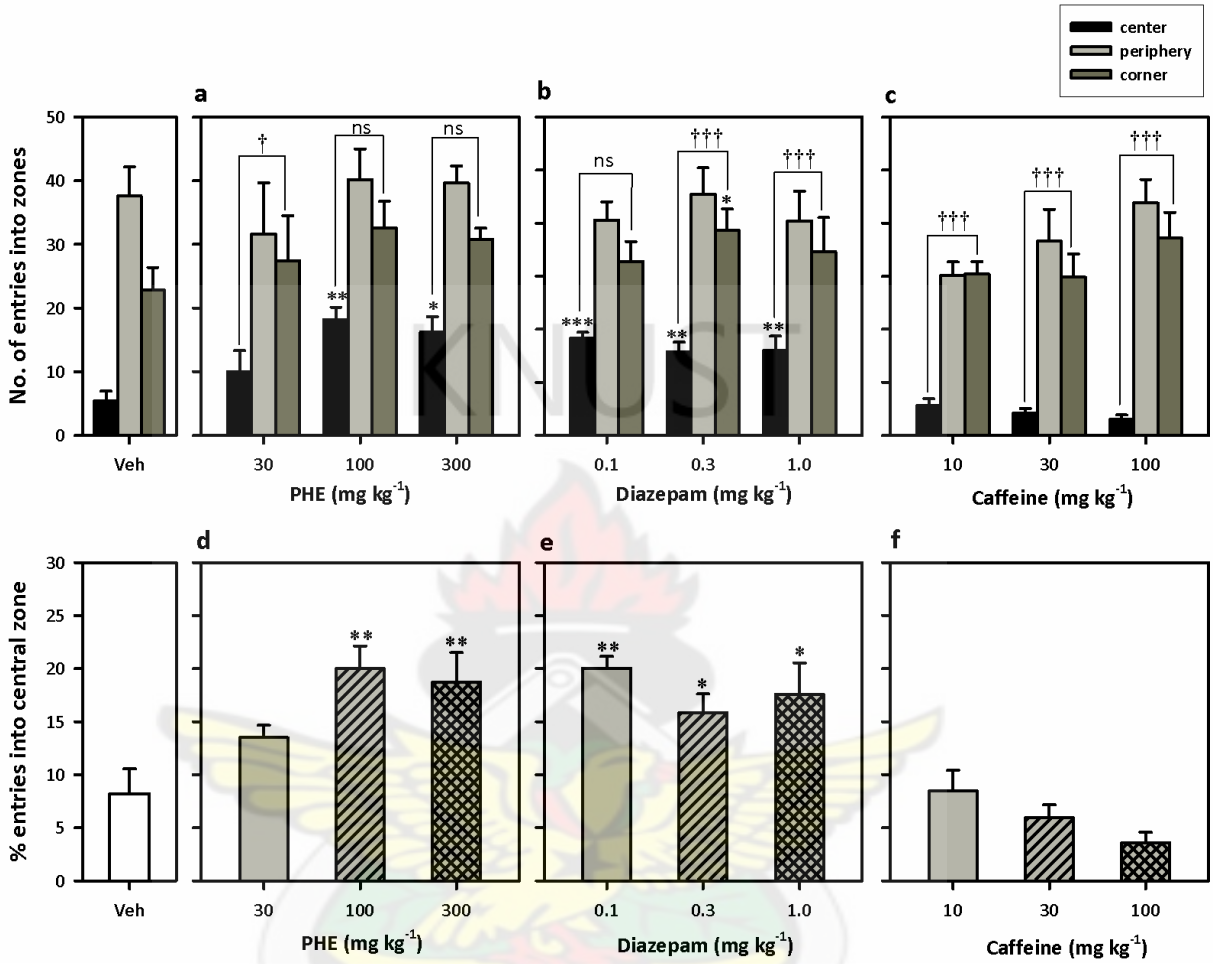

Figure 5.4 Effects of acute PHE (30, 100 and 300 mg kg^{-1}), diazepam (0.1, 0.3, 1.0 mg kg^{-1}) and caffeine (10, 30 and 100 mg kg^{-1}) treatment on the number of zonal entries for PHE (a), diazepam (b), caffeine (c) and % entries into central zone for PHE (d), diazepam (e), caffeine (f) in the open field test. Data are presented as group means ±SEM. Significantly different from control: *P<0.05, **P<0.01, ***P<0.001 by Newman-Keuls test and significant difference when the zonal entries where compared to each other: $^{\dagger}P$<0.05, $^{\dagger\dagger}P$<0.01, $^{\dagger\dagger\dagger}P$<0.001 (two-way repeated measures ANOVA followed by Bonferroni's *post hoc* test).

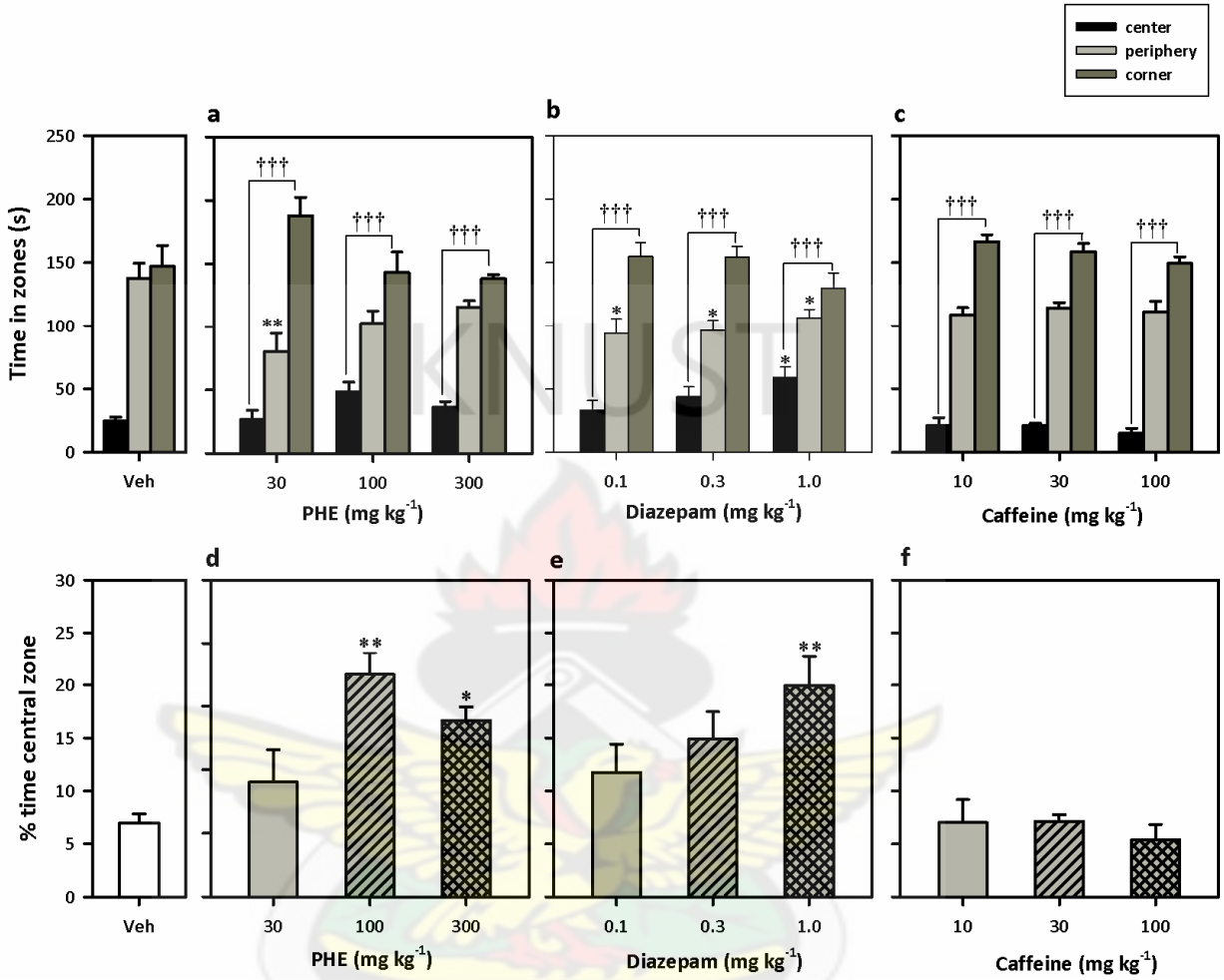

Figure 5.5 Effects of acute PHE (30, 100 and 300 mg kg^{-1}), diazepam (0.1, 0.3, 1.0 mg kg^{-1}) and caffeine (10, 30 and 100 mg kg^{-1}) treatment on the total time spent in zones for PHE (a), diazepam (b), caffeine (c) and % time spent in central zone for PHE (d), diazepam (e), caffeine (f) in the open field test. Data are presented as group means ±SEM. Significantly different from control: $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ by Newman-Keuls test and significant difference when the zonal entries where compared to each other: $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$, $^{\dagger\dagger\dagger}P<0.001$ (two-way repeated measures ANOVA followed by Bonferroni's *post hoc* test).

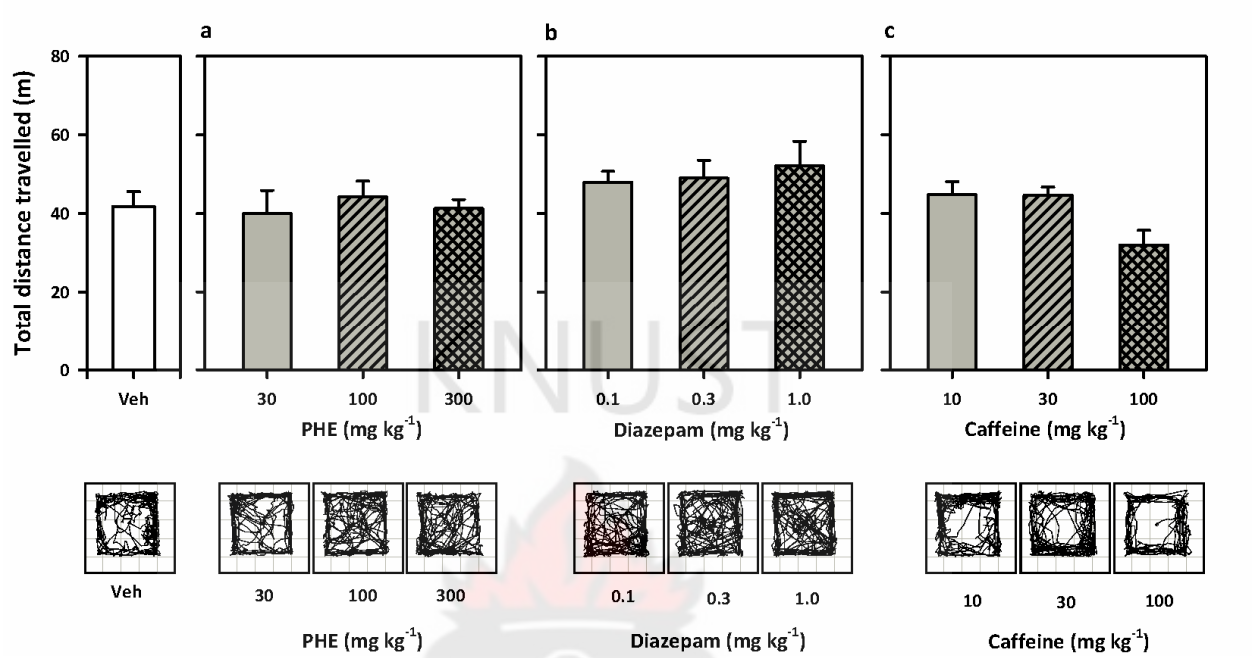

Figure 5.6 Effects of acute PHE (30, 100 and 300 mg kg^{-1}), diazepam (0.1, 0.3 and 1.0 mg kg^{-1}) and caffeine (10, 30 and 100.0 mg kg^{-1}) on total distance travelled in the open field test. Data are presented as group means ±SEM. Significantly different from control (*Ctrl*): **P*<0.05, ***P*<0.01, ****P*<0.001 by Newman-Keuls test. Line plots (lower panels) 3D plots were generated from the time and XY data obtained (*see Materilas and Methods*) using SigmaPlot Version 10 (Systat Software Inc., Point Richmond, CA, USA).

Light / dark box test

In the light/dark test, acute administration of PHE (30-300 mg kg^{-1} *p.o*) induced anxiolytic-related effects like diazepam by significantly increasing the time spent in the lit box ($F_{3,20}$=12.200; P<0.0001) and decreased the time spent in the dark box ($F_{3,20}$=13.551; P<0.0001) (fig. 5.7d). There were no significant changes in the frequency of entries into the light ($F_{3,20}$=1.337; P=0.2905) as well as the dark compartment ($F_{3,20}$=1.234; P=0.3249) of the box (fig. 5.7a). There was a significant difference ($F_{1,30}$=33.66; P=0.0002) in the duration of entries into the light and dark box but concentration does not affect the result ($F_{3,30}$=0.01; P=0.9983).

Caffeine (10-100 mg kg^{-1} i.p) significantly decreased ($F_{3,20}$= 4.839; P=0.0108) the time spent by mice in the lit box and increased significantly ($F_{3,20}$= 4.839; P=0.0108) the time spent in the dark box (fig. 5.7f). The frequency of entry into the light and darks compartments also increased significantly ($F_{3,20}$=6.310; P=0.0035, $F_{3,20}$= 4.373; P=0.0160 respectively) (fig. 5.7c). There was significant difference ($F_{1,30}$=250.99; P<0.0001) in the duration of entries into the light and dark areas but this was not dependent on the concentration ($F_{3,30}$=0.00; P=1.000).

By contrast, diazepam (0.1-1.0 mg kg^{-1} i.p) induced anxiolytic-related measures in the light/dark test (fig. 5.7b & e). Diazepam significantly increased the duration of time spent in the lit box ($F_{3,20}$=26.73; P<0.0001) and decreased the time spent in the dark box ($F_{3,20}$= 26.73; P<0.0001) (fig. 4e). Frequencies of entries into the light and dark compartments were however not significantly different from each other ($F_{3,20}$= 2.420; P=0.0961) ($F_{3,20}$= 2.079; P=0.1352) (fig. 5.7b). There was a significant difference ($F_{1,30}$=65.13; P<0.0001) in the duration of entries into the light and dark compartments but concentration does not affect the result ($F_{3,30}$=0.00; P=1.000).

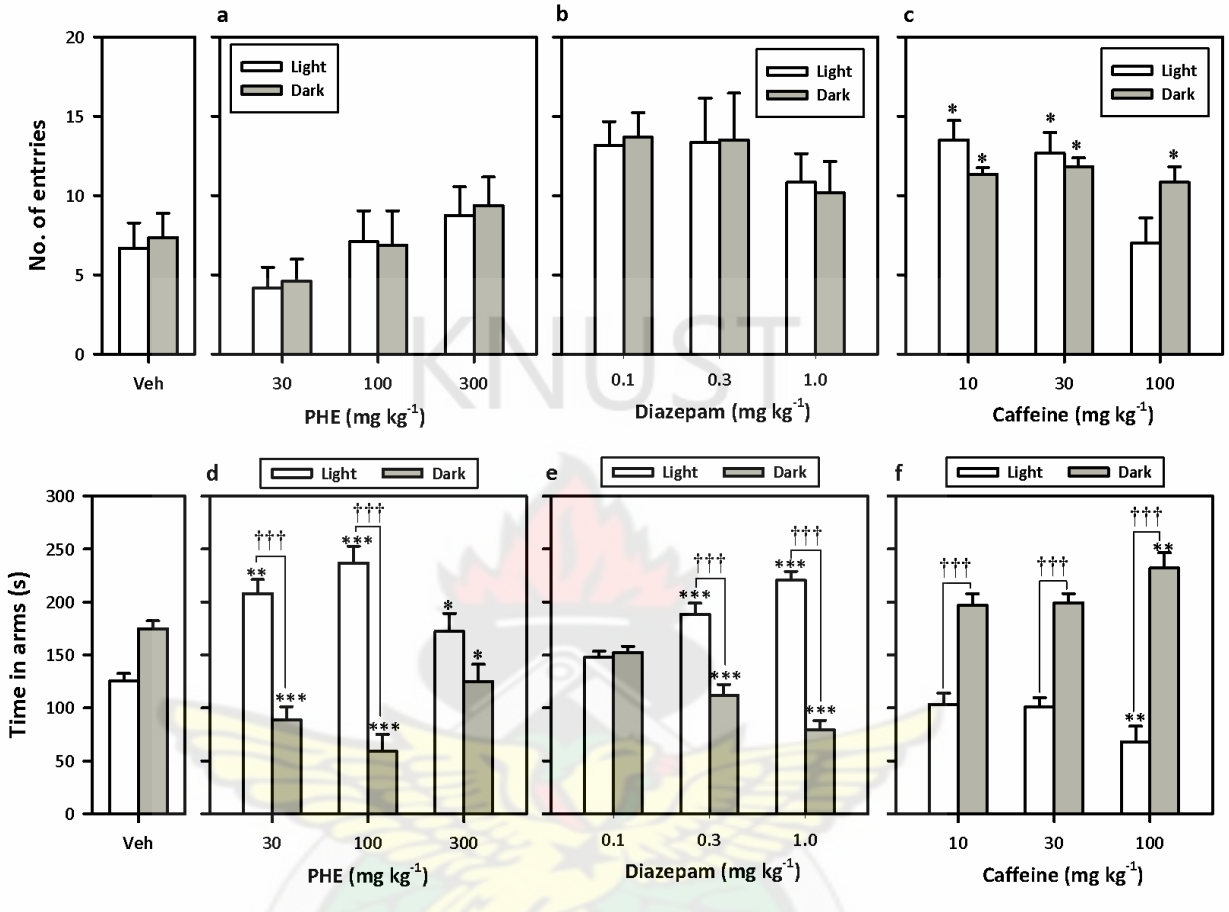

Figure 5.7 Effects of acute PHE (30, 100 and 300 mg kg^{-1}), diazepam (0.1, 0.3, 1.0 mg kg^{-1}) and caffeine (10, 30 and 100 mg kg^{-1}) treatment on number of compartmental entries for PHE (a), diazepam (b), caffeine (c) and on the time spent in compartment for PHE (d), diazepam (e), and caffeine (f) in the light/dark test. Data are presented as group means ±SEM. Significantly different from control (*Ctrl)*: $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ by Newman-Keuls test and significant difference when open compartment and closed compartment where compared: $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$, $^{\dagger\dagger\dagger}P<0.001$ (two-way repeated measures ANOVA followed by Bonferroni's *post hoc* test)

Elevated plus maze test

The effects of *P. hirsuta,* diazepam and caffeine on conventional elevated plus-maze parameters are shown in figs. 5.8, 5.9 and 5.10.

Administration of *P. hirsuta (*30–300 mg kg^{-1}) significantly increased open arm activity by increasing the percentage number of entries ($F_{3,16}$=1.33, *P*=0.2987) (fig. 5.8d) as well as the percentage time spent in the open arm of the elevated plus maze ($F_{3,16}$=4.41, *P*=0.0192) (fig.5.9d). *P. hirsuta* also significantly reduced risk assessment by decreasing both the percentage protected stretch attend postures ($F_{3,16}$=2.49, *P*=0.0976) (fig. 5.10a) and percentage protected head dips ($F_{3,16}$=4.10, *P*=0.0245) (fig. 5.10d) measures from the closed arm. Diazepam (0.1-1 mg kg^{-1}) also increased the percentage entries and time spent in the open arm of the EPM ($F_{3,16}$=2.04, *P*=0.1486) and ($F_{3,16}$=0.76, *P*=0.5334) respectively (figs. 5.8e and 5.9e). Percentage protected stretch attend postures ($F_{3,16}$=2.35, *P*=0.1113) (fig. 5.10b) and percentage protected head dips ($F_{3,16}$=5.74, *P*=0.0073) (fig. 5.10e) were also significantly reduced, a confirmation of its anxiolytic activity. Caffeine (10-100 mg kg^{-1}), an agent that induces anxiety, significantly increased open arm avoidance by decreasing the percentage entries ($F_{3,16}$=1.28, *P*=0.3152) (fig. 5.8c) and time spent ($F_{3,16}$=1.99, *P*=0.1564) (fig. 5.9c) respectively in the open arm of the EPM test. Caffeine treatment however increased the percentage protected stretch attend postures ($F_{3,16}$=1.22, *P*=0.3354) (fig. 5.10c) and percentage protected head dips ($F_{3,16}$=5.48, *P*=0.0087) (fig. 5.10f) indicated an increase in risk assessment behavior.

Exposure to both the extract (fig. 5.11a) ($F_{3,16}$=2.44, *P*=0.1018) and diazepam (fig. 5.11b) ($F_{3,16}$=1.36, *P*=0.2903) did not have any significant effect on the total distance travelled in the EPM compared to the vehicle treated animals. However, treatment with caffeine caused a significant ($F_{3,16}$=5.80, *P*=0.0070) decrease in the total distance travelled confirming its anxiogenic nature (fig. 5.11c). Comparing the 3D line plots in fig 5.11, PHE and diazepam treated animals seemed to have made a greater number of visits into the open arms than the closed arms of the EPM which is

indicative of their anxiolytic properties. By contrast, caffeine treated animals however made more closed arms entries than open arm entries.

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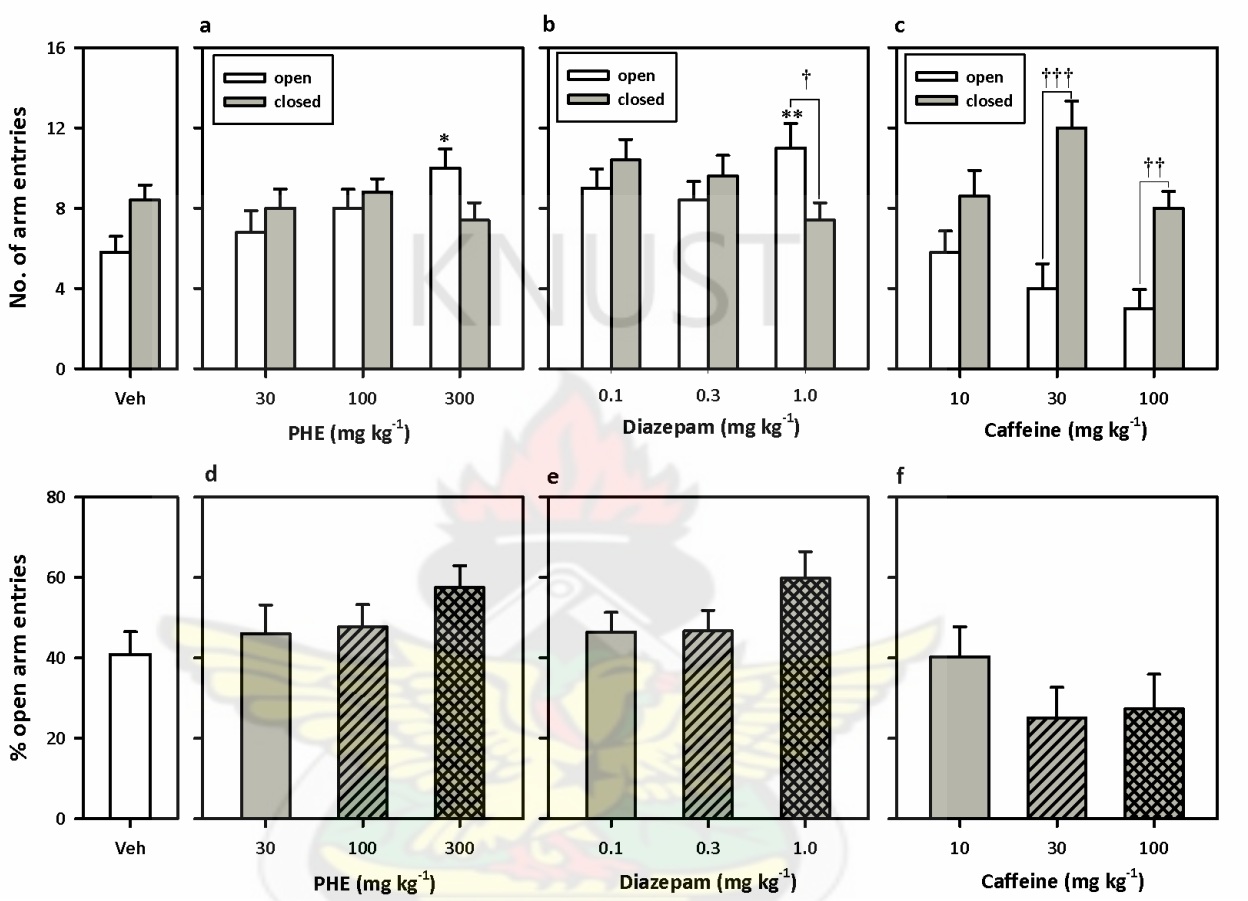

Figure 5.8 Effects of acute PHE (30, 100 and 300 mg kg^{-1}), diazepam (0.1, 0.3, 1.0 mg kg^{-1}) and caffeine (10, 30 and 100 mg kg^{-1}) treatment on the number of arm entries for PHE (a), diazepam (b), caffeine (c) and % number of open arm entries for PHE (d), diazepam (e), caffeine (f) in the elevated plus maze test. Data are presented as group means ±SEM. Significantly different from control: $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ by Newman-Keuls test and significant difference when the zonal entries where compared to each other: $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$, $^{\dagger\dagger\dagger}P<0.001$ (two-way repeated measures ANOVA followed by Bonferroni's *post hoc* test).

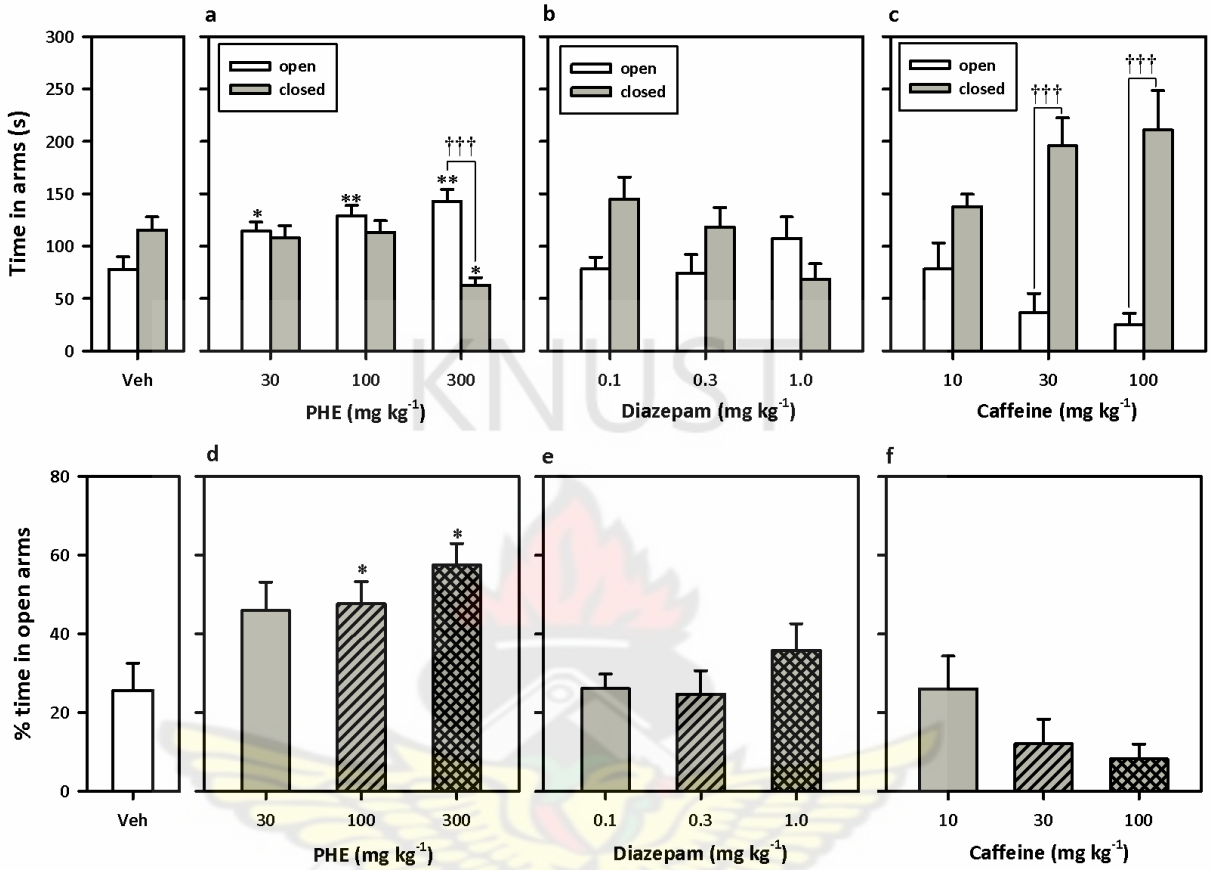

Figure 5.9 Effects of acute PHE (30, 100 and 300 mg kg^{-1}), diazepam (0.1, 0.3, 1.0 mg kg^{-1}) and caffeine (10, 30 and 100 mg kg^{-1}) treatment on the time spent in various arms for PHE (a), diazepam (b), caffeine (c) and % time spent in the open arm for PHE (d), diazepam (e), caffeine (f) in elevated plus maze test. Data are presented as group means ±SEM. Significantly different from control: *$P<0.05$, **$P<0.01$, ***$P<0.001$ by Newman-Keuls test and significant difference when the zonal entries where compared to each other: $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$, $^{\dagger\dagger\dagger}P<0.001$ (two-way repeated measures ANOVA followed by Bonferroni's *post hoc* test).

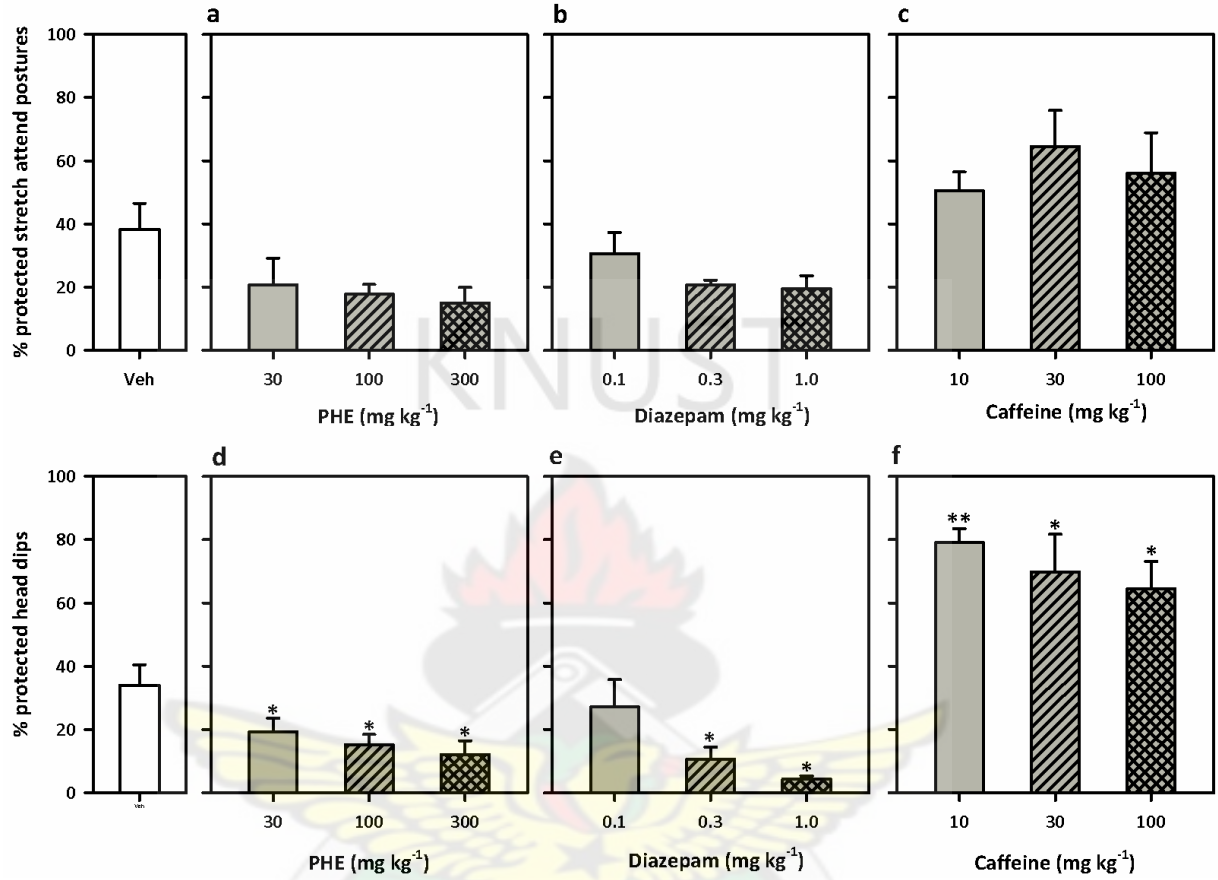

Figure 5.10 Effects of acute PHE (30, 100 and 300 mg kg^{-1}), diazepam (0.1, 0.3, 1.0 mg kg^{-1}) and caffeine (10, 30 and 100 mg kg^{-1}) treatment on the % protected stretch attend postures for PHE (a), diazepam (b), caffeine (c) and on the % protected head dips for PHE (d), diazepam (e), caffeine (f) in elevated plus maze test. Data are presented as group means ±SEM. Significantly different from control: **P*<0.05, ***P*<0.01, ****P*<0.001 by Newman-Keuls test

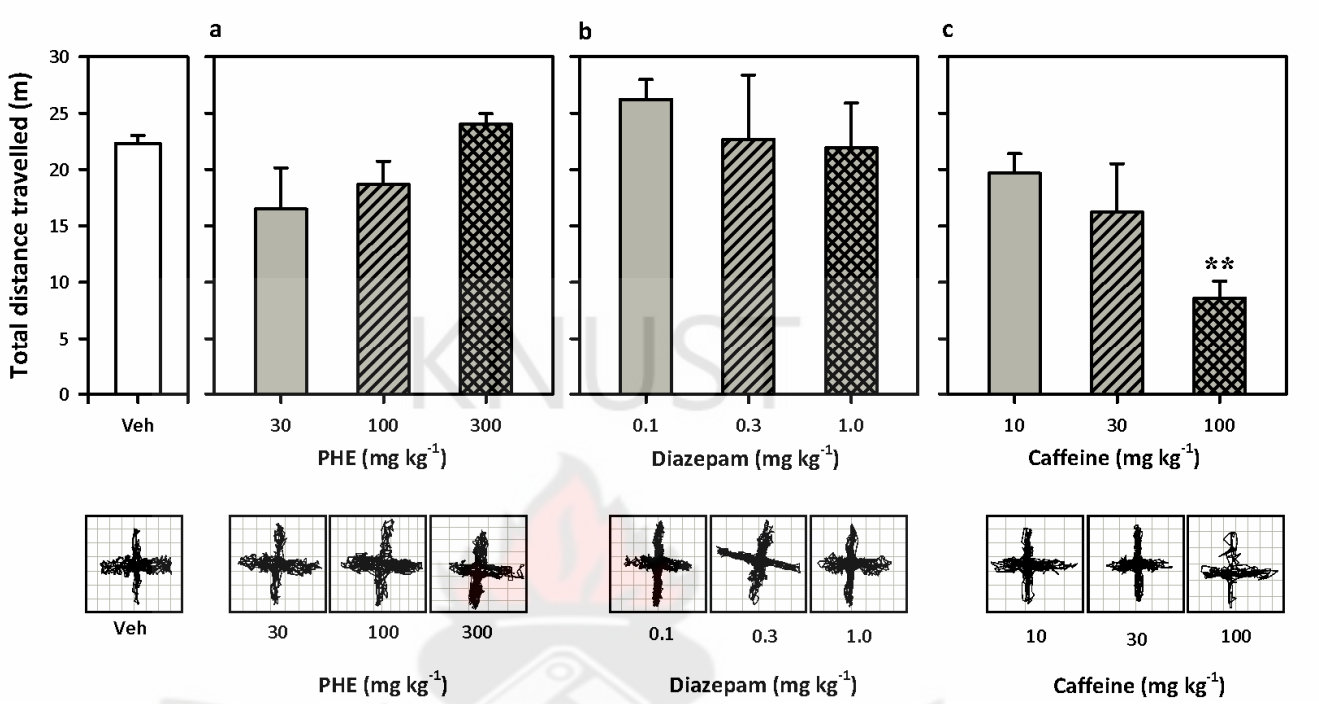

Figure 5.11Effects of acute PHE (30, 100 and 300 mg kg^{-1}), diazepam (0.1, 0.3 and 1.0 mg kg^{-1}) and caffeine (10, 30 and 100.0 mg kg^{-1}) on total distance travelled in the open field test. Data are presented as group means ±SEM. Significantly different from control (*Ctrl*): **P*<0.05, ***P*<0.01, ****P*<0.001 by Newman-Keuls test. Line plots (lower panels) 3D plots were generated from the time and XY data obtained (*see Materilas and Methods*) using SigmaPlot Version 10 (Systat Software Inc., Point Richmond, CA, USA).

Effect of flumazenil on anxiolytic properties of PHE and diazepam

Statistical analysis showed that PHE (30–300 mg kg^{-1}) as well as diazepam (0.3 mg kg^{-1}) increased both the percentage number of open arm entries (PHE $F_{3,16}$=4.116; P=0.0242) (diazepam $F_{4,4}$=3.142; P=0.0419) (fig. 5.12a) and the percentage time spent on open arms (PHE $F_{3,16}$=6.792; P=0.0036) (diazepam $F_{4,4}$=1.713; P=0.3169) (fig. 5.12b) significantly compared to control. The effect of benzodiazepine antagonist flumazenil alone was not significantly different from the control animals in all experiments (fig. 5.12). The percentage number of open arm entries ($F_{4,4}$=4.021; P=0.0283) (fig. 5.12a) as well as the percentage time spent on open arms ($F_{4,4}$=3.752; P=0.0117) (fig. 5.12b) were significantly decreased when flumazenil was injected intraperitoneally 15 min before administration of diazepam. However, i.p. injection of flumazenil did not affect significantly the percentage number of entries ($F_{3,16}$=2.597; P=0.0883) induced by PHE administration (fig. 5.12a) but it was able to decrease significantly the percentage time spent on the open arms ($F_{3,16}$=4.0395 P=0.0195) (fig. 5.12b).

Similar effects were observed for the percentage numbers of protected stretch attend postures and head dips (fig. 5.12c and 5.12d). The percentage numbers of protected stretch attend postures and head dips for flumazenil-treated animals alone was not significantly different from control. After administration of flumazenil, the numbers of protected stretch attend postures ($F_{1,8}$=19.11 P=0.0024) (fig. 5.12c) as well as head dips ($F_{1,8}$=43.92 P=0.0002) (fig. 5.12d) were significantly increased for diazepam in the presence of flumazenil compared to diazepam alone.

Just like diazepam, concomitant administration of flumazenil and PHE caused a significant increase in both the percentage numbers of protected stretch attend postures ($F_{1,24}$=24.04 P=0.0012) (fig. 5.12c) and the protected number of head dips ($F_{1,8}$=24.04 P=0.0012) (fig. 5.12d).

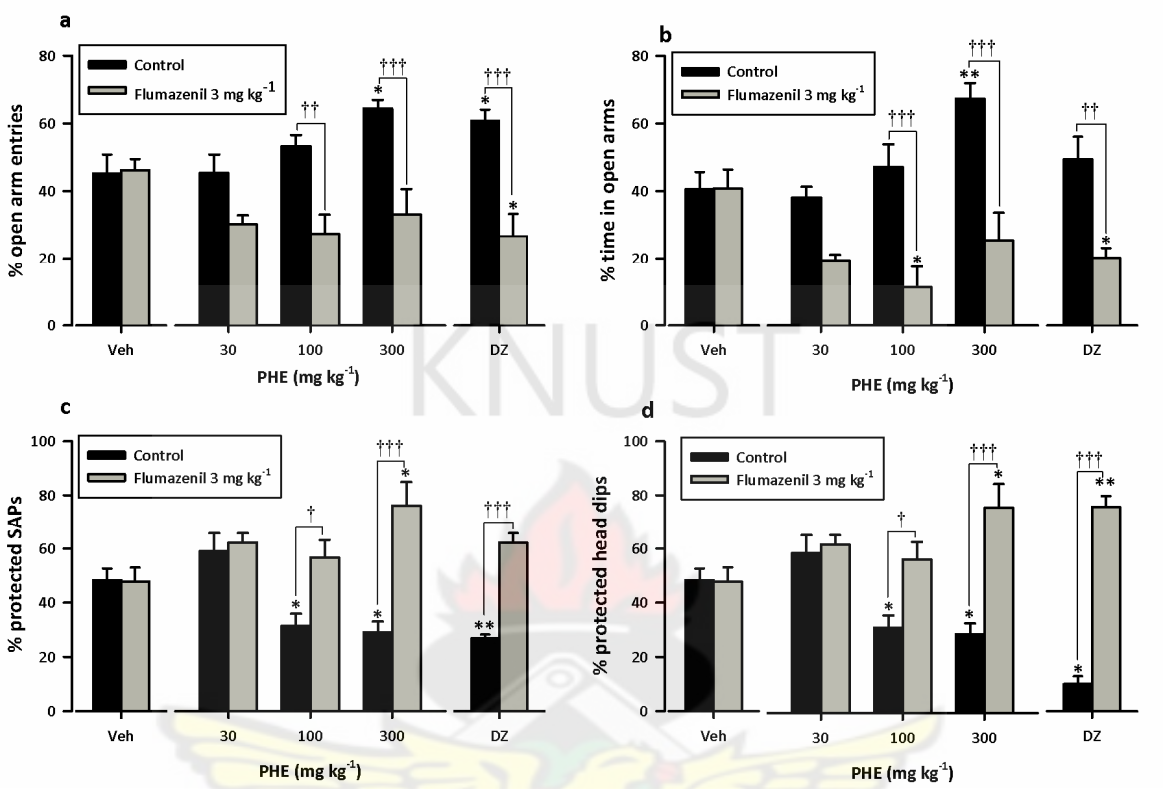

Figure 5.12 Effects of acute PHE (30, 100 and 300 mg kg^{-1}) and diazepam (0.3 mg kg^{-1}) treatment 15 min after pretreatment with flumazenil (3 mg kg^{-1}, ip.) on the % number of open arm entries (a), % time spent in the open arm (b), % protected stretch attend postures (c) and on the % protected head dips (d) in the elevated plus maze test. Data are presented as group means ±SEM. significantly different from control: $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ by Newman-Keuls test and significant difference when the zonal entries where compared to each other: $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$, $^{\dagger\dagger\dagger}P<0.001$ (two-way repeated measures ANOVA followed by Bonferroni's *post hoc* test).

5.2.3.6 DISCUSSION

Ethnomedical and pharmacological knowledge about this plant would allow us to presume that it has a depressant activity on CNS, which could be used to decrease anxiety or depression states in patients. This study has shown anxiolytic activity in the ethanolic leaf extract of *Palisota hirsuta*, as assessed by the open field, light/dark box and elevated plus maze tests. The effect of the plant extract was qualitatively similar to that of diazepam, an anti-anxiety agent. Behavioral models used in the study are based on unconditioned responses to stimuli which are thought to be indicative of generalized anxiety symptoms in humans (Crawley, 1999; Ohl, 2005).

The open field test is utilized to evaluate the animal emotional state. PHE increased the percentage of corner entries, an index of anxiety, as well as the percentage time spent in the central portion of the arena just like diazepam. Both the extract and diazepam caused a reduction in peripheral movement or thigmotaxis without having much effect on the total distance covered. The open-field model examines anxiety-related behavior characterized by the normal aversion of the animal to an open, brightly lit area (Asano, 1986; Choleris *et al.*, 2001; Mechan *et al.*, 2002). Thus, animals removed from their normal environment and placed in a novel environment express anxiety and fear, by showing alteration in all or some parameters, such as decreases in ambulation and exploration time in the center of the open field with increased peripheral movement or thigmotaxis (Bhattacharya, 1994; Bhattacharya and Mitra, 1991). These parameters are attenuated by classical anxiolytics, and potentiated by anxiogenic agents. Open field activity, therefore, represents a valid measure of "anxiety-like" behavior in drug-treated and genetically manipulated animals (Choleris *et al.*, 2001; Prut and Belzung, 2003).

The light/dark test is an ethologically-based approach-avoidance conflict test and it is sensitive to drugs that affect anxiety (Chaouloff *et al.*, 1997; Costall *et al.*, 1989a; Crawley *et al.*, 1997; Ohl, 2005). In this experiment, PHE treated – mice, just like diazepam, spent a significantly more time in the lit chamber of the box than control animals. However, both drugs failed to have any significant effect on the frequency

of lit chamber entries. According to Young and Johnson, (1991), the time spent in the illuminated compartment, rather than the number of transitions, is the most consistent and usual parameter for evaluating anxiolytic activity, while Lepicard *et al.*, (2000) affirmed that the number of transitions reflected both anxiety and exploration, whereas the time spent in the light area was a stronger indication in the study of anxiety emphasizing that the latter is the most robust indicator in the anxiety study and that the first is also a sign of exploratory activity.

The elevated plus maze is considered to be an etiologically valid animal model of anxiety because it uses natural stimuli (fear of a novel, brightly lit open space and fear of balancing on a relatively narrow, raised platform) that can induce anxiety in humans (Dawson and Tricklebank, 1995; Imaizumi and Onodera, 2000; Jung *et al.*, 2000). This test has been demonstrated to be bi-directionally sensitive to both anxiolytic drugs; in particular benzodiazepines (Handley and Mithani, 1984; Lister, 1987; Pellow *et al.*, 1985), as well as compounds which induce anxiety in man (Lister, 1987; Pellow *et al.*, 1985; Pellow *et al.*, 1986). Generally, an anxiolytic agent increases the number of entries into and the time spent in the open arms of the EPM. In agreement with previously published reports, diazepam increased the percentage time spent in open arms and open arm entries (Helton *et al.*, 1998; Moser, 1989). In the present study, oral administration of an extract prepared from the leaves of *Palisota hirsuta* induced an anxiolytic-like effect in mice, since it increased the percentage number of entries and the percentage time spent on open arms of the EPM test.

In addition to using the spatio-temporal indicators of anxiety in the EPM, ethological measures of ''risk assessment'', such as stretched–attend postures and head-dipping, which have been validated and shown by factor analysis to be a more predictive determinant of anxiety were also used (Rodgers *et al.*, 1997; Rodgers and Johnson, 1995). Both PHE and diazepam were able to markedly decrease the percentage protected forms of both stretch attend postures and head dipping indicating reduced anxiety/fear related behaviors. Exposure of animals to

the extract did not have much effect on the total distance travelled in the EPM. However, comparing the total distance travelled in the EPM to that in the open field, the animals seems to move more in the open field than in the EMP which can be indicative of the aversiveness of the EPM. Pretreatment with flumazenil, a specific antagonist of the benzodiazepine site in the $GABA_A$ benzodiazepine receptor complex, was able to reverse the anxiolytic effect induced by both diazepam and the extract indicating that the effects are mainly mediated via the GABAergic system (Rowlett *et al.*, 2001).

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5.2.3.7 CONCLUSION

Results presented here indicated that the ethanolic leaf extract of *P. hirsuta* exhibits anxiolytic effect possibly through the GABAergic system just like diazepam.

Chapter 6

TOXICITY STUDIES

6.1 INTRODUCTION

Toxicological studies are a basic requirement for drugs intended for human consumption. It has been recommended that all natural products used in therapeutics must be subjected to safety tests by the same methods for new scientific drugs (Agbedahunsi *et al.*, 2004; Franzotti *et al.*, 2000; Khalil *et al.*, 2006; Witaicenis *et al.*, 2007). Acute toxicity studies investigate the toxic effects produced by a single large dose of a drug and this information about a drug is required in the establishment of a safety profile for a drug (Veerappan *et al.*, 2006). The results from acute toxicity studies help in the evaluation of any possible hazardous effects of a new drug or a drug which is in use with no documentation of its systemic toxicity (Singh *et al.*, 1987).

6.2 METHODS

6.2.1 ACUTE TOXICITY

ICR mice (20-25 g) of both sexes were randomly selected and divided into five groups of five mice in each group. They were fasted overnight and high doses of *P. hirsuta* extract (1.0-5.0 g kg^{-1}) were orally administered to the group of mice respectively. The control group received 10 ml kg^{-1} *p.o.* of saline. The mice were observed over 24 h after treatment for any change in behavior or death as well as possible lowest lethal dose and highest non-lethal dose.

6.2.2 SUBACUTE TOXICITY

P. hirsuta (3.0-0.3 g kg^{-1}) was administered to Sprague-Dawley rats of both sexes (100-150 g), 5 per group, daily for 14 consecutive days. Group A, the control, received 10 ml kg^{-1} *p.o* of saline daily. Group B, C and D were treated with extract (3.0, 1.2 and 0.3 g kg^{-1} *p.o.*) respectively daily. The extract was prepared such that not more than 2 ml was given orally. The animals were monitored closely for signs of toxicity. The rats were sacrificed on the fifteenth day by cervical dislocation, the jugular vein was cut and blood flowed freely. An

amount of 1.5 ml of blood was collected in a vial containing 2.5 µg of ethylene diamine tetra acetic acid (EDTA) as an anticoagulant for haematological assay and 3.5 ml of the blood was collected into a plain Vacutainer™ tube without anticoagulant. The blood was centrifuged at 500 *g* for 15 min and serum was collected and stored in a freezer until assayed for biochemical parameters.

6.2.2.1 Effect of extract on haematological parameters

Haematological parameters including red blood cells (RBC), white blood cells (WBC), haematocrit (HCT), platelets (PLT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC) were determined by an automatic analyzer (CELL-DYN 1700, Abbot Diagnostics Division, Abbot Laboratories, Abbot Park, Illinois, USA).

6.2.2.2 Effect of extract on serum biochemical parameters

Parameters that were determined include: levels of the liver enzymes- aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), •-glutamyltranspeptidase (GGT), as well as serum determination of total bilirubin (T-BIL), direct bilirubin (D-BIL), indirect bilirubin (I-BIL), total-protein, albumin, blood urea nitrogen (BUN) and creatinine. These were performed using an automatic analyzer ATAC 8000 Random Access Chemistry System (Elan Diagnostics, Smithfied, RI, USA).

6.2.2.3 Effect of extract on organ weights in rats

Selected organs including the spleen, liver, kidney and stomach were excised quickly, trimmed of fat and connective tissue, blotted dry and weighed on a balance. Body weight of the rats was taken on day 0 and 14. The organ-to-body weight index (OBI) was calculated as the ratio of organ weight and the animal body weight x 100. Appearance and behavior pattern were recorded daily and any abnormalities in food and water intake were registered.

6.2.2.4 Histopathological examination

Portions of the tissue from liver, kidney, spleen and stomach were used for histopathological examination. Tissues were fixed in 10% buffered formalin

(pH 7.2) and dehydrated through a series of ethanol solutions, embedded in paraffin and routinely processed for histological analysis. Sections of 2 µm thickness were cut and stained with haematoxylin-eosin for examination. The stained tissues were observed through an Olympus microscope (Model BX51, Olympus America Inc., Center Valley, PA) and photographed by a chare-couple device (CCD) camera.

6.2.3 Analysis of data

Data were presented as mean ± SEM except for the histopathological examinations. Significant differences between means of groups were determined by one-way ANOVA using GraphPad Prism for Windows version 4.02 (GraphPad Software, San Diego, CA, USA).

6.3 RESULTS

6.3.1 Acute Toxicity

All the mice survived throughout the study period (24 h). During observation, the animals did not exhibit any sign of decreased mobility, respiratory distress (gasping), convulsions or death.

6.3.2 Sub acute Toxicity

All the rats survived throughout the 14 days. Furthermore, no remarkable signs of toxicity were observed either immediately or during the post-treatment period even at the highest dose of 3.0 g kg^{-1}. There were also no changes noted in behavior, activity, posture, or external appearance that were considered to be test drug related.

6.3.2.1 Effect of extract on haematological parameters

(Table 6.1) There were generally no significant difference noted between the treated and the control groups for the parameters measured.

6.3.2.2 Effect of extract on serum biochemical parameters

(Table 6.2) There were generally no statistically significant differences for the biochemical parameters measured.

6.3.2.3 Effect of extract on animal body weight and organ weights in rats

Rats in all experimental groups gained weight over the course of this study. Overall, mean body weights were comparable for extract treated groups and control with no significant difference ($P > 0.05$ in all comparison) (figure 6.1). There were generally no statistically significant differences noted in organ-to-body weight index (OBI) between treated and control groups (Figure 6.2).

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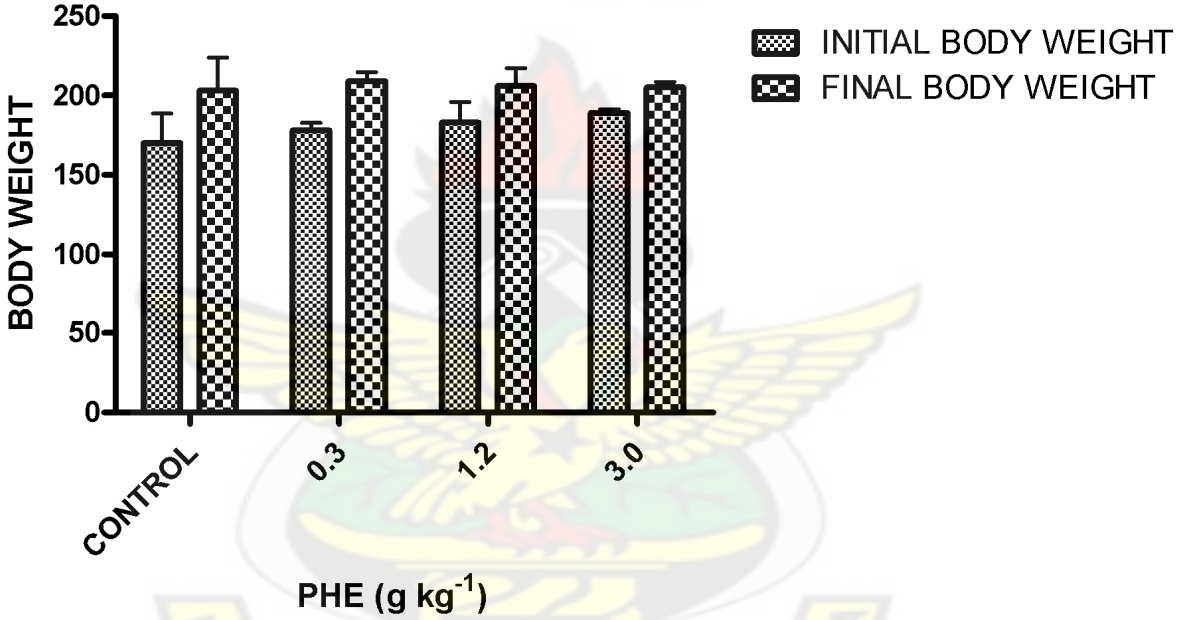

Figure 6.1 Effects of *P.hirsuta* extract (0.3, 1.2 and 3.0 g kg^{-1}) on body weight of rats before and after 14 days of exposure. Data are presented as group means ±S.E.M. significantly different from vehicle by Newman-Keuls test.

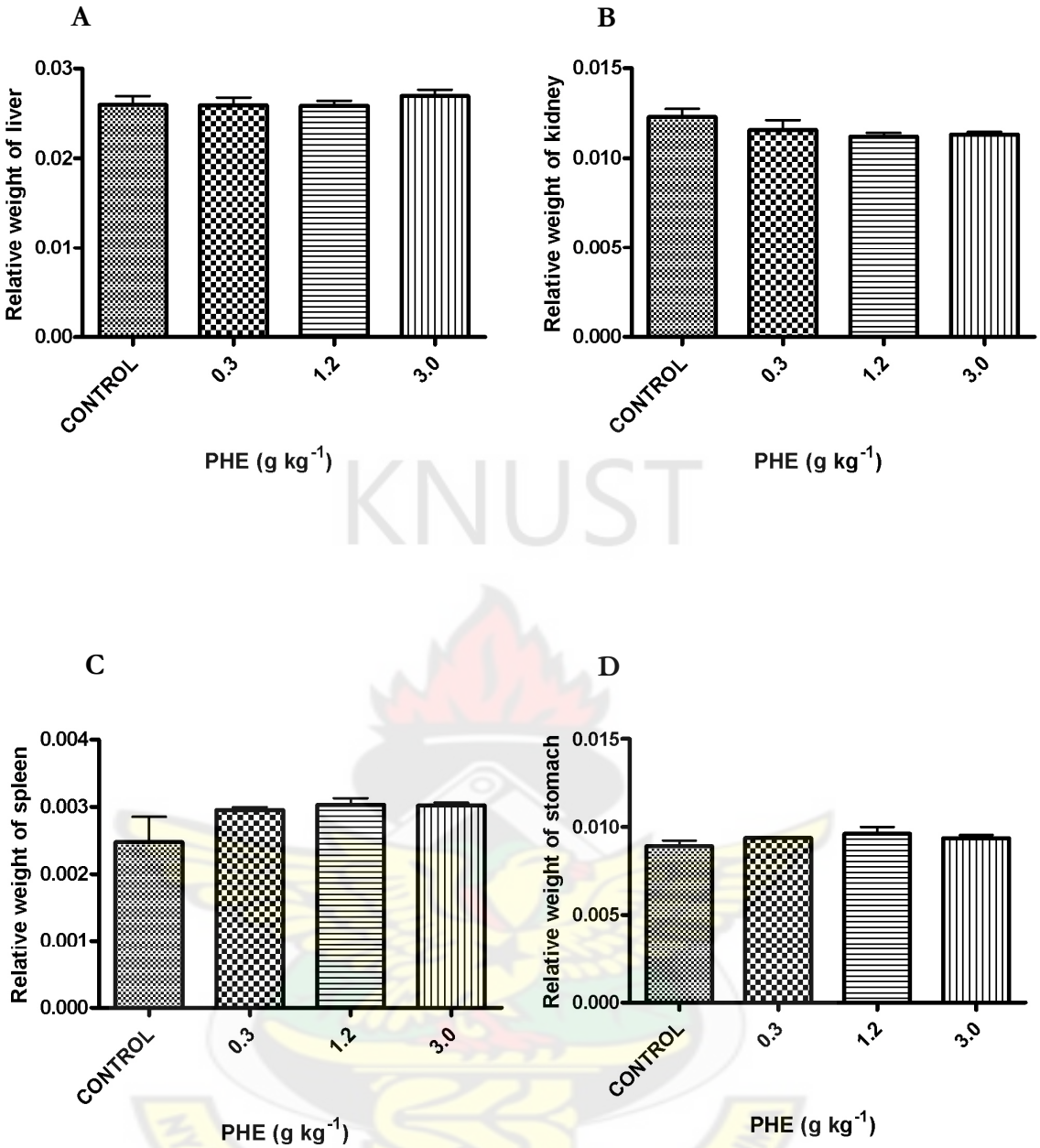

Figure 6.2 Relative weights of liver, kidney, spleen and stomach in rats treated with *P. hirsuta* (0.3, 1.2 and 3.0 g kg^{-1}) (A, B C&D) respectively. Data are presented as group means ± SEM significantly different from vehicle by Newman-Keuls test.

Table 6.1Haematology mean ± SEM following 14 days of exposure to PHE in Sprague-Dawley rats.

	Palisota hirsuta extract (g kg^{-1})					
Test (units)	0	0.3	1.2	3	F test	P Value
WBC (K/ul)	11.62±1.69	10.66±0.81	8.78±0.58	9.54±0.94	$F_{3,16}$=1.3190	0.3028
LYM (%)	6.76±0.86	6.54±0.75	5.68±0.78	5.88±0.85	$F_{3,16}$=0.4004	0.7504
MID (%)	3.58±0.73	3.04±0.62	2.52±0.12	2.60±0.43	$F_{3,16}$=0.8567	0.4835
GRAN (%)	1.30±0.51	1.10±0.52	0.60±0.13	1.06±0.35	$F_{3,16}$=0.5247	0.6715
RBC (M/ul)	6.33±0.35	6.56±0.15	6.44±0.22	6.35±0.20	$F_{3,16}$=0.1905	0.9013
HGB(g/dl)	12.92±1.16	13.08±0.66	13.34±0.76	13.62±0.48	$F_{3,16}$=0.1462	0.9306
HCT (%)	33.28±1.86	35.10 ±0.83	32.66±1.50	34.66±1.59	$F_{3,16}$=0.5872	0.6322
MCV(fL)	55.96±1.01	57.30±0.95	56.64±0.78	53.78±0.95	$F_{3,16}$=2.7310	0.0782
MCH (pg)	19.98±0.86	20.00±0.04	19.92±0.46	20.52±0.81	$F_{3,16}$=0.1552	0.9248
MCHC (g/dl)	34.12±1.10	32.82±0.92	33.92±1.09	33.36±1.23	$F_{3,16}$=0.2896	0.8323
RDW (%)	13.54±0.76	12.58±0.39	13.20±0.65	13.02±0.26	$F_{3,16}$=0.5230	0.6726
Platelets (K/ul)	706.80±34.33	762.40±28.55	720.10±14.50	677.60±48.15	$F_{3,16}$=1.0990	0.3784

Table 6.2Serum biochemistry mean ± SEM following 14 days of exposure to PHE in Sprague-Dawley rats.

	Palisota hirsuta extract (g kg^{-1})					
parameters	0	0.3	1.2	3	F Test	*Pvalue*
Alanine trans. (U/L)	68.40±16.68	71.60±14.56	60.60±12.39	63.21±14.04	$F_{3,16}$=0.118	0.9485
ALP (U/L)	524.20±17.60	512.70±8.94	522.64±17.97	509.40±16.09	$F_{3,16}$=1.016	0.3796
Aspartate trans (U/L)	156.61±8.01	180.61±14.55	161.00 ±8.19	163.60±7.78	$F_{3,16}$=1.094	0.3803
GGT (U/L)	3.60±1.08	4.62±0.51	4.00 ±1.52	6.22±1.37	$F_{3,16}$=0.964	0.4336
Total.protein (g/dl)	52.40±13.13	65.80±2.71	39.20±16.04	64.80±1.20	$F_{3,16}$=1.423	0.2728
Albumen (g/dl)	33.60±1.29	34.62±1.29	26.40 ±6.68	34.00±0.58	$F_{3,16}$=1.231	0.3308
T-Bilirubin (µmol/L)	4.52±1.16	3.72±0.35	2.78 ±0.76	4.32±1.10	$F_{3,16}$=1.911	0.1686
Direct bil. (µmol/L)	2.18±0.34	1.33±0.09	1.42±0.43	1.58±0.19	$F_{3,16}$=2.090	0.1419
I-BIL (µmol/L)	2.34±0.85	2.38 ±0.42	1.36 ±0.64	2.74±0.41	$F_{3,16}$=0.959	0.4361
BUN (mmol/L)	6.94±1.15	7.74±1.61	7.02±0.13	6.12±1.80	$F_{3,16}$=1.225	0.3330
Creatinine (µmol/L)	48.60 ±1.12	47.92±6.68	52.94 ±2.57	49.42±3.60	$F_{3,16}$=1.523	0.2468

6.3.3 Histopathological Studies

No test drug-related changes were observed in this study. For all the rats in the treated and control groups, the morphological structure of the livers, capsule and hepatic lobule were normal and no necrosis or denaturation was found. No infiltration of inflammatory cells observed in the portal area and no hyperplasia was found in connective tissues (Plate 6.2). The morphological structure of renal glomerulus was also found normal for each rat in the study. Neither renal glomerulus nor epithelia of renal capsule were thickened (Plate 6.1). There were no pathologic changes observed for the spleens of both control and treated animals. There were no signs of spleenomegaly including no signs of smooth muscle actin expression (Plate 6.3). All stomach samples showed normal zymogenic cells, parietal cells and normal grooves in the mucosa. There was neither atrophy nor inflammatory cell infiltration present in any of the samples (plate 6.4).

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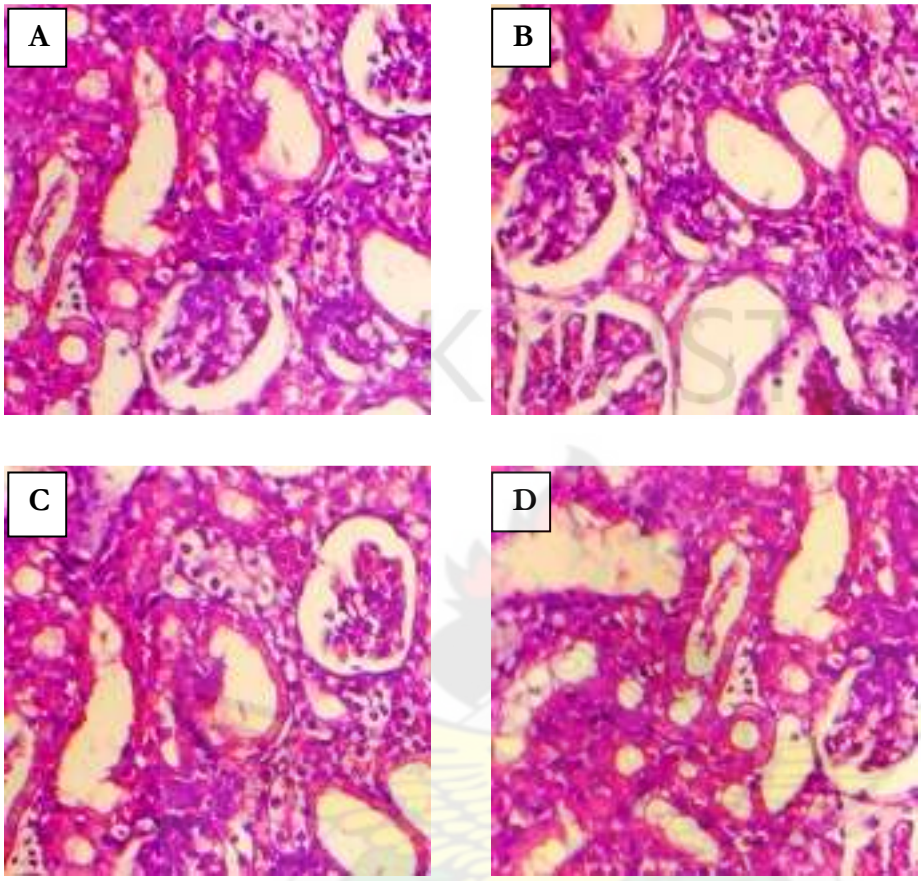

Plate 6.1 Photomicrograph of a transverse section of the kidneys of control (A) and *P. hirsuta* treated groups (0.3, 1.2 and 3.0 g kg^{-1}) (B, C & D) respectively for 14 days repeated dose (H & E, x 40, n = 5).

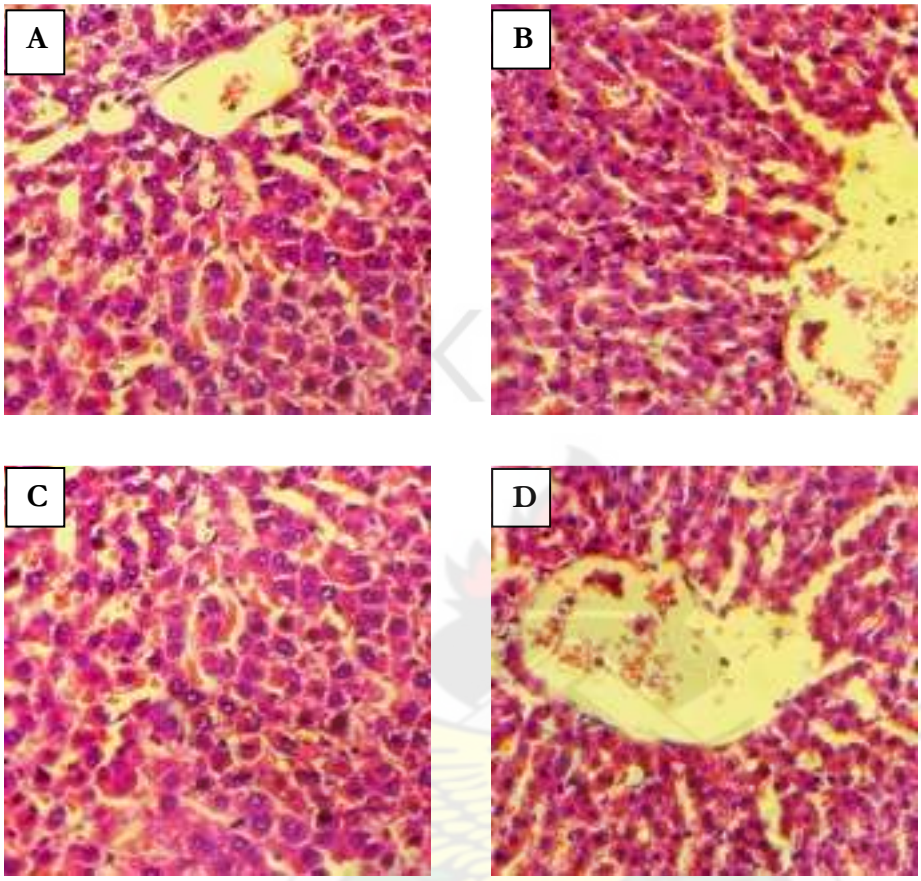

Plate 6.2 Photomicrograph of a transverse section of the livers of control (A) and *P. hirsuta* treated groups (30, 100 and 300 mg kg^{-1}) (B, C & D) respectively for 14 days repeated dose (H & E, x 40, n = 5).

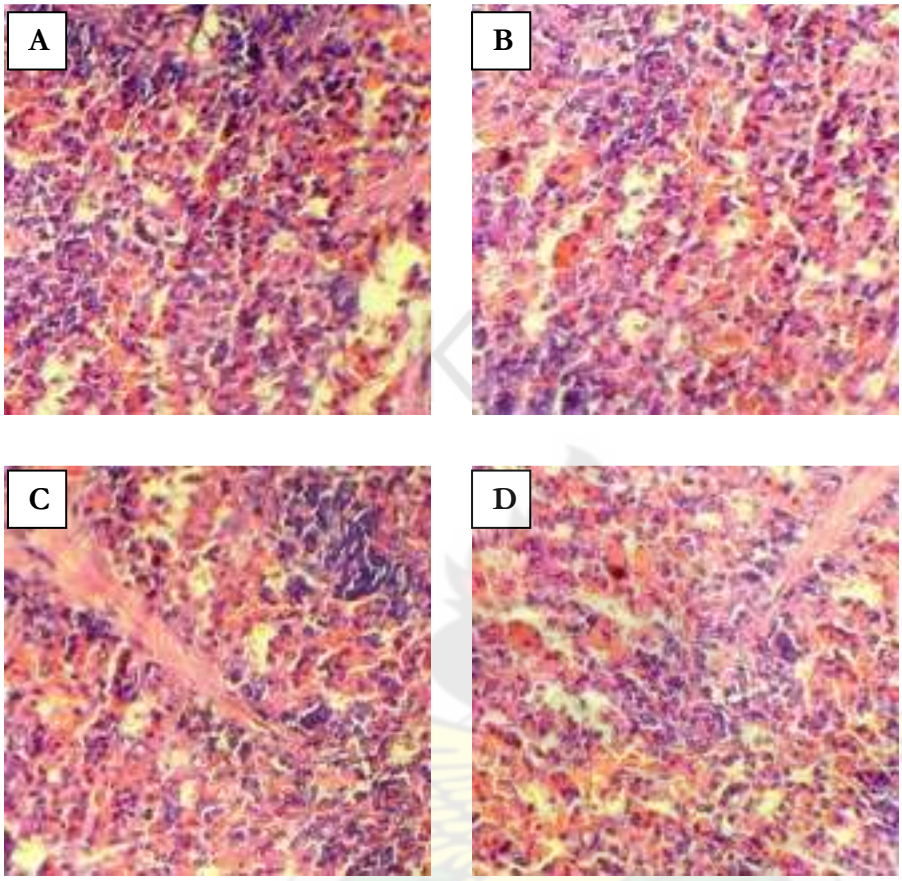

Plate 6.3 Photomicrograph of a transverse section of the spleens of control (A) and *P. hirsuta* treated groups (0.3, 1.2 and 3.0 g kg^{-1}) (B, C & D) respectively for 14 days repeated dose (H & E, x 40, n = 5).

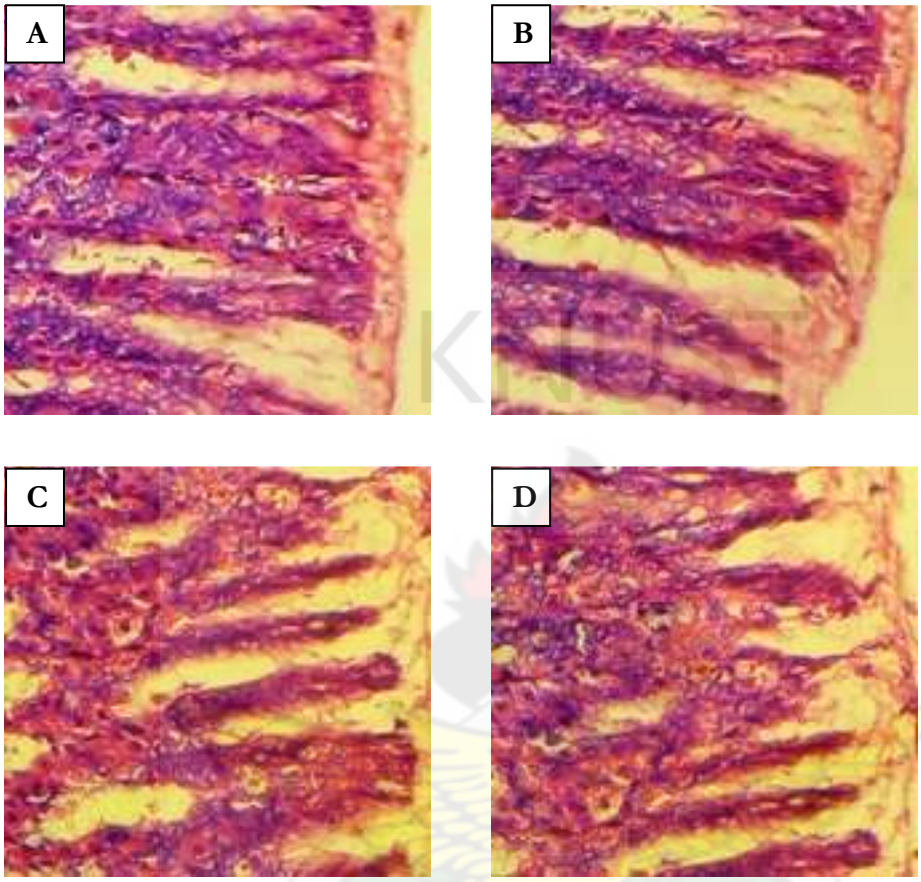

Plate 6.4 Photomicrograph of a transverse section of the stomach of control (A) and *P. hirsuta* treated groups (0.3, 1.2 and 3.0 g kg^{-1}) (B, C & D) respectively for 14 days repeated dose (H & E, x 40, n = 5).

6.4 DISCUSSION

When PHE was administered to Sprague Dawley rats at 300 - 3000 mg kg^{-1} (*p.o.*) body weight per day for 14 days, there were no significant adverse toxicological effects attributable to the treatment. The no-observable- adverse-effect level (NOAEL) for PHE was found to be more than 3000 mg kg^{-1} body weight per day when administered orally for 14 consecutive days. This is indicative that, the LD_{50} for extract is above 3000 mg kg^{-1}.

In toxicological experiments involving animals, decrease in body weight is an indicator of adverse effects as the animals that survive any toxicity test cannot lose more than 10% of the initial body weight (Raza *et al.*, 2002; Teo *et al.*, 2002). Monitoring of body weight gain and food consumption in drug studies can be an indicator of overall animal health (Borzelleca, 1996). Changes in organ weights are also indices of toxicity in animals which are readily determined in short-term toxicity tests. There is a very high possibility that herbal constituents and preparations, when ingested into the body may be toxic to important organs such as the kidney, liver, spleen, stomach, and lungs because of their diverse roles in the human body. The absence of any significant differences in the body weight and weights of the liver, kidney, spleen and stomach provides support for the safety of the extract.

The criteria for assessing histopathological changes include necrosis, cloudy swelling, fatty infiltration of cells, inflammatory infiltrations among other parameters. Cells, which are unable to adapt to stimuli exhibit a variety of morphological changes, first seen ultrastructurally and later as visible light microscopic abnormality. Cells that have failed to adapt to metabolic stress, cease to produce structural proteins and begin to have difficulty in supplying energy to preserve electrolyte gradients to sustain other membrane functions. Light microscopic examination of cells typically shows fluid accumulation in cells, which make them pale-staining or vacuolated. This is described as cloudy swelling or hydropic degeneration. Cells failing to metabolize fatty acid

accumulate lipid within cytoplasmic vacuoles giving rise to term fatty change. If the injury is irreversible, there is a progressive failure of key structural and metabolic components leading to death of cells; this is termed necrosis (Wheater, 1990). There were no findings at the end of the study in either macroscopic or histopathologic examinations of the liver, kidney, spleen and stomach that indicated that any of these effects were related to treatment with the test material. No test drug-related changes were observed in this study. All changes were considered normal background lesions in this strain and age of rat.

Blood is a very important tissue in the body and forms the main medium of transport for many drugs and xenobiotics in the body. With almost all foreign compounds distributed via the bloodstream (Timbrel, 2000), the components of the blood such as red blood cells, white blood cells, haemoglobin and platelets are at least initially exposed to significant concentrations of toxic compounds. Damage to and destruction of the blood cells results in a variety of sequelae such as a reduction in the oxygen carrying capacity of the blood if the cells affected here are the red blood cells. The haematological system carries a higher predictive value (91%) for toxicity in humans when assays involve rodents and non rodents (Olson *et al.*, 2000) and as such makes the assessment of blood very relevant to the evaluation of risk. Moreover, certain medicinal herbal preparations have been reported to adversely affect various blood components causing conditions like haemolytic anaemia and thrombocytopenia (Gandolfo *et al.*, 1992; King and Kelton, 1984; Synder *et al.*, 1977; Yunis *et al.*, 1980). *P. hirsuta* had no marked changes in the haematological indices.

Liver function was determined in order to detect possible hepatic dysfunction, tissue damage or changes in biliary excretion evoked by prolonged exposure to the extract. The liver is the major site for the metabolism of most chemicals and it has the ability to metabolize a large number of drugs including herbal medicines and this may predispose it to toxicity since metabolism does not always result in detoxification. Sometimes more toxic compounds are produced which may in turn affect the liver. The liver is also involved in intermediary

metabolism and synthesis of several substances including most of the proteins in the body. When a compound precipitates in the canalicular lumen, there is an interference with the production and flow of bile which may cause damage to the biliary system and surrounding hepatocytes and cause the leakage of liver enzymes into the blood. Blood supply to the liver also exposes it to relatively high concentrations of toxic substances absorbed from the gastrointestinal tract.

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) determination is the most common means of detecting liver damage. Plasma levels of these enzymes are raised several folds in the first 24 hours after damage. The liver produces most of the plasma proteins in the body. This includes albumin and globulin. After 14 days of *P. hirsuta* extract administration, the amount of total proteins and albumin in the serum compared to vehicle treated animals were not significantly different which is very indicative that the extract did not interfere with the synthetic ability of the liver.

Bilirubin is the main pigment that is formed from the breakdown of haeme in red blood cells. It is conjugated in the liver and then secreted into the bile. Hence increased levels of bilirubin in the plasma may result from an increase in its production, a decrease in its conjugation, a decrease in its secretion by the liver, or a blockade of the bile ducts. In cases of increased production, or decreased conjugation, the unconjugated or indirect form of bilirubin is elevated. A rise in serum levels of unconjugated bilirubin indicates pre hepatic or hepatic jaundice whereas a rise in conjugated bilirubin indicates post hepatic jaundice. When the bile ducts are obstructed, there is a buildup of direct bilirubin. This escapes from the liver and ends up in the blood increasing plasma levels which is indicative of significant damage to hepatocytes (Akdogan *et al.*, 2003). Serum bilirubin is thus considered a true test of liver function, since it reflects the liver's ability to take up, process, and secrete bilirubin into the bile. Hence, since there were no marked changes in direct,

indirect and total bilirubin fractions after treatment with the extract it indicates that the extract does not alter hepatic metabolism or biliary excretion.

The liver has a variety of enzymes to synthesize and breakdown amino acids and to interconvert energy storage molecules. These enzymes include Gamma glutamyl transferase (GGT), alkaline phosphatase (ALP), aspartate aminotransferase (AST) and alanine aminotransferase (ALT). Plasma levels of these enzymes are usually low. Whenever there is any significant damage to the hepatocytes, plasma levels of these enzymes are elevated (Alder *et al.*, 1981; Kallner *et al.*, 1989; Stonard *et al.*, 1995). Several reports have revealed that abnormal levels of the enzymes in plasma are usually indicative of the hepatic cellular injury in experimental animals (Biasi *et al.*, 1991; Knook *et al.*, 1995; Miao *et al.*, 1990; Mondini *et al.*, 2006; Murray, 1998; Parola *et al.*, 1992).

The levels of liver enzymes were decreased following administration of the extract indicating that liver tissue damage was not induced. Other biochemical markers such as plasma levels of total bilirubin, total proteins, globulin and albumin in animals exposed to the extract over 14 days period did not see any marked changes clearly demonstrating that liver function was preserved in these animals.

Similarly, blood urea nitrogen (BUN) and creatinine were used as indicators for assessing renal function for possible nephrotoxicity of chronic treatment with *P. hirsuta* extract. There was no significant difference in blood samples from animals exposed to PHE and vehicle treated animals, indicating that renal function was unaffected by PHE treatment.

6.5 CONCLUSION

Based on this study, it can be concluded that the extract is safe in the animals used since it did not show any overt effect in any of the parameters examined in the study.

Chapter 7

GENERAL DISCUSSION

The present study has demonstrated that the ethanolic leaf extract of *Palisota hirsuta* has significant inhibitory effect on both acute and chronic inflammation. It also exhibited anti-nociceptive potential as well as anti-pyretic activity. The study has also established the anti-depressant and anxiolytic properties of the extract in addition to its anti-oxidant properties. Also, this study has demonstrated the acute and sub acute safety profile of the extract in rats.

Based on the effect on carragenan-induced acute inflammation, the extract may inhibit or interfere with the production of some inflammatory mediators, especially prostaglandins (Muniappan and Sundararaj, 2003; Vinegar *et al.*, 1987). The role of prostaglandins in the generation of fever is also well established (Romanovsky *et al.*, 2005) and as such the extract showing significant anti-pyretic activity further supports the inhibitory effects of the extract on prostaglandin synthesis. This speculation is further strengthened by the role of the plant extract in peripheral analgesia in which prostaglandins are well known to play a major role (Rosland *et al.*, 1990; Tonussi and Ferreira, 1994). However, most agents that are known to affect prostaglandin synthesis also have the potential of damaging the mucosal lining of the stomach (Wolfe *et al.*, 1999; Wong *et al.*, 2005) and also causing nephrotoxicity (Engelhardt and Trummlitz, 1990; Loewen, 2002; Mirshafiey *et al.*, 2005). Histology of the stomach after sub-acute toxicity studies did not show any difference between drug-treated and control animals. In addition, the extract did not show any overt effect on the kidneys; ruling out nephrotoxicity. This makes the findings in this study unique in that, even though the extract is acting similarly to most NSAIDS as an anti-inflammatory, anti-pyretic and an analgesic, it could be free from two of its worrisome side effects gastric ulceration and nephrotoxicity as it did not affect the integrity of the stomach and/or the kidneys.

One major drawback of centrally acting analgesics in current clinical practice especially morphine is the development of tolerance. In this study, the extract

exhibited potent central analgesic activity which may be mediated partially or wholly to the stimulation of peripheral opioid receptors through the activation of the nitric oxide-cyclic GMP- ATP-sensitive K^+ (NO/cGMP/K^+ATP)-channel pathway similar to that of morphine but without the development of tolerance. This puts the extract in a very promising position since the development of tolerance associated with morphine sometimes limits its use in the management of chronic painful conditions.

Cognitive and emotional factors have been shown to interact with the ascending regulation of pain transmission in the spinal cord (Woolf, 2004). The neuromatrix theory suggests an interaction between brain activities and pain processing in neural networks (Melzack, 1999). Several midbrain/ hindbrain areas that are involved in fear, anxiety, mood regulation and autonomic responses are associated with pathways that are activated by painful stimuli (Millan, 2002). Although it is a common belief that anxiety will increase pain, research has demonstrated no consistent relationship between anxiety and pain. However, anxiety that is relevant or related to pain may increase the patient's perception of pain. *P. hirsuta* leaf extract which has proven to be an analgesic both centrally and peripherally as well as an anxiolytic will therefore be a better candidate in the management of pains especially chronic pain.

Just as anxiety is associated with pain because of concerns and fear about the underlying disease, epidemiologic studies indicate that depression is also a common co morbidity accompanying chronic pain states. Aside the analgesic activity exhibited by the extract, it also showed a clear antidepressant effect qualitatively similar to imipramine in both the forced swimming and tail suspension tests strengthen further its usefulness in the management of chronic pain states. Moreover, the clinical effectiveness of antidepressants as analgesics have recently been confirmed in preclinical studies using various tests of nociceptive activity, which use thermal, mechanical, electrical or chemical stimuli (Duman *et al.*, 2004; Otsuka *et al.*, 2001; Rojas-Corrales *et al.*, 2003; Schreiber *et al.*, 1999), as well as in animal models of chronic pain (Anjaneyulu and Chopra, 2004; Marchand *et al.*, 2003a; Zarrindast *et al.*, 2000). They are

believed to interact with the pain pathways in several ways and the effect is separated from the anti-depressant action (Arnold *et al.*, 2005). These further emphasize the possible superiority of the extract over the opioids in pain management especially in the treatment of major depression-pain related disorders.

The probable usefulness of this plant extract in the treatment of arthritis either as monotherapy or in combination with other standard drugs was also established. Furthermore, it also had potent antioxidant properties. The ability of polyphenols (electron rich compounds) to engage in electron-donation reactions with oxidizing agents like superoxide and hydrogen peroxide which are produced in large amounts 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). Although the effects of the extract on the free radical levels *in vivo* was not investigated in this study, reports that the anti-inflammatory effect of several compounds is closely related to their antioxidant properties such as their ability to protect against lipid peroxidation (Costa *et al.*, 2004), supports the assertion that the antioxidant properties of the *Palisota* extract may contribute in part to its anti-arthritic effects. Moreover, systemic inflammation is also known to be accompanied by changes in body temperature (Romanovsky *et al.*, 2005; Yesilada and Kupeli, 2007) and with the extract exhibiting potent anti-pyretic activity as well also signifies its role in the management of some of the systemic effects of inflammation like fever.

Depression and arthritis take an immense toll on individual and public health. Studies conducted in the United States as well as other developed and developing countries consistently show that depression is 2 to 3 fold more prevalent among patients with arthritis relative to patients with no arthritis (Bair *et al.*, 2003; Shih *et al.*, 2006; Stang *et al.*, 2006). When depression accompanies arthritis, patients show higher activity limitation related to arthritis and increased healthcare use and medical cost (Moussavi *et al.*, 2007)

which is similar to the observed increases in disability and healthcare cost when depression is comorbid with other chronic medical conditions (Katon, 2003; Simon *et al.*, 2005). Despite efficacious and accessible treatment options, poor outcomes of persistent depression and arthritis, particularly arthritic pain are common. With PHE showing potent anti-arthritic as well as anti-depressant effects, it is likely that PHE will give a better treatment outcome by improving arthritis- related pain and function, as well as better general health and quality of life as shown in a randomized trial involving depressed individuals with comorbid arthritis in primary care settings (Lin *et al.*, 2003).

The roles of constitutive and inducible nitric oxide synthase (NOS) isoforms in inflammation have been extensively studied (Ialenti *et al.*, 1992; Ialenti *et al.*, 1993; Weinberg *et al.*, 2007). It has been suggested that NO plays a role in the early stages of inflammation as a mechanism to decrease and limit the process by inhibiting white cell activation (Kubes *et al.*, 1991) and platelet aggregation (Moncada *et al.*, 1990) and by inducing vasodilatation (Kajekar *et al.*, 1995). NO has also been identified to play a likely role in down-regulating the activity of osteoclasts (MacIntyre *et al.*, 1991). Furthermore, NOS inhibitors have been shown to potentiate bone resorption and enhance bone loss in animals (Tsukahara *et al.*, 1996). Based on the premise that the extract is able to activate the nitric oxide pathway as part of its analgesic mechanism, it is possible that this activation can also be responsible in part to the role of the extract in modulating both acute and chronic inflammation as well decreasing bone loss and resorption.

Oxidative stress is known to be primarily or secondarily involved in the pathogenesis of major depression (de Kloet *et al.*, 2005; Michel *et al.*, 2007) and as such several authors have established the co-existence of increased oxidative stress with symptoms of depression in patients, as evidenced by defective plasma antioxidant defenses in association with enhanced susceptibility to lipid peroxidation (Bilici *et al.*, 2001; Khanzode *et al.*, 2003; Maes *et al.*, 2000; Ozcan *et al.*, 2004; Sarandol *et al.*, 2007; Tsuboi *et al.*, 2006). The role of endogenous antioxidant status in the therapeutic actions of chronic antidepressant

treatments has been clearly established and they are widely prescribed for the treatment of stress and stress-related depression and anxiety (Diamond and Rose, 1994). Some antidepressant drugs have been demonstrated to up-regulate gene expression and activity of the important neuroprotective antioxidant enzyme superoxide dismutase (SOD) (Bilici *et al.*, 2001; Kolla *et al.*, 2005; Li *et al.*, 2000; Sarandol *et al.*, 2007; Zafir and Banu, 2007). Even though the antioxidant effect of the extract *in vivo* was not established in this study, it exhibited significant antidepressant activity which can be dependent in part to the potent antioxidant activity shown by the extract *in vitro* in this study.

Behavioral and biochemical data indicate the contribution of the dopamine (DA) neurotransmitter system in the pathophysiology of anxiety and depression (Rogoz *et al.*, 2002a; Rogoz *et al.*, 2002b). It is also well established that stress activates the mesocorticolimbic DA system, and increases extracellular DA in the nucleus accumbens septi and medial prefrontal cortex, inducing anxiolytic-like behavioral effects (Cabib and Puglisi-Allegra, 1994; Salamone, 1994). There is also evidence that stress induced increases in DA metabolism can be attenuated by anti anxiety drugs, such as diazepam (Decker and McGaugh, 1991). Animal studies (Costall *et al.*, 1987; Pich *et al.*, 1986) showed that D_2 receptor antagonists such as haloperidol present anxiolytic-like effects. It has been demonstrated that mice without functional D_3 receptors show a reduced anxiety in the open field and elevated plus maze tests (Steiner *et al.*, 1997). Recent data (Rogoz *et al.*, 2004) suggest that preferential receptor agonists may play a role in the therapy of anxiety and/or depression. In addition, continual administration of antidepressants, including imipramine, significantly augmented D_3 receptor mRNA expression, in the nucleus accumbens, and improved the activity of central mesolimbic D_2 and D_3 receptors (Maj *et al.*, 1998). Based on these, the probable involvement of dopaminergic mechanisms in the CNS effects exhibited by the extract in this study cannot be overlooked.

It must however be pointed out that, PHE contains several secondary metabolites and therefore apart from the speculated GABAergic and

dopaminergic involvement in the anxiolytic and anti depressant effects, other mechanisms and neurotransmitters may be involved such as, serotonergic, and glutamatergic neurotransmissions. Further experiments, may be necessary therefore to confirm the exact mechanism/s involved in these CNS effects.

Even though the extract did not show any overt effect in any of the parameters examined in the acute and sub acute toxicity studies, both chronic toxicity studies results and results from acute toxicity studies however help in the evaluation of any possible hazardous effects of a new drug or a drug which is in use with no documentation of its systemic toxicity like PHE.

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Chapter 8

CONCLUSIONS AND RECOMMENDATIONS

8.1 CONCLUSIONS

The results of this novel study have provided evidence to support the use of *P. hirsuta* leaves as an analgesic and anti inflammatory/anti-arthritic agent in traditional medicine. These effects might be partially or wholly due to:

- Possible inhibition or interference with the production of some inflammatory mediators, especially prostaglandins
- Its potent antioxidant properties as a reducing agent, a free radical scavenger, and a potent inhibitor of lipid peroxidation
- Stimulation of peripheral and/or central opioid receptors through the activation of the nitric oxide-cyclic GMP- ATP-sensitive K^+ ($NO/cGMP/K^+ATP$)-channel pathway without the development of tolerance to its use
- Its anxiolytic effects possibly through the activation of the GABAergic system
- Its anti-depressant properties qualitatively similar to imipramine by affecting levels of noradrenaline and dopamine

The toxicological finding of this study indicates that, the ethanolic leaf extract of *P. hirsuta* is safe for consumption and more importantly, even though the extract is acting similarly to most opioids and NSAIDS as an anti-inflammatory, anti-pyretic and an analgesic, it did not affect the integrity of the stomach and/or the kidneys.

8.2 RECOMMENDATIONS

- Effect of the extract in other pain models like neuropathic pain should be investigated.
- Effect of the extract in other models of arthritis example, collagen-induced and urate-induced polyarthritis should be investigated.
- In vivo antioxidant properties of the extract should be conducted.
- Fractionation of crude extract and isolation of specific active compounds responsible for the identified pharmacological effects.
- Mechanism(s) of actions should be further investigated.
- Acute and sub acute toxicity in other species of rats and higher animals together with sub chronic and chronic toxicity studies should be conducted.

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APPENDIX

PHARMACOLOGICAL METHODS

PREPARATION OF CARRAGEENAN SUSPENSION

A 2% carrageenan suspension was prepared by sprinkling small amounts of the powder (200 mg) evenly over the surface of 10 ml of 0.9% NaCl solution and left to soak between additions. It was then left for 2-3 hours before use.

PREPARATION OF COMPLETE FREUND'S ADJUVANT (CFA)

50 mg heat-killed Mycobacterium tuberculosis [strains C, DT and PN (mixed) obtained from the Ministry of Agriculture, Fisheries and Food, U.K] was finely grounded in a mortar using a pestle. Liquid paraffin was added gradually to make 20 ml of 5 mg ml-1 suspension.

PREPARATION OF PHOSPHATE BUFFER

Sodium dihydrogen phosphate monohydrate (8.942 g) and disodium hydrogen phosphate heptahydrate (9.433 g) were dissolved in 500 ml distilled water to make 0.2M sodium phosphate buffer, pH 6.6.

Sodium dihydrogen phosphate monohydrate (1.558 g) and disodium hydrogen phosphate heptahydrate (10.374 g) were dissolved in 500 ml distilled water to make 0.1M sodium phosphate buffer, pH 7.4.

FOOT VOLUME MEASUREMENT

A liquid column containing water was placed on a balance. When an object is immersed, the liquid applies a force F to attempt its expulsion. Physically, F is the weight (W) of the volume of liquid displaced by that part of the object inserted into the water. A balance was used to measure this force (F=W). Therefore, the partial or entire volume of any object, for example the inflamed foot of a chick, can be calculated thus, using the specific gravity of the immersion liquid, at equilibrium mass/specific gravity = volume (V). Since

water was used as the immersion liquid in this case, the mass or weight of the foot inserted in the water will be the same as its volume.the extent of oedema at time t (measured as V) will be $v_1 - v_0$. The foot being measured was kept away from contacting the wall of the column containing the water whilst the value on the balance was being read.

CALCULATION

Percentage increase in foot volume = $(V_t - V_o)/V_o \times 100$

Where, V_t is the foot volume at time t (after injection).

Vo is the foot volume before injection. (0 h)

DRUG PREPARATION AND ADMINISTRATION

A 2% w/v suspension of tragacanth in 0.9% NaCl was prepared by mixing thoroughly the tragacanth powder in saline with a stirrer. This was used to suspend the plant extract. All the other drugs were prepared by diluting the stock with 0.9% NaCl. Generally, drug concentration were made such that the required dose was always given in equivalent volumes not exceeding a total volume of 0.4ml. for oral administration and 0.2ml for intraperitoneal route.