ANTI-NOCICEPTIVE AND ANTI-INFLAMMATORY EFFECTS OF AN ETHANOLIC EXTRACT OF THE AERIAL PARTS OF *HILLERIA LATIFOLIA* (LAM.) H. WALT. (PHYTOLACCACEAE)

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DECLARATION

The experimental work described in this thesis was carried out at the Department of Pharmacology, Faculty of Pharmacy and Pharmaceutical Sciences, College of Health Sciences, KNUST in the period from 2007 to 2010. This work has not been submitted for any other degree.

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ABSTRACT

Pain and inflammation remain a real and current problem in clinical medicine and require new agents with improved efficacy for more effective therapy. The aerial parts of the perennial herb *Hilleria latifolia* (Lam.) H. Walt. (Family: Phytolaccaceae) are used in Ghanaian traditional medicine for the treatment of various painful and inflammatory conditions. The present study examined the anti-nociceptive, anti-inflammatory, some neurobehavioural properties as well as toxicity of an ethanolic extract of the aerial parts of *Hilleria latifolia* in animal models.

Preliminary phytochemical screening of the *Hilleria latifolia* extract (HLE) revealed the presence of saponins, tannins, glycosides, steroids, terpenoids as well as small amounts of flavonoids and alkaloids.

HLE (10–300 mg kg^{-1}, *p.o.*), together with morphine and diclofenac (positive controls), showed significant anti-nociceptive activity in chemical (acetic acid-induced abdominal writhing, glutamate, formalin and capsaicin tests) and thermal (tail immersion test) behavioural pain models in rodents. The anti-nociceptive effect exhibited by HLE in the formalin test was partly or wholly reversed by the systemic administration of naloxone (a non-selective opioid receptor antagonist), theophylline (a non-selective adenosine A_1/A_2 receptor antagonist) and atropine (a non-selective muscarinic receptor antagonist). Cyproheptadine (a 5-HT_{2A} receptor antagonist), ondansetron (a 5-HT_3 receptor antagonist), yohimbine (a selective α_2-adrenoceptor antagonist), nifedipine (L-type Voltage-gated calcium channel blocker), glibenclamide (an ATP-sensitive K^+ channel inhibitor) and N^G-L-nitro-arginine methyl ester /L-NAME (a NO synthase inhibitor), however, did not significantly block the anti-nociceptive effect of the extract. HLE, unlike morphine, did not induce tolerance to its anti-nociceptive effect in the formalin test after chronic administration; morphine tolerance did not also cross-generalize to the anti-nociceptive effects of HLE. Interestingly also, chronic concomitant administration of HLE and morphine significantly suppressed the development of morphine tolerance. Hexamethonium antagonised the neuronal nicotinic effects of HLE on isolated guinea pig ileum preparation. Together, these results indicate that HLE produces dose-related anti-nociception in several models of chemical and thermal pain—without tolerance induction—through mechanisms that may involve an interaction with adenosinergic, nicotinic cholinergic, muscarinic cholinergic and opioidergic pathways.

Oral administration of HLE (10-300 mg kg^{-1}, *p.o.*), either pre-emptively or curatively, significantly inhibited carrageenan-induced foot oedema in 7-day old chicks with maximal inhibitions of 38.11±5.55 % (pre-emptive) and 30.91±4.66 % (curative). Similarly, the NSAID diclofenac (10-100 mg kg^{-1}, i.p.) dose-dependently reduced the oedema by 59.33±10.82 % and 42.87±7.46 % respectively for pre-emptive and curative treatments. Dexamethasone (0.3-3 mg kg^{-1}, i.p.), a steroidal anti-inflammatory agent inhibited the oedema with maximal effect of 42.77±7.64 % (pre-emptive) and 36.60±6.76 % (curative).

In the Freund's adjuvant induced-arthritis model in rats, HLE as well as the positive controls, dexamethasone and methotrexate, showed significant anti-arthritic properties when applied to established adjuvant arthritis. Oral administration of HLE (10-300 mg kg^{-1} *p.o.*) significantly reduced oedema in the ipsilateral paw of rats with a maximal inhibition of 32.64± 2.74 %. HLE (10-300 mg kg^{-1} *p.o.*), however, did not significantly affect joint oedema or systemic arthritic spread which is usually indicated by the inhibition of the spread of the oedema from the ipsilateral to the contralateral paw. The DMARD methotrexate (0.1-1 mg kg^{-1}, i.p.) and the steroidal anti-inflammatory agent dexamethasone (0.3-3 mg kg^{-1}, i.p) reduced very significantly the total polyarthritic oedema as well as the spread of the arthritis from the ipsilateral to the contralateral paws of the treated animals.

In all the *in vitro* antioxidant tests performed, with the exception of the total phenol assay and total antioxidant capacity, *n*-propyl gallate was used as the reference antioxidant. The extract (0.03-1 mg ml^{-1}) exhibited Fe^{3+} reducing activity (EC_{50}=2.071±0.782 mg ml^{-1}), scavenged DPPH (EC_{50} =0.2269±0.037 mg ml^{-1}) and prevented lipid peroxidation (IC_{50} =0.1122±0.010 mg ml^{-1}). *N*-propyl gallate showed similar effects like the extract but was more potent. The total phenol content of HLE was estimated to be 29.40±1.09 mg tannic acid equivalent/g of HLE while the total antioxidant capacity was 55.16±13.60 mg ascorbic acid equivalent/g of HLE. These findings reveal that the extract has antioxidant properties which may partly account for its anti-inflammatory activity.

Neurobehavioural properties of HLE were evaluated in various behavioural paradigms—elevated plus maze (EPM), the light/dark box, forced swimming test (FST), tail suspension test (TST) and pentobarbitone sleeping time test. HLE (10-300 mg kg^{-1}, *p.o.*) displayed anxiolytic activity similar to diazepam in all the anxiety models used by significantly increasing the number of inter-compartment transitions and time spent in the lit area of the light/dark box as well

as significantly increasing open arm entries, percentage open arm entries and percentage open arm time in the EPM. The extract (10-300 mg kg^{-1}, *p.o.*) also exhibited antidepressant effects by reducing the duration of immobility in both the FST and TST. The extract (10-300 mg kg^{-1}, *p.o.*) neither modified motor performance in the beam walk test nor caused CNS depression in the pentobarbitone sleeping time test. These results suggest that the extract has anxiolytic and antidepressant effects.

Acute and sub-acute toxicity tests were conducted by the oral route in rats. During the experiment, no deaths were observed in any groups and there were no remarkable changes in general appearance, as well as in food and water consumption. The LD_{50} of HLE was estimated to be above 3000 mg kg^{-1}. The no-observed-adverse-effect level (NOAEL) of *H. latifolia* was 300 mg kg^{-1}. No significant changes were observed in haematological parameters, body weights and organ/body weight ratios. There were, however, significant changes in some serum biochemical parameters (plasma proteins and serum bilirubin) of extract-treated groups compared to control. No significant histopathological changes were noted in the kidneys, stomach, liver and spleen of rats at extract doses up to 1200 mg kg^{-1}. Based on these findings, it can be inferred that HLE is relatively non-toxic in rats but has the potential to cause toxicity at high dose levels—demanding that caution be taken when using *H. latifolia* for medicinal purposes.

Putting all together, this study has shown that the ethanolic extract of *Hilleria latifolia* aerial parts has anti-nociceptive, anti-inflammatory, antioxidant, anxiolytic and antidepressant activities. The extract also has low oral toxicity but should be used with caution.

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ABBREVIATIONS

5-HT	5-Hydroxytryptamine
AIA	Adjuvant-induced arthritis
ALP	Alkaline phosphatase
ALT	Alanine transaminase
AMPA	α-Amino-3-hydroxyl-5-methylsoxazole-4-propionic acid
AST	Aspartate transaminase
CFA	Complete Freund's Adjuvant
CNS	Central nervous system
COX	Cyclooxygenase
DA	Dopamine
DEX	Dexamethasone
DH	Dorsal horn
DMARD	Disease modifying anti-rheumatoid drugs
DNA	Deoxyribonucleic acid
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
EPM	Elevated plus-maze
FST	Forced swimming test

GABA	Gamma-aminobutyric acid
GAFCO	Ghana Agro Food Company
GI	Gastrointestinal
HLE	*Hilleria latifolia* extract
HIV	Human immunodeficiency virus
i.p	Intraperitoneal
IASP	International Association for the Study of Pain
IFA	Incomplete Freund's adjuvant
IFN	Interferon
IL	Interleukin
KNUST	Kwame Nkrumah University of Science and Technology
L-NAME	N^G-nitro-arginine methyl ester
LD_{50}	Dose of drug which kills 50% of the population
LOAEL	Lowest-observed-adverse-effect level
LOX	Lipoxygenase
MDA	Malondialdehyde
MET	Methotrexate
MPE	Maximum possible effect
NE	Norepinephrine
NF-κB	Nuclear factor-κB
NMDA	*N*-methyl *D*-aspartate
NO	Nitric oxide

NOAEL	No-observed-adverse-effect level
NSAID	Non-steroidal anti-inflammatory drugs
OA	Osteoarthritis
p.o	Per os
PAMPS	Pathogen-associated molecular patterns
PG	Prostaglandin
PAF	Platelet activating factor
PUFA	Polyunsaturated fatty acids
RA	Rheumatoid arthritis
ROS	Reactive oxygen species
SSRI	Selective serotonin reuptake inhibitor
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substance
TCA	Trichloroacetic acid
TNF	Tumour necrosis factor
TRPC	Transient receptor potential C
TRPV	Transient receptor potential vanilloid
TST	Tail suspension test
TTX-R	Tetrodotoxin resistant
TTX-S	Tetrodotoxin sensitive
UV	Ultraviolet

Chapter 1

INTRODUCTION

1.1 GENERAL INTRODUCTION

Inflammation and pain are the most common reasons why patients seek advice from health professionals. They represent important medical and economic costs for the community (Jagerovic *et al.*, 2002). Current therapies, despite their proven efficacy in alleviating symptoms and providing relief, all have considerable side effects. Anti-inflammatory agents including NSAIDs and steroids cause considerable gastrointestinal and renal damage amongst other side effects. Analgesics, aside NSAIDs, such as opioids also cause significant adverse effects like respiratory depression, emesis, constipation, tolerance and addiction. This challenges of current therapy together with the fact that many patients especially pain sufferers are not satisfied with their pain care makes the search for new analgesic and anti-inflammatory agents that can more effectively treat pain and inflammation an important area in drug research.

Throughout the ages humans have relied on nature for their basic needs and not least, their medicines. Natural products particularly medicinal plants have been used as either prophylactic or therapeutic arsenal to restore and maintain health, and have formed the basis of sophisticated traditional medical systems that have been in existence for thousands of years. The earliest written documentation of knowledge on medicinal properties of plants is found on Assyrian cuneiform clay tablets dated about 2000 B.C. The Egyptian culture already used a range of medicinal plants which were described in the Ebers papyrus (1550 B.C.). In India, the traditional medicine was documented in the Ayurveda in about 900 B.C., and the first written records on the system of Traditional Chinese Medicine appeared around the same time (Potterat and Hamburger, 2008). In Europe, the knowledge on medicinal plants reached an early summit with Hippocrates (5th Century B.C.), Dioscorides (1st Century A.D.) and Galen (2nd Century A.D.). The knowledge was transmitted and expanded by the Arab scholars of the 10th to 12th Century A.D., such as Avicenna and Ibn al-Baitar. In Europe, this knowledge was recorded in numerous herbals that were published from the Middle Age onwards (Sneader, 2005; Potterat and Hamburger, 2008). At present, there are more than 85,000 plant species that have been acknowledged for medical use globally (Balunas and Kinghorn, 2005; Liu and Wang, 2008).

Traditional medicine (TM), especially in the developing world, has made very much use of natural products. According to World Health Organization (WHO), 80% of the world's people—primarily those of developing countries—rely on traditional medicine for their primary health care needs (WHO, 2003). This is particularly true for Ghana, where it is estimated that there is one (1) traditional doctor to approximately four hundred (400) people as opposed to one allopathic or orthodox doctor to twelve thousand (12000) people (Ghana Herbal Pharmacopoeia, 1992). The people in developing countries depend on TM, because it is cheaper and more accessible than orthodox medicine (Sofowora, 1982; Tabuti *et al*., 2003). Traditional medicine is also more acceptable because it blends readily into peoples' socio-cultural life. Whiles herbal medicines remain the traditional form of medicine in many developing countries; it is increasingly gaining popularity in the developed world, which hitherto relied mostly on allopathic medicine. This recent growing interest in the West for herbal medicines may be attributed to the general perception that they are safer, producing fewer side effects.

Many traditional medicines have promising potential in the management of pathological conditions currently plaguing man including pain and inflammatory disorders. Many, however, though increasingly been used, remain untested and their use is not monitored. As a result, scientific knowledge of their effectiveness and potential side effects is limited. This makes identification of the safest and most effective therapies and promotion of their rational use more difficult. The study of plant species used traditionally should, therefore, still be seen as a fruitful research strategy to provide new and important leads against various pharmacological targets and to help humanity, especially the developing world who depend heavily on traditional medicines, more effectively fight their diseases.

Medicinal plants for the management of pain and inflammation are rich in Ghanaian traditional medical folklore but lack scientific evidence supporting their use. This, coupled with the need for more safe and effective analgesic and anti-inflammatory agents, was the most significant motivation for research into the analgesic and anti-inflammatory properties of *Hilleria latifolia* in this study.

1.2 THE PLANT *HILLERIA LATIFOLIA*

Botanical name: *Hilleria latifolia* (Lam.) H. Walt.

Family: Phytolaccaceae

Local names:

Ewe: Akople, Avegboma (i.e. forest spinach), Boe, Kukluigbe ("pepper herb");

Fante/Twi: Anafranaku;

Ga: Nyabataa kplai

Figure 1.1 The *Hilleria latifolia* plant

1.2.1 Description

It is a perennial herb, woody below and 30-120 cm high (Fig. 1.1). The leaves are ovate-elliptic, to 15 cm long, 6 cm broad, obtuse or sub-acute at the base, acutely acuminate, entire, with

numerous short lines of crystals resembling appressed hairs on lower surface. There are lateral nerves about 6 cm on each side. Petiole is 3-7 cm long whilst flowers are pink or white in slender racemes up to 13 cm long. The fruits are reticulate, glabrous, ellipsoid-globose, about 2 mm in diameter (Dokosi, 1998; Mshana *et al*., 2000).

1.2.2 Ecological and Geographical Distribution

It is common in cultivated ground and along forest paths near villages in the forest regions of Ghana. It can be found along the West African cost, from Sierra Leone to Cameroun. *H. latifolia* is a native of South America but has naturalised in many parts of tropical Africa and the Mascarenes (Dokosi, 1998).

1.2.3 Traditional Uses

Various parts of *H. latifolia* are popular in Africa for many medicinal as well as non-medicinal uses.

1.2.3.1 Medicinal Uses

The medicinal uses of *H. latifolia* can be broadly considered as follows:

a) **Pain and inflammation**: In Ghana, the juice squeezed from heated leaves is dropped into the ear for earache. Fine powder from grounded leaves is rubbed into skin incisions in the treatment of rheumatism. Also, the crushed leaves are applied locally for feverish pains, stiffness and violent headache (Kerharo and Bouquet, 1950). The leaves, added to those of *Piper guineense*, are applied to the body as a remedy for general oedema (Dokosi, 1998).
b) **Infections, infestations and skin disorders**: The crushed leaves are used in the Congo as a lotion for different kinds of skin diseases including scabies and small pox (Bouquet, 1969; Schmelzer and Gurib-Fakim, 2008). The sap is also used as ear drops to treat ear infections (Schmelzer and Gurib-Fakim, 2008). Also, in Ghana, a poultice of fresh leaves or roots is applied to boils. A leaf decoction, in small doses is given for leprosy (Dokosi, 1998). The herb is boiled alone or in palm-oil soup and drunk as a remedy for Guinea-worms and for urethral discharges (Iwu, 1993). In Nigeria, the leaves are eaten in soup to treat gonorrhoea (Schmelzer and Gurib-Fakim, 2008). The crushed plant is also applied to breast cancer (Schmelzer and Gurib-Fakim, 2008).

c) **Respiratory disorders**: A paste made from grounded flowers is taken together with fresh orange juice in the treatment of asthma (Mshana *et al.*, 2000). A leaf decoction is taken to treat coughing of blood (Schmelzer and Gurib-Fakim, 2008)

d) **Gastrointestinal disorders**: In Cote d' Ivoire, a leaf decoction is taken or administered by enema to treat ascites and food poisoning, as it causes violent purging (Schmelzer and Gurib-Fakim, 2008). The decoction of the leaves and twigs is used in Ghana for the treatment of jaundice; it is also used as a steambath for the same purpose (Iwu, 1993).

e) **Haematological disorders**: The leaf sap is considered a haemostatic (Schmelzer and Gurib-Fakim, 2008).

1.2.3.2 Non-Medicinal Uses

H. latifolia is useful as an indicator of suitable soil and climatic conditions for the growth of cocoa and coffee. The dried seeds are used as a relish to the meat of the tortoise (Dokosi, 1998). In Narok District of Kenya, the Maasai people use the blackened stems for drawing eyebrows (Schmelzer and Gurib-Fakim, 2008).

1.2.4 Toxicity Reports from Traditional Use

There is controversial information concerning the toxicity of *H. Latifolia*. In Ghana, the seeds are said to be fatal to sheep and goats. The plant is also poisonous to large edible forest snails (*Achatina*). In Cote d'Ivoire, *H. latifolia* is considered to be poisonous to both humans and animals, symptoms being a large swelling of the stomach and immediate putrefaction and death. Fruits are said to be fatal to sheep and goats and to cause diarrhoea in horses in northern Nigeria (Dokosi, 1998). However, in Nigeria and Cameroon the leaves are eaten as a vegetable or in soup. The dried fruits are also eaten as a relish in Ghana (Schmelzer and Gurib-Fakim, 2008).

1.2.5 Previous Work on H. latifolia

There is little information on the phytochemistry and pharmacological activities of *H. latifolia* in established scientific literature. It has been reported that a crude extract of the stem bark caused significant mortality *in vitro* of adults and microfilariae of *Onchocerca volvulus* (Titanji *et al.*, 1987).

The leaves have also been reported to contain per 100 g edible portion: water 84.3 g, energy 184 kJ (44 kcal), fat 0.8 g, carbohydrate 7.8 g, Ca 349 mg, Fe 4.1 mg, ascorbic acid 22 mg (Leung *et al.*, 1968).

1.3 PAIN

Pain is a perception, and one of the outputs of a system in higher animals—the nociceptive system—which itself is a component of the overall set of controls responsible for homeostasis. It is defined by the International Association for the Study of Pain (IASP) as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage" (Merskey *et al.*, 1979). Pain constitutes an alarm that helps protect the organism: it both triggers reactions and induces learned avoidance behaviours, which may decrease whatever is causing the pain and, as a result, may limit the (potentially) damaging consequences.

Pain has at least three functions (Le Bars *et al.*, 2001):

1. to warn the individual of the existence of real tissue damage;
2. to warn the individual of the probability that tissue damage is about to occur by realizing that a stimulus has the potential to cause such damage; and
3. to warn a social group of danger as soon as it exists for anyone of its members.

Pain may vary in intensity (mild, moderate, or severe), quality (sharp, burning, or dull), duration (transient, intermittent, or persistent), and referral (superficial or deep, localized or diffuse). Pain can be essentially divided into two broad categories: acute (adaptive) or chronic (maladaptive).

Acute pain, which lasts from seconds to days, is defined by the IASP as "pain of recent onset and probable limited duration. It usually has an identifiable temporal and causal relationship to injury or disease" (Merskey and Bogduk, 1994). It generally subsides with removal of the stimulus (cause) and healing. It may be associated with heightened arousal leading to tachycardia, tachypnoea, and anxiety. Acute pain is also referred to as adaptive pain since it serves to protect the individual from further injury or promote healing.

Chronic pain may or may not be related to an easily identified pathophysiologic phenomenon and may be present for an indeterminate period. Chronic pain conditions are caused by ongoing disease states or tissue damage that result in sensitization of primary afferent and spinal cord neurons. This sensitization results in an increased sensitivity to both noxious (hyperalgesia) and non-noxious (allodynia) stimuli that are frequently difficult to treat with current pharmacological or surgical approaches (Koltzenburg, 1998). In contrast to acute pain, chronic pain typically lasts from months to years (at least six months) and "commonly persists beyond the time of healing of an injury" (Merskey and Bogduk, 1994). Chronic pain, also known as

maladaptive pain, is an expression of the pathologic operation of the nervous system; it is pain as disease. Though there is often no increased sympathetic response, chronic pain is associated with depression and decreased function.

1.1.1 Types of Pain

Pain has been classified into several different types according to their pathogenesis: nociceptive, inflammatory, neuropathic and functional (Fig. 1.2). Commonly, pain syndromes come with different mixtures of pain types.

1.3.1.1 Nociceptive pain

This is an acute pain sensation evoked by activation of nociceptors located in non-damaged skin, viscera and other organs in the absence of sensitization. It protects tissue from being (further) damaged because withdrawal reflexes are usually elicited. The pain is typically well localized, constant and often with an aching or throbbing quality (Smith, 2003).

1.3.1.2 Inflammatory pain

Inflammatory pain occurs as a consequence of hypersensitivity that arises in inflamed tissue following sensitization of peripheral nerve terminals (Smith, 2003). The body now changes focus from protecting against painful stimuli to protecting the injured tissue. Inflammatory pain serves to prevent contact or movement of the injured part until healing is complete, thus reducing further damage.

1.3.1.3 *Neuropathic pain*

Neuropathic pain is the result of an injury or disease or dysfunction of a nerve or group of nerves in the peripheral or central nervous system (Tremont-Lukats *et al.*, 2000). Examples include post herpetic (or post-shingles) neuralgia, reflex sympathetic dystrophy/causalgia, components of cancer pain, phantom limb pain and peripheral neuropathy. Damaged neurones can have spontaneous discharges resulting in action potentials that may also produce a neuropathic form of pain. The pain frequently has burning, lancinating or electric shock qualities. Persistent allodynia is also a common characteristic of neuropathic pain. The pain may persist for months or years beyond the apparent healing of any damaged tissues (Macres and Richeimer, 2000). Neuropathic pain tends to be less responsive to treatment with opioids, but

may respond well to other drugs such as anticonvulsants and antidepressants. Usually, neuropathic problems are not fully reversible, but partial improvement is often possible with proper treatment (Guevara-Lopez *et al.*, 2004).

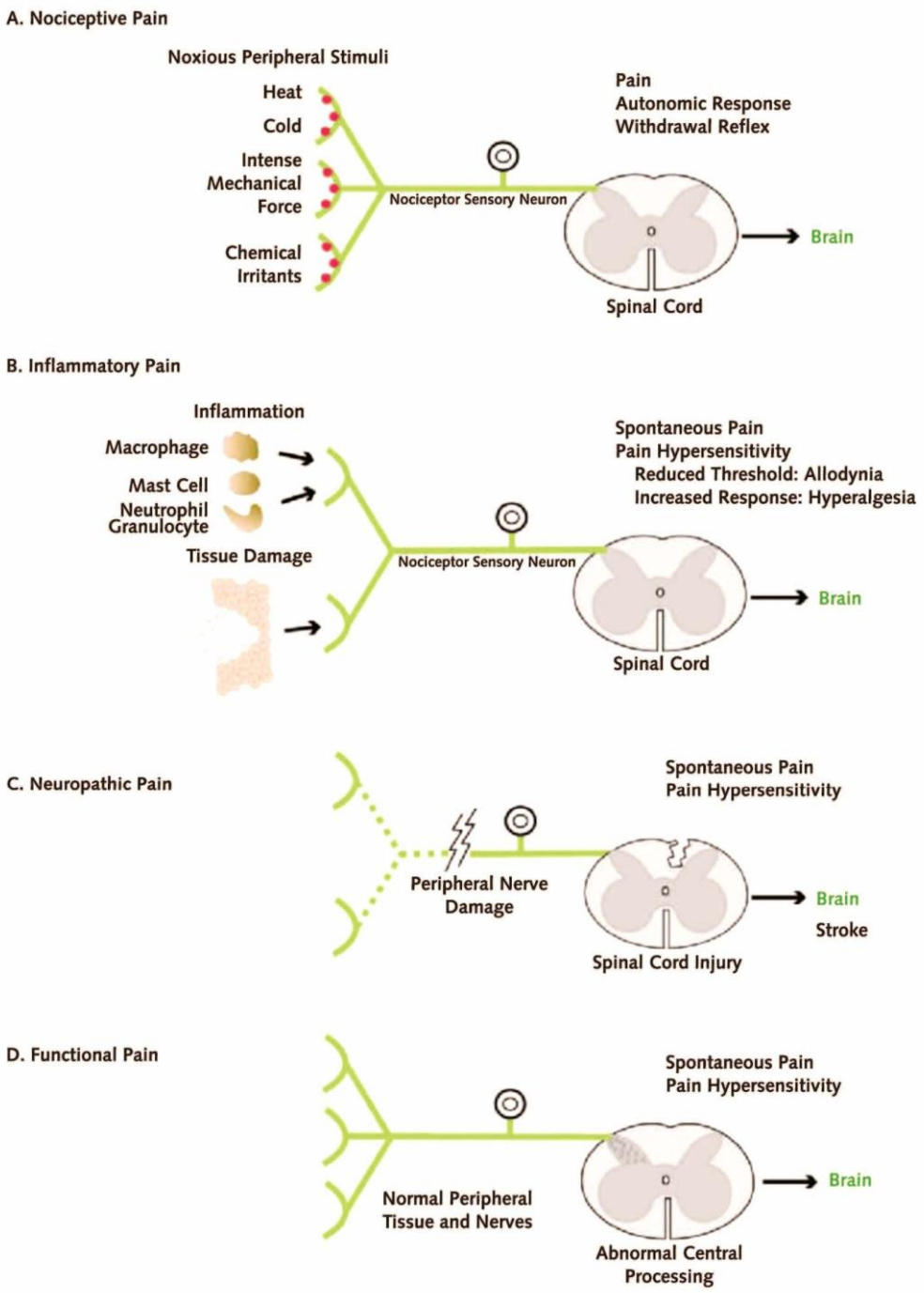

Figure 1.2 The four primary types of pain (Adapted from Woolf, 2004)

1.3.1.4 Functional pain

Functional pain is an evolving concept. In this form of pain sensitivity, no neurologic deficit or peripheral abnormality can be detected. The pain is due to an abnormal responsiveness or function of the nervous system, in which heightened gain or sensitivity of the sensory apparatus amplifies symptoms. Several common conditions have features that may place them this category: for example, fibromyalgia, irritable bowel syndrome, some forms of non-cardiac chest pain, and tension-type headache (Woolf, 2004).

1.1.2 Neurobiology of pain

Nociception is the encoding and processing of noxious stimuli in the nervous system that can be measured with electrophysiological techniques. Neurons involved in nociception form the nociceptive system. This system extends from the periphery through the spinal cord, brain stem, and thalamus to the cerebral cortex, where the sensation is perceived.

1.1.2.1 Peripheral Mechanisms

Peripheral mechanisms of pain begin with the primary afferent nociceptors that respond to mechanical, thermal, and chemical stimuli. Neuronal subtypes sense and transmit distinct information about actual stimuli. The myelinated Aδ-fibers transmit mechanothermal information (phasic pain with sharp, pricking quality) while unmyelinated C-fiber nociceptors are polymodal (tonic pain with burning, itching, aching quality) and represent the majority of nociceptors (Clark and Treisman, 2004). In addition to polymodal nociceptors, joint, skin and visceral nerves contain Aδ and C fibers (silent nociceptors/mechano-insensitive nociceptors) that are not activated until they are sensitised to mechanical and thermal stimuli during inflammation (Schaible, 2006). Nociceptors project to spinal cord and further to the brain stem or to the thalamocortical system that produces the conscious pain response upon noxious stimulation.

1.1.2.1.1 Peripheral Sensitization

Nociceptor sensitization underlies the phenomenon of peripheral hyperalgesia that results in an increase in the perception of and response to pain.

Tissue damage or inflammatory insults intensify pain experience by increasing the sensitivity of nociceptors to both thermal and mechanical stimuli. The excitation threshold of polymodal

nociceptors drop such that even normally innocuous stimuli can activate them (allodynia). Silent nociceptors are recruited adding significantly to the inflammatory nociceptive input to the spinal cord. Chemical mediators including ATP, bradykinin (BK), serotonin (5-HT), epinephrine, prostaglandin E_2 (PGE_2), nerve growth factor (NGF) and substance P (SP) are released from axon terminals, damaged skin, inflammatory cells and the microvasculature surrounding the injury site (Fig. 1.3a). The injury site is typically very acidic owing to the increased concentration of protons in the immediate area. Each of the chemical mediators binds to its high-affinity cognate receptor, present on nociceptive afferent terminals (Fig. 1.3a). The nociceptor-specific receptor for the irritant capsaicin, TRPV1 is also present on terminals and transduces noxious thermal stimuli.

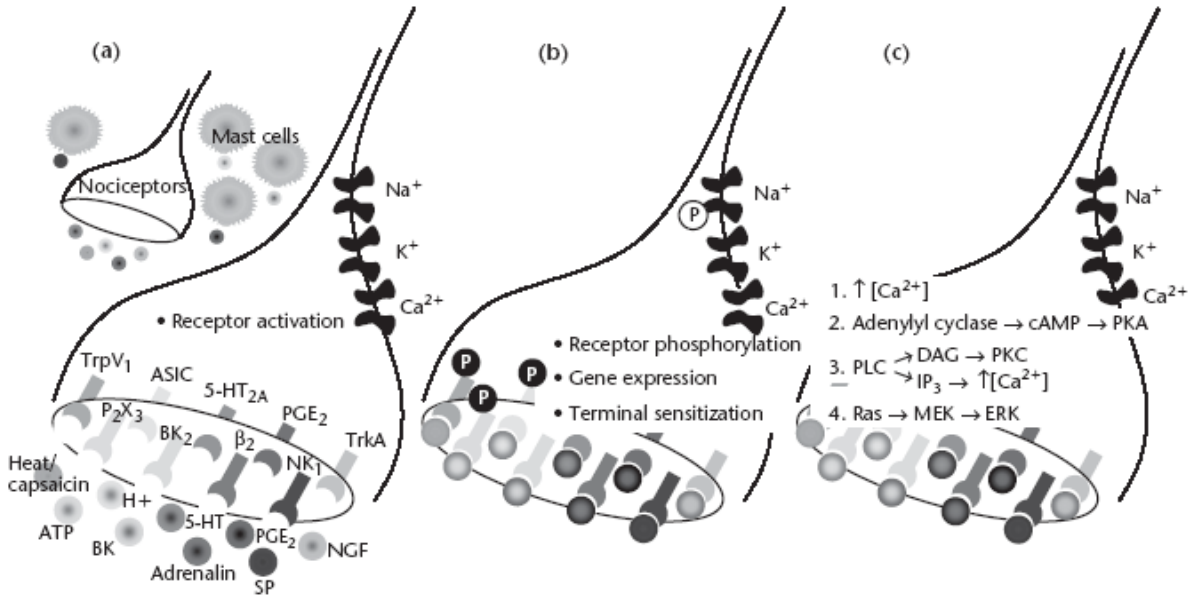

Figure 1.3 Mechanisms by which the peripheral apparatus of the nociceptive pathway (the primary afferent), exacerbates pain sensation (Adapted from Cafferty, 2005).

Receptor activation results in terminal sensitization or plasticity, either immediately via a post-translational mechanism (e.g. receptor phosphorylation TRPV1, P_2X_3 or ion channel phosphorylation PGE_2 or BK-mediated Na^+ phosphorylation) or over a prolonged time course which requires gene expression (NGF) (Fig. 1.3b). The pathways activated by these ligands include elevating intracellular Ca^{2+} (ASIC, P_2X_3, TRPV1), activating G-protein-coupled receptors (PGE_2, BK, β_2) and subsequently elevating cAMP then PKA or elevating intracellular

Ca^{2+} via PLC or the Ras-MEK–ERK/MAP-kinase pathway (NGF) (Fig. 1.3c). These pathways converge to alter the excitability of the nociceptor, ultimately lowering its threshold for activation and resulting in an increased pain sensation (Riedel and Neeck, 2001; Cafferty, 2005).

Nociceptors are also sensitised during nerve injury leading to the development of neuropathic pain. When a nerve is damaged (by stretch, compression or transection), a complex reaction ensues peripherally that alters the neurochemistry of the damaged axons. There is an alteration of gene expression within the damaged fibres. This disruption of homeostasis shifts the phenotype of the damaged pathways from one of the transduction and transmission of sensory information, to one that must accomplish survival and regeneration. Sodium channels increase in number and appear in novel locations with altered subtype profiles (i.e., downregulation of TTX-R Na^+ channels, $Na_V1.8$ and $Na_V1.9$, and an upregulation of type III TTX-S Na^+ channel, $Na_V1.3$.), peptide production increases, the end of the nerve fibre sprouts, sensitivity to mechanical stimulation and noradrenaline increases, and the nerve fires spontaneously and with increased evoked activity (Clark and Treisman, 2004; Cafferty, 2005).

1.1.2.2 Central Mechanisms

Pain transmission depends on the balance of inhibitory and facilitatory influences acting on the neural circuits of the somatosensory system. Integration of these influences occurs at multiple levels of the CNS including the spinal cord, brain stem and multiple cortical regions.

1.1.2.2.1 Spinal Mechanisms

Further regulation of pain occurs at the level of the spinal synapse, the lowest level of the central nociceptive system. The primary afferent nociceptors terminate in laminae I, II, and V of the dorsal horn. The neuronal organisation of the spinal cord determines characteristic features of pain, e.g. the projection of pain into particular tissues. The second-order neurons project to the thalamus, periaqueductal grey, hypothalamus, amygdala as well as a variety of other higher structures including several regions of the cortex. Rather than a simple relay, these afferents organize the data from the peripheral fibres into a new format. These afferents can be classified into nociceptive-specific (high threshold) or wide dynamic range (convergent neurons). The nociceptive-specific neurons are located more superficially in the dorsal horn and respond only to noxious stimuli (Aδ and C fibre stimulation) while wide dynamic range neurons are more deeply located and respond to all types of stimuli (Aβ, Aδ and C fibre inputs)(Clark

and Treisman, 2004; Schaible, 2007). Nociceptive-specific neurons may be involved in the sensory-discriminative aspects of pain, whereas wide dynamic range neurons participate in the affective-motivational components of pain (Bolay and Moskowitz, 2002).

Many neurophysiological studies helped in providing evidence that pain can be modulated depending upon the balance of activity between nociceptive and other inputs. But the gate theory of pain (Melzack and Wall, 1965; Melzack, 1999) has remarkably revolutionised thinking regarding pain mechanisms—pain is not the inevitable consequence of activation of a specific pain pathway beginning at the C-fibre and ending at the cerebral cortex but its perception is a result of the complex processing (Fig. 1.4) of patterns of activity within the somatosensory system (Bennet, 2005).

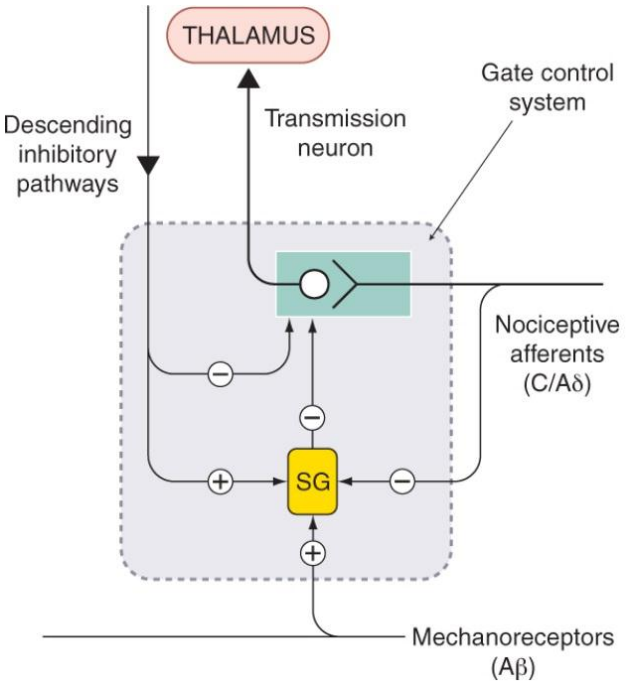

Figure 1.4 **Schematic diagram of the gate control system**. This system regulates the passage of impulses from the peripheral afferent fibres to the thalamus via transmission neurons originating in the dorsal horn. Neurons in the *substantia gelatinosa* (*SG*) of the dorsal horn act to inhibit the transmission pathway. Inhibitory interneurons are activated by descending inhibitory neurons or by non-nociceptive afferent input. They are inhibited by nociceptive C-fibre input, so the persistent C-fibre activity facilitates excitation of the transmission cells by either nociceptive or non-nociceptive inputs. This autofacilitation causes successive bursts of activity in the nociceptive afferents to become increasingly effective in activating transmission neurons (Adapted from Rang *et al.*, 2007).

Numerous transmitters and receptors mediate the processing of noxious information within the spinal cord. Transmitter actions have either fast kinetics (e.g. action of glutamate and ATP at ionotropic receptors) or slower kinetics (in particular neuropeptides that act through G protein-coupled metabotropic receptors). Actions at fast kinetics evoke immediate and short effects on neurons, thus encoding the input to the neuron, whereas actions at slow kinetics modulate synaptic processing. Glutamate is a principal transmitter of primary afferent and dorsal horn neurons, activating ionotropic AMPA, kainate and NMDA receptors. Other excitatory transmitters include ATP and neuropeptides such as SP, CGRP, VIP, neurotensin, CCK, TRH, CRH and pituitary adenylate cyclase-activating polypeptide (Schaible, 2007).

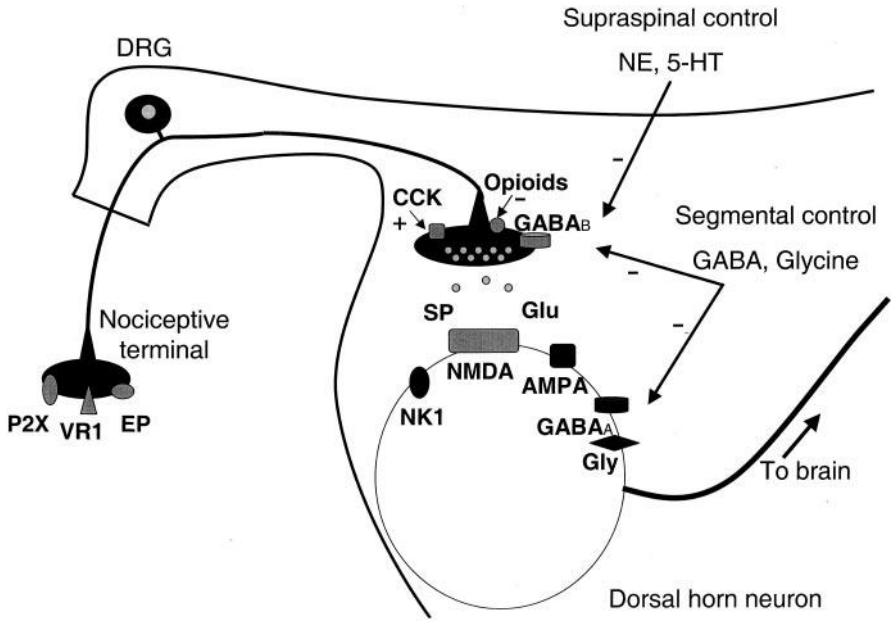

Figure 1.5 **Nociceptive stimulation of the dorsal horn (DH).** CCK = cholecystokinin; SP = substance P; NMDA=N-methyl-D-aspartate; AMPA=α-amino-3-hydroxy-5-methyl-4-isoxazolepropionicacid; GABA = ɣ-aminobutyric acid; NE=norepinephrine; 5-HT=serotonin; EP=epinephrine; DRG=dorsal root ganglia; Glu = glutamate; Gly = glycine (Adapted from Bolay *et al.*, 2002).

Transmission in the somatosensory system can be suppressed within the DH as a result of segmental and descending inhibitory controls. This inhibition can occur (Fig. 1.5) at the pre-synaptic level on the primary afferent terminal or post-synaptically on the DH neurone. Inhibitory neurotransmitter systems within the DH include GABA, glycine, 5-HT, adenosine,

endogenous cannabinoids and the endogenous opioid peptides. The opioid system in particular plays a crucial role in regulating pain transmission. Opioid receptors (μ, δ, κ) are concentrated in the superficial dorsal horn, and in particular μ and δ receptors are located in interneurons and on primary afferent fibres. Opioids inhibit the transmission of nociceptive information by reducing neurotransmitter release from the terminals of nociceptive afferents and causing hyperpolarisation of DH neurones, hence reducing their excitability (Bennet, 2005; Schaible, 2007).

1.1.2.2.1.1 CENTRAL SENSITISATION (SPINAL HYPEREXCITABILITY)

In the course of inflammation and nerve damage neurons in the superficial, deep and the ventral cord show pronounced changes of their response properties, a so-called central sensitization. This sensitization, a simple form of learning and synaptic plasticity, can be described as an increased response to neuronal input following noxious stimuli. Typical changes in the spinal cord neurons include wind-up (progressive increases in neuronal activity throughout the stimulus duration), facilitation (magnification and prolongation of the duration of neuron response), action potential threshold reduction, receptive field expansion, oncogene induction, and long term potentiation (strengthening of synaptic transmission efficacy after activity across the synapse)(Clark and Treisman, 2004; Schaible, 2007).

The mechanisms of central sensitization are complex, and it is likely that different pain states are characterized at least in part by specific mechanisms, although some of the mechanisms are involved in all types of central sensitization (Schaible, 2007).

Analogous to the situation in the periphery, interactions between different transmitters determine the state of excitability of the system and eliminating any one single transmitter molecule or receptor may well have little effect on nociception unless that entity has a dominant role. So far, the only prime substrate identified is the NMDA receptor for glutamate. This glutamate-gated ion channel is a powerful switch and coincidence detector, which requires a specific combination of events for its activation. Once these conditions are met, intracellular interactions between the various cascades that are instigated continue the process of central sensitization (Fig. 1.6) (Carpenter and Dickenson, 2005).

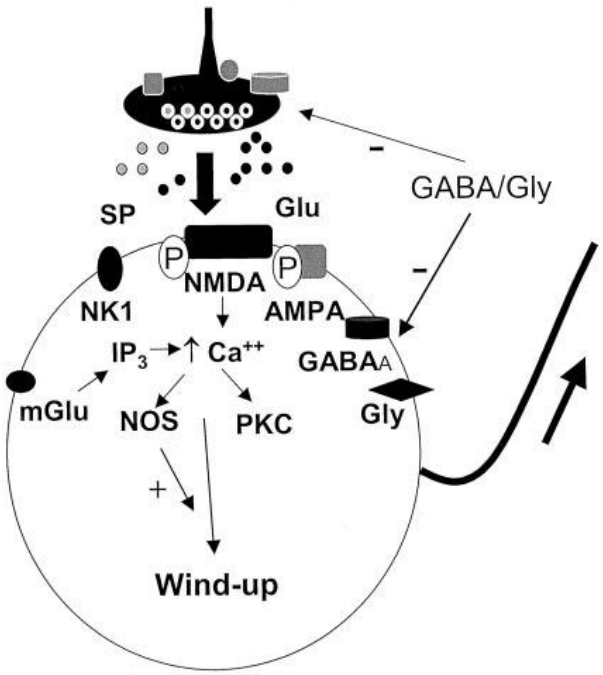

Figure 1.6 **Glutamate and central sensitisation**. Low-frequency stimulation of nociceptors by mildly noxious stimuli releases glutamate from the central terminals of primary afferent neurons terminating in laminae I, II, and V. Glutamate, acting on postsynaptic AMPA receptors, causes fast excitatory postsynaptic potentials (EPSPs) and rapid depolarization in postsynaptic cells. At rest, the NMDA receptor channel is closed due to magnesium blockade. Under pathologic conditions, NMDA and AMPA receptors are recruited. During intense or sustained noxious stimulation (high-frequency discharge), substance P (via the NK1 receptor) and glutamate are co-released, causing sustained slow EPSPs (lasting tens of seconds), temporal summation, and removal of the magnesium blockade of the NMDA calcium channel. As a result of NMDA receptor activation, the intracellular calcium level increases and calcium also enters the postsynaptic cell via voltage-gated calcium channels (not shown). Metabotropic glutamate receptors (mGlu) are coupled to inositol triphosphate and calcium release. As a consequence of the longer depolarization and calcium entry noted above, activated PKC phosphorylates NMDA receptors and enhances NMDA receptor currents. Consequently, nociceptive inputs increase the excitability of dorsal horn neurons (Adapted from Bolay *et al.*, 2002).

1.1.2.2.2 Supraspinal Mechanisms (Ascending Tract, Descending Inhibition and Facilitation)

Second order neurons project to supraspinal structures in the ascending tracts of the contralateral anterolateral spinal cord (spinothalamic, spinoreticular, spinomesencephalic) although not all fibres decussate and a latent ipsilateral pathway is present (Clark and Treisman, 2004). Although there is no absolute clear anatomical separation in the ascending nociceptive transfer systems to the supraspinal targets by which the global sensation of pain is finally modulated and experienced, two dimensions of pain can be distinguished: the sensory-

discriminative, and the affective-cognitive/affective-motivational component (Riedel and Neeck, 2001). The former deals with the perception and detection of noxious stimuli *per se* depending on their intensity, location, duration, temporal pattern and quality; the latter comprises the relationship between pain and mood, the attention to and memory of pain, the capacity to cope with and tolerate pain and its rationalization (Riedel and Neeck, 2001) .

The conscious pain response is produced by the thalamocortical system. The lateral thalamocortical system consisting of relay nuclei in the lateral thalamus and the areas SI and SII in the post-central gyrus represent the sensory-discriminative (temporal and spatial) aspects of pain. In these regions innocuous and noxious stimuli are discriminated (Treede *et al.* 1999). It has, however, being suggested recently that brain mechanisms supporting discrimination of sensory features of pain extend far beyond the somatosensory cortices and involve frontal regions traditionally associated with affective processing and the medial thalamocortical system (Oshiro *et al.*, 2007). Affective-motivational features of pain are produced in the medial thalamocortical system, which consists of relay nuclei in the central and medial thalamus, the anterior cingulate cortex (ACC), the insula and the prefrontal cortex (Treede *et al.*, 1999; Vogt, 2005). These brain structures are part of the limbic system, and the insula may be an interface of the somatosensory and the limbic system. Other cingulate regions are involved in response selection (they have projections to the spinal cord and the motor cortices) and the orientation of the body towards innocuous and noxious somatosensory stimuli. Most of the other subcortical structures (e.g. basal ganglia, hypothalamus, amygdala and cerebellum) are postulated to function in the transmission of nociception and perception of pain (Schaible, 2007).

From brain stem nuclei, impulses "descend" onto the spinal cord and influence the transmission of pain signals at the dorsal horn (Fig. 1.7). Concerning descending inhibition, the periaqueductal grey matter (PAG) is a key region. It projects to the rostral ventromedial medulla (RVM), which includes the serotonin-rich nucleus raphe magnus (NRM) as well as the nucleus reticularis gigantocellularis pars alpha and the nucleus paragigantocellularis lateralis (Fields *et al.*, 1991), and it receives inputs from the hypothalamus, cortical regions and the limbic system. Neurons in RVM then project along the dorsolateral funiculus (DLF) to the dorsal horn. Opioids induce analgesia by acting upon PAG and RVM in addition to the spinal dorsal horn. RVM seems to mediate anti-nociception and facilitation of pain transmission and so spinal bulbospinal loops are significant in setting the gain of spinal processing (Schaible, 2007).

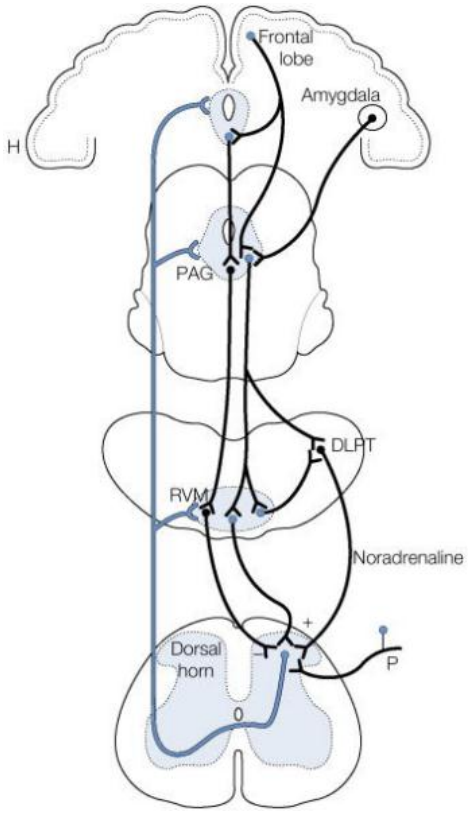

Figure 1.7 **Schematic representation of ascending afferent sensory input (left) and descending inhibitory modulatory output (right).** Regions of the frontal lobe and amygdala project directly and via the hypothalamus (H) to the PAG. The PAG in turn controls spinal nociceptive neurons through relays in the RVM and the dorsolateral pontine tegmentum (DLPT). The RVM contains both serotonergic and non-serotonergic projection neurons; the DLPT provides noradrenergic innervation of the dorsal horn. The RVM exerts bidirectional control over nociceptive transmission in the dorsal horn (Adapted from Fields *et al.*, 2006)

A particular form of descending inhibition of wide dynamic range (WDR) neurons is the "diffuse noxious inhibitory control" (DNIC). When a strong noxious stimulus is applied to a given body region, nociceptive neurons with input from that body region send impulses to structures located in the caudal medulla (caudal to RVM), and this triggers a centrifugal inhibition (DNIC) of nociceptive WDR neurons located throughout the neuraxis (Le Bars *et al.*, 1979; Schaible, 2007).

Descending facilitatory mechanisms arise from medullary sites such as the dorsal reticular nucleus and potentiate nociception through spinal dorsal horn neurons (Clark and Treisman, 2004).

1.1.3 Depression and Pain

Pain and depression are often linked, and several studies have indicated that pain and depression share common neurochemical mechanisms (Blackburn-Munro and Blackburn-Munro, 2001, Suzuki *et al.*, 2004). Clinical depression is common in patients with persistent chronic pain: 30–54% (Banks and Kerns, 1996). Conversely, pain is among the most common physical symptoms in patients with depression, and a common complaint reported to specialists (Leo, 2005). Relapses into a depressive state are more common in such patients and make total symptom remission difficult, closing a vicious cycle: depression–pain–depression.

Antidepressants are, however, prescribed for pain due to their specific analgesic, rather than mood altering effects. The presence of a distinct effect on pain is borne out by a number of observations:

- Doses necessary to improve pain are often lower than those used to treat depression (Goldstein *et al.*, 2005).
- At these doses the onset of analgesic activity is more rapid than any antidepressant activity (Goldstein *et al.*, 2005; Hirschfeld *et al.*, 2005).
- Analgesic efficacy is usually obtained in non-depressed patients and does not correlate with improvement in mood in depressed patients (Mico *et al.*, 2006).
- The drugs are useful in acute and experimental pain (Fishbain, 2000).

The analgesic efficacy of antidepressants is due to their ability to block central nervous system monoamine uptake (particularly serotonin and noradrenaline) pre-synaptically as well as their effects on post-synaptic adrenoceptors (Fig. 1.8). Thus, antidepressants augment descending monoaminergic anti-nociceptive pathways from the midbrain periaqueductal grey and medulla (nucleus raphe magnus, NRM) (Stannard, 2005).

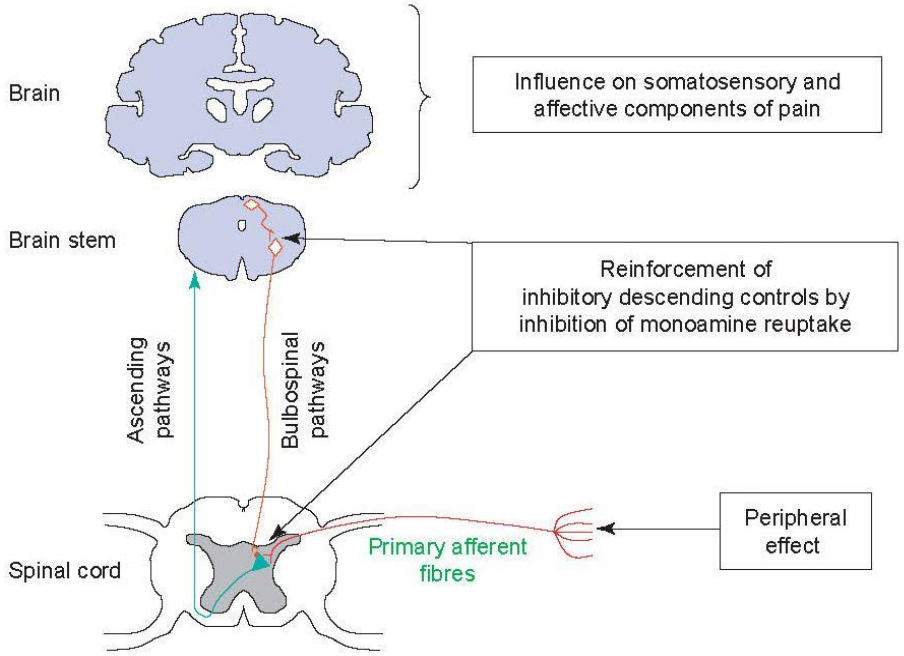

Figure 1.8 Postulated sites of the analgesic action of antidepressants (Adapted from Mico *et al.* 2006).

1.3.2 Pharmacological Management of Pain

1.3.2.1 Drugs in Clinical Use

The principal analgesics currently used clinically are the non-steroidal anti-inflammatory drugs (NSAIDs) and the opioids. They are the most popular and have long been used in pain management. NSAIDs (e.g. diclofenac) are effective for mild to moderate pain, giving way to the opioids (e.g. morphine) in severe cases. They are, however, not effective in all pain syndromes, allowing for (adjuvant) analgesics including local anaesthetics, serotonin receptor ligands, anticonvulsants and antidepressants to be used. Local anaesthetics (e.g. lidocaine) are used broadly to prevent/reverse acute pain and to treat symptoms of chronic pain. Serotonin receptor ligands (e.g. sumatriptan) are also being used for the management of acute migraine and cluster headache. The anticonvulsants (e.g. carbamazepine, lamotrigine, gabapentin, etc) and antidepressants (e.g. amitryptyline) are also useful in the treatment of neuropathic pain.

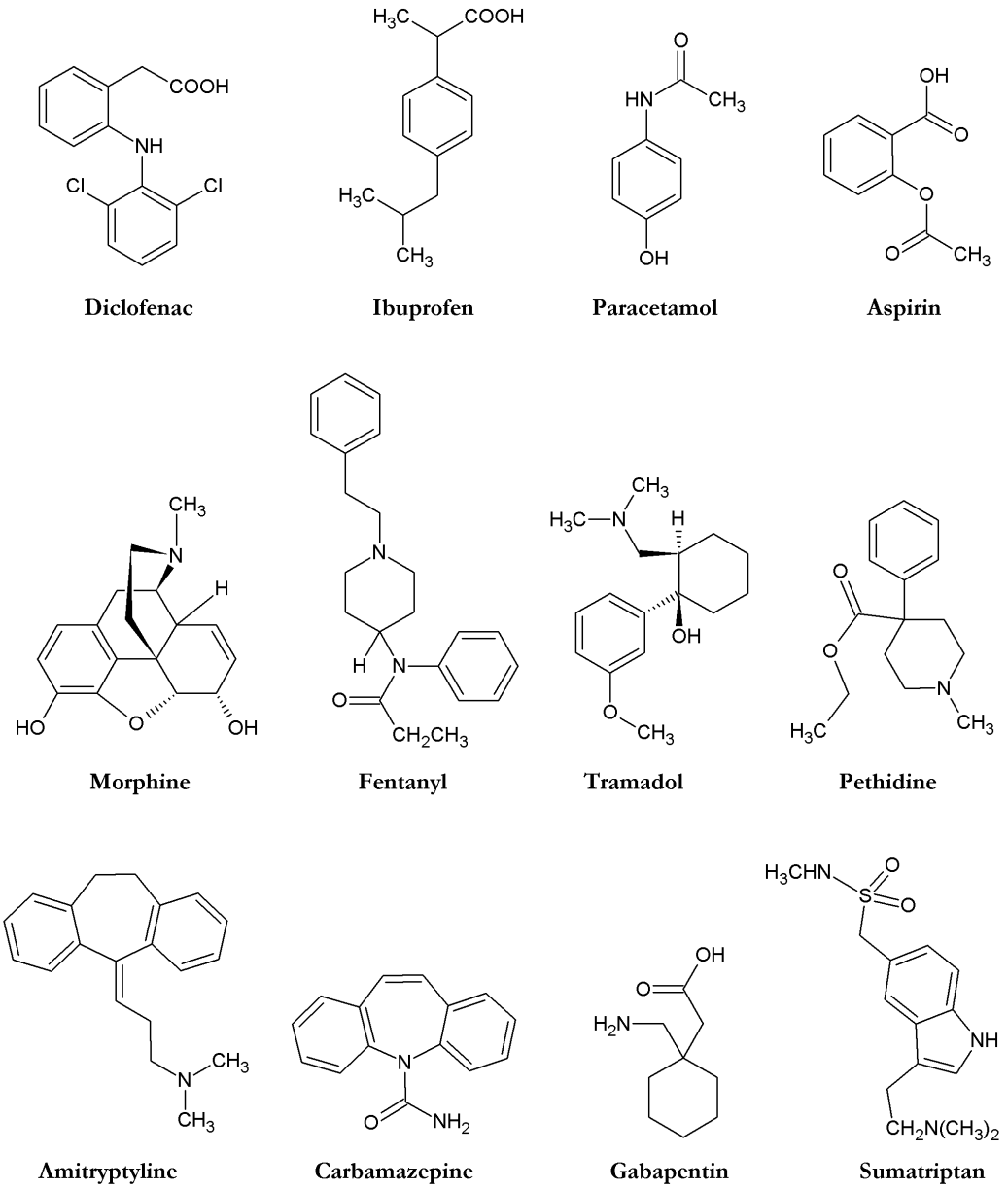

Figure 1.9 Examples of some commonly used drugs in the treatment of pain

1.3.2.2 Compounds in Preclinical Development

The drugs currently in clinical use are not without limitations. They present a lot of adverse effects and hence the need for safer and more effective analgesics.

With increasing knowledge of the various chemical mediators and signalling pathways responsible for pain sensation, many new approaches to the control of pain are being

developed. Under current preclinical development are many potential analgesics. These include neuropeptide and kinin antagonists, glutamate receptor ligands, α_2-adrenergic agonists, cholinergic agonists and cannabinoids.

1.3.2.3 Future Targets in Analgesia Research

Adenosine and ATP receptors, ion channels and protein kinases may well be potential targets for the treatment of pathological pain due to their immense role in nociception.

1.3.3 Animal Models of Nociception

Models for assessing analgesic activity in animals have been devised for both behavioural and non-behavioural manifestations of pain/nociception. Since the most reliable signs of pain are physical ones (Le Bars *et al.*, 2001), behavioural models have become more popular in analgesia research.

Behavioural models of nociception are widely used in analgesia research and have been rigorously characterized for their validity and reproducibility. The absence of verbal communication in animals, however, makes the models still limited because the measurement of pain is often an approximation. Measurement involves a high degree of subjectivity between the responsiveness of different animals and the observer's quantification of these behaviours. Thus, the subjectivity of these types of observations can produce a high degree of experimental bias. These common pitfalls can be remedied by designing experiments with the proper controls:

- Experimenter blindness (i.e. the observer is unaware of any treatment the animal has received).
- Use of proper negative control or sham groups.
- Using a single observer throughout the course of an experiment.

1.3.3.1 Models of Acute Pain

Currently, the commonly used behavioural tests of acute nociception can be considered broadly into those for phasic nociception (use of short-duration stimuli) and those for tonic/persistent nociception (use of long duration stimuli) (Le Bars *et al.*, 2001).

1.3.3.1.1 Phasic Nociception

These tests involve the use of short-duration stimuli (in order of seconds) and have somatic rather than visceral sites of stimulation. They can be classified by the nature of the stimulus into thermal (Hot plate test, Plantar/Hargreaves test, Tail flick test and Tail immersion test), mechanical (Von Frey hair/monofilaments assays, Tail clip test and Paw pressure test) and electrical (Tail, Paw or Dental pulp stimulation) (Le Bars *et al.*, 2001).

1.3.3.1.2 Persistent/Tonic Nociception

The basic feature of models of persistent or tonic nociception is that they involve a single injection of a neuroactive (algogenic) compound that will stimulate nociceptive fibres for a prolonged period. They are not models for chronic pain because their duration is only in the order of some tens of minutes. The main types of behavioural test based on such stimuli use intradermal (e.g. formalin test) or intraperitoneal (e.g. acetic acid-induced writhing test) injections. There are also other tests that involve injection into hollow organs (Le Bars *et al.*, 2001).

1.3.3.2 Models of Chronic pain

Common chronic inflammatory pain models include those involving the injection of carrageenan, turpentine, iodoacetate, Freund's complete adjuvant or other compounds into some part of experimental animals (especially the knee or ankle joint) to produce protracted allodynia/hyperalgesia, lasting for several days to weeks.

Several animal models of neuropathic pain have been developed to reflect the aberrant sensitivity to thermal and mechanical stimuli known to occur in humans with neuropathic pain. Each of these neuropathic models evokes a unique set of physiological and anatomical changes at the level of the nerve, the dorsal root ganglia and the spinal cord. However, they have all been found to produce robust and reliable changes in nociceptive behaviours in response to both thermal and mechanical stimulation. The models include chronic constriction injury (CCI), partial sciatic nerve injury (Seltzer model), spinal nerve ligation ('Chung' model), inflammatory injury (neuritis model), diabetic neuropathic (e.g. streptozocin diabetic neuropathy) and chemotherapy-induced neuropathic (e.g. vincristine-induced neuropathy) models (Kerr *et al.*, 2005).

1.4 INFLAMMATION

Inflammation is the basic mechanism available for repair of tissue after an injury (evoked by noxious agents including infections, antibodies, or physical injuries) and consists of a cascade of cellular and microvascular reactions that serve to remove damaged and generate new tissue (Schmid-Schönbein, 2006). The ability to mount an inflammatory response is essential for survival in the face of environmental pathogens and injury; in some situations and diseases, the inflammatory response may be exaggerated and sustained without apparent benefit and even with severe adverse consequences.

No matter the initiating stimulus, the classical signs of inflammation are *calor* (heat), *dolor* (pain), *rubor* (redness), *tumor* (swelling) and *functio laesa* (loss of function) (Kumar *et al.*, 2010). The first four classical signs were first listed by Celsus whereas *loss of function* was added later by Galen (Goldsby *et al.*, 2006) even though the attribution is disputed and the origination of the fifth sign has also been ascribed to Rudolf Virchow (Kumar *et al.* 2010).

Inflammatory responses occur in three distinct temporal phases, each apparently mediated by different mechanisms: an acute phase characterized by transient local vasodilation and increased capillary permeability; a delayed, sub-acute phase characterized by infiltration of leukocytes and phagocytic cells; and a chronic proliferative phase, in which tissue degeneration and fibrosis occur (Burke *et al.*, 2006).

Inflammation may be acute or chronic, depending on the nature of the stimulus and the effectiveness of the initial reaction in eliminating the stimulus or the damaged tissues. Chronicity of inflammatory response does not, however, directly infer pathology.

1.4.1 Acute Inflammation

Acute inflammation is a rapid host response (rapid onset, typically in minutes and of short duration, lasting for hours or a few days) that serves to deliver leukocytes and plasma proteins, such as antibodies, to sites of infection or tissue injury (Fig. 1.10). Acute inflammation has three major components:

a) ***Alterations in vascular calibre that lead to an increase in blood flow***. This results mainly from arteriolar dilation and opening of capillary beds induced by mediators such as histamine.

b) **Structural changes in the microvasculature that permit plasma proteins and leukocytes to leave the circulation.** Increased vascular permeability results in the accumulation of protein-rich extravascular fluid, which forms the exudate. Plasma proteins leave the vessels, most commonly through widened interendothelial cell junctions of the venules.

c) **Emigration of the leukocytes from the microcirculation, their accumulation in the focus of injury, and their activation to eliminate the offending agent**. Circulating leukocytes, initially predominantly neutrophils, adhere to the endothelium via adhesion molecules [including the E-, P-, and L-selectins, intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and leukocyte integrins], traverse the endothelium, and migrate to the site of injury under the influence of chemotactic agents (including cytokines e.g. IL-8; components of the complement system, e.g. C5a; and arachidonic acid metabolites, mainly leukotriene B_4) (Hicks and Sackeyfio, 1972, Sackeyfio and Yamyolia, 1977; Kumar *et al.*, 2010).

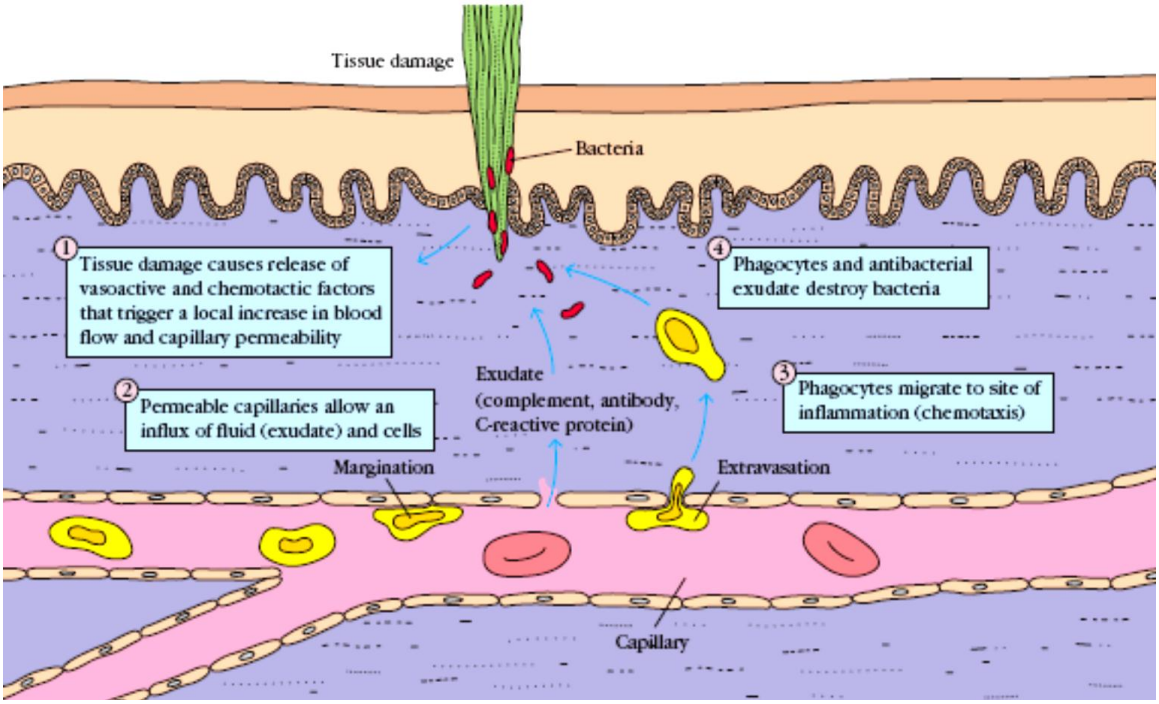

Figure 1.10 Major events in acute inflammatory response (Adapted from Goldsby *et al.*, 2006).

Acute inflammatory reaction is usually triggered by a variety of stimuli including infections (bacterial, viral, fungal, parasitic) and microbial toxins, tissue necrosis (through ischaemia, trauma and physical and chemical injury, hypoxia), hypoxia, foreign bodies and immune reactions (Kumar *et al.*, 2010).

Acute inflammation subsides upon successful elimination of the offending stimulus leading to either a complete resolution or healing by connective tissue replacement (fibrosis). However, it can progress to a chronic inflammatory phase if the acute response fails to clear the invaders.

1.4.2 Chronic Inflammation

Chronic inflammation is inflammation of prolonged duration (weeks or months) in which inflammation, tissue injury, and attempts at repair coexist, in varying combinations. It may follow acute inflammation, or begin insidiously, as a low-grade, smouldering response without any manifestations of an acute reaction. This latter type of chronic inflammation is the cause of tissue damage in some of the most common and disabling human diseases, such as rheumatoid arthritis, atherosclerosis, tuberculosis, and pulmonary fibrosis. It has also been implicated in the progression of cancer and Alzheimer disease (Kumar *et al.*, 2010)

Chronic inflammation is a result of either the persistence of the injurious agent or some interference with the normal process of healing. Persistent infections by microorganisms that are difficult to eradicate, such as mycobacteria, and certain viruses, fungi, and parasites often leads to chronic inflammation by evoking a delayed-type hypersensitivity. Chronic inflammation plays an important role in immune-mediated inflammatory diseases that are caused by excessive and inappropriate activation of the immune system e.g. rheumatoid arthritis and multiple sclerosis. Prolonged exposure to potentially toxic exogenous and/or endogenous agents also leads to chronic inflammation as is evidenced in silicosis and atherosclerosis (Kumar *et al.*, 2010).

Chronic inflammation, in contrast to acute inflammation which is manifested by vascular changes, oedema, and predominantly neutrophilic infiltration, is characterized by infiltration with mononuclear cells (including macrophages, lymphocytes and plasma cells), tissue destruction, induced by the persistent offending agent or by the inflammatory cells and attempts at healing by connective tissue replacement of damaged tissue, accomplished by

proliferation of small blood vessels (angiogenesis) and, in particular, fibrosis (Kumar *et al.*, 2010). Although neutrophils are characteristic of acute inflammation, many forms of chronic inflammation, lasting for months, continue to show large numbers of neutrophils, induced either by persistent microbes or by mediators produced by activated macrophages and T lymphocytes. The products of activated macrophages serve to eliminate injurious agents such as microbes and to initiate the process of repair, and are responsible for much of the tissue destruction, one of the hallmarks of chronic inflammation.

1.4.3 Systemic Responses in Inflammation

In addition to the local changes in an inflammatory area, there are often general systemic manifestations of inflammatory disease including fever, leucocytosis and the release from the liver of acute-phase proteins. These include C-reactive protein, α_2-macroglobulin, fibrinogen, α_1-antitrypsin and some complement components. Although it is not certain what the function of many of these components are, they all seem to have antimicrobial actions. Cortisol is also increased and exerts an important counter-regulatory effect on the inflammatory response (Rang *et al.*, 2007).

1.4.4 The Inflammatory Response

1.4.4.1 Inducers and Sensors of Inflammation

Inflammatory inducers and sensors are signals that initiate the inflammatory response. Inducers of inflammation can be exogenous or endogenous. Exogenous inducers can be classified into two groups: microbial and non-microbial. Microbial inducers can be pathogen-associated molecular patterns (PAMPs) or virulence factors (Medzhitov and Janeway, 1997; Sokol *et al.*, 2008). PAMPs, is a limited and defined set of conserved molecular patterns that are carried by all microorganisms of a given class (whether pathogenic or commensal). In contrast to PAMPs, virulence factors are restricted to pathogens. Exogenous inducers of inflammation that are of non-microbial origin include allergens, irritants, foreign bodies and toxic compounds (Rizki and Rizki, 1992; Dostert *et al.*, 2008)

Endogenous inducers of inflammation are signals produced by stressed, damaged or otherwise malfunctioning tissues. The identity and characteristics of these signals are poorly defined. But they probably belong to various functional classes according to the nature and the degree of

tissue anomalies on which they report (Bianchi, 2007; Rock and Kono, 2008). Another class of endogenous inducer, more relevant to chronic inflammatory conditions includes crystals of monosodium urate and calcium pyrophosphate dihydrate, AGEs (advanced glycation end products) and oxidized lipoproteins (such as high-density lipoproteins and low density lipoproteins). The formation of such crystals is facilitated in certain connective tissues, which provide an appropriate surface for crystal nucleation (Rock and Kono, 2008).

1.4.4.2 Mediators of Inflammation

Inflammatory mediators are substances derived from plasma, blood cells or tissues and have biological properties that cause or enhance the signs and symptoms of inflammation. Plasma contains four major mediator-producing systems (kinin, coagulation, complement, fibrinolytic) which interact in defined manners to generate phlogistic compounds. Other mediators are cell derived and, within the cells of origin, may be preformed and stored in granules (histamine in mast cells, cationic proteins in neutrophils) or may be newly synthesized by the cells (interleukin-1, leukotrienes, platelet-activating factor). The importance of these distinctions lies in part in the rapidity of release of the molecules, but also in therapeutic approaches that may be taken to modify their effects (Larsen and Henson, 1983).

1.4.4.2.1 Mediators from Plasma

In inflammation, fluid exudates from blood vessels contain a variety of mediators which influence the cells in the vicinity and the blood vessels themselves. These include components for four proteolytic enzyme cascades: the complement system, the coagulation system, the fibrinolytic system, and the kinin system. The components of these cascades are proteases that are inactive in their native form but that are activated by proteolytic cleavage, each activated component then activating the next. The activation of these components give rise to more inflammatory mediators (Rang *et al.*, 2007).

The complement system (Fig. 1.11) comprises nine major components, designated C1 to C9. Activation of the cascade leads to various events, one of which is the enzymatic splitting of C3, giving rise to various peptides including C3a, C3b , C5a, which have various roles in histamine release, opsonisation, lysis of bacteria and chemotaxis. The coagulation and fibrinolytic systems (Fig. 1.11) have important roles in limiting the extent of the infection. The kinin system (Fig.

1.11), another very important enzyme cascade yields several mediators, in particular bradykinin, relevant to inflammation (Rang *et al.*, 2007).

Bradykinin (BK) is a nonapeptide formed by proteolytic cleavage of a plasma α-globulin, kininogen, by kallikrein in the kinin cascade pathway. It is converted by kininase I to an octapeptide, BK_{1-8} (des-Arg^9-BK), and inactivated by kininase II (angiotensin-converting enzyme, ACE) in the lung. Bradykinin causes vasodilatation and increased vascular permeability. Its vasodilator action is partly a result of generation of PGI_2 and release of NO. It is a potent pain-producing agent, and its action is potentiated by the prostaglandins. Bradykinin also has spasmogenic actions on intestinal, uterine and bronchial smooth muscle (Rang *et al.*, 2007).

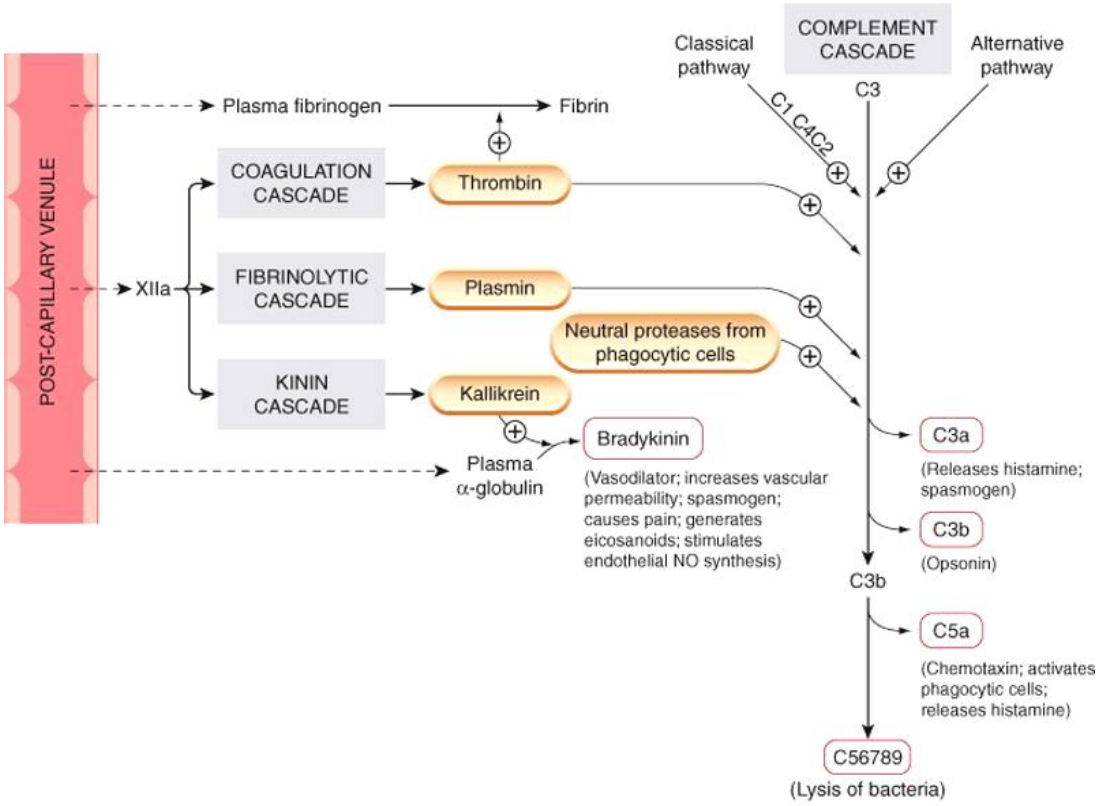

Figure 1.11 **The four proteolytic enzyme cascades in plasma.** The enzyme cascades are activated when plasma leaks out into the tissues as a result of the increased vascular permeability of inflammation. Complement components are indicated by C1, C2, etc (Adapted from Rang *et al.*, 2007).

1.4.4.2.2 Mediators from Cells

Various cell-mediated processes occur during inflammation with the goal of containing and eradicating local injury. Of the cells involved in inflammation, some (vascular endothelial cells, mast cells and tissue macrophages) are normally present in tissues, while others (platelets and leucocytes) gain access from the blood. Many substances are derived from these cells which play important mediatory roles in inflammation. Principal among them include eicosanoids, vasoactive amines, bradykinin, PAF, NO, neuropeptides and the cytokines.

Eicosanoids: Eicosanoids—arachidonate metabolites, including prostaglandins, prostacyclin, thromboxane A_2, leukotrienes, lipoxins and hepoxylins—are not stored but are produced by most cells when a variety of physical, chemical, and hormonal stimuli activate acyl hydrolases that make arachidonate available (Burke *et al.*, 2006). They are implicated in the control of many physiological processes, and are among the most important mediators and modulators of the inflammatory reaction.

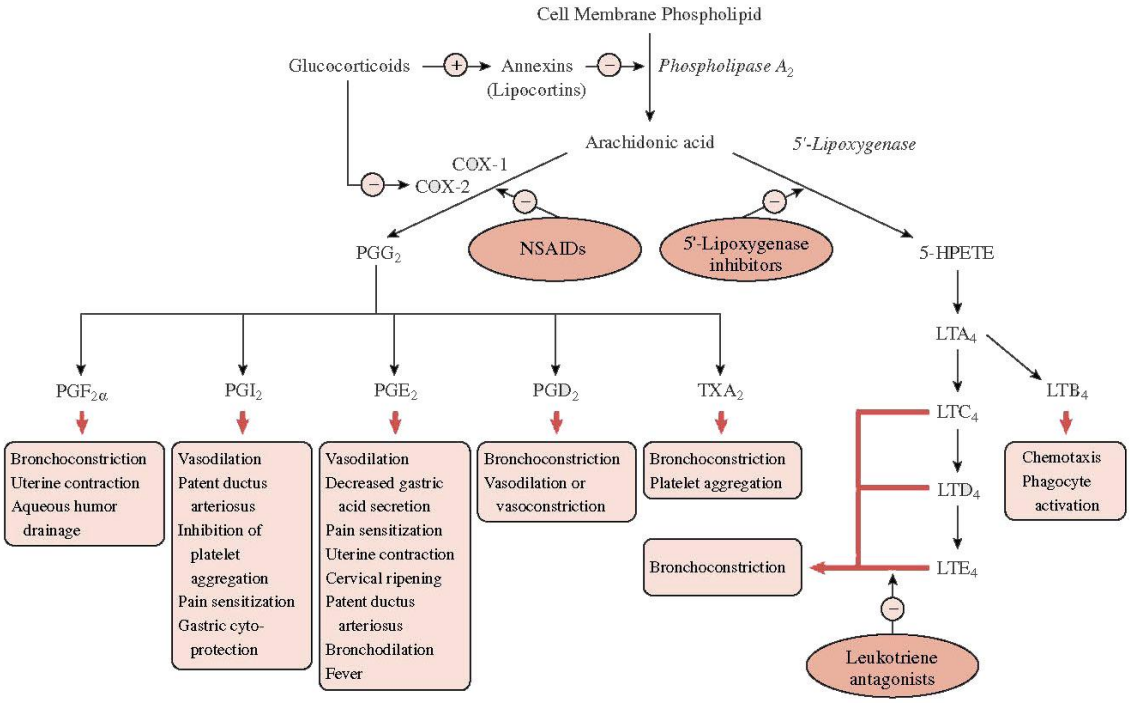

Figure 1.12 The eicosanoids synthesis pathway (Adapted from Craig & Stitzel, 2004)

The eicosanoids are synthesised from arachidonic acid (Fig. 1.12) released directly from phospholipids by phospholipase A_2, or by a two-step process involving phospholipase C and diacylglycerol lipase. Arachidonate is metabolised by cyclo-oxygenases (COX) to prostanoids, or by lipoxygenases (LOX) to leukotrienes which are two most important groups of eicosanoids in inflammation (Rang *et al.*, 2007).

Vasoactive Amines: Histamine and 5-HT are low molecular weight amines that are important inflammatory mediators. Histamine, a decarboxylation product of histidine, is stored in mast cells and released in mast cell activation. Its biological effects include vasodilatation and enhanced permeability of postcapillary venules. 5-HT stored in the dense bodies of platelets, enhances microvascular permeability and fibrosis by promoting collagen synthesis by fibroblast (Cassim *et al.*, 2002).

Cytokines: Cytokines are protein or polypeptide mediators synthesised and released by cells of the immune system during inflammation. More than 100 cytokines have been identified, and the superfamily is generally regarded as comprising interleukins, chemokines, interferons, colony-stimulating factors, growth factors and TNFs. Cytokines act locally by autocrine or paracrine mechanisms binding to and activating specific, high-affinity receptors on target cells that, in most cases, are up-regulated during inflammation. Except for chemokines, which act on G-protein-coupled receptors, most cytokines act on kinase-linked receptors, regulating phosphorylation cascades that affect gene expression, such as the JAK/STAT pathway. In addition to their own direct actions on cells, some cytokines amplify inflammation by inducing formation of other inflammatory mediators. Others can induce receptors for other cytokines on their target cell, or engage in synergistic or antagonistic interactions with other cytokines. Cytokines have been likened to a complex signalling language, with the final response of a particular cell involved being determined by the strength and number of different messages received concurrently at the cell surface (Rang *et al.*, 2007).

Platelet-Activating Factor: PAF is believed to be an important mediator in both acute and chronic allergic and inflammatory phenomena. PAF is not stored in cells but is synthesized in response to stimulation. PAF is biosynthesised from acyl-PAF in a two-step process (Fig. 1.13). Platelets stimulated with thrombin and most inflammatory cells can release PAF under the right circumstances. By acting on specific receptors, PAF produces vasodilatation and increased vascular permeability. It is a potent chemotaxin for neutrophils and monocytes, and recruits

eosinophils into the bronchial mucosa in the late phase of asthma. It can activate PLA_2 and initiates eicosanoid synthesis (Rang *et al.*, 2007).

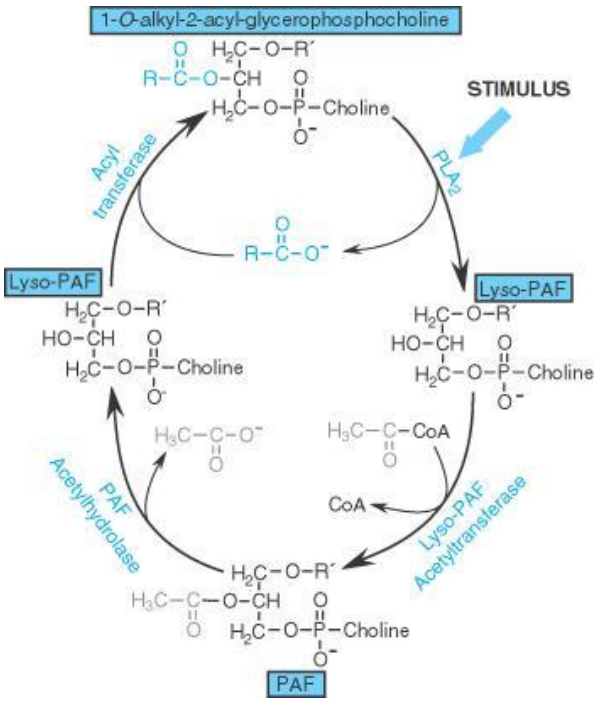

Figure 1.13 Synthesis and degradation of platelet-activating factor.

Nitric Oxide: NO has mainly pro-inflammatory effect; it increases vascular permeability and prostaglandin production, and is a potent vasodilator.

Neuropeptides: Neuropeptides released from sensory neurons cause neurogenic inflammation. The main peptides involved are SP, neurokinin A (NKA) and CGRP. SP and NKA (members of the tachykinin family) act on mast cells, releasing histamine and other mediators, and producing smooth muscle contraction and mucus secretion, whereas CGRP is a potent vasodilator (Rang *et al.*, 2007).

1.4.5 Pharmacological Management of Inflammation

The principal anti-inflammatory drugs in clinical use are the non-steroidal anti-inflammatory drugs (NSAIDs) and steroidal anti-inflammatory drugs (glucocorticoids). These conventional

anti-inflammatory agents, though effective, are associated with significant side effects and complications especially on systemic usage. Second generation COX-2 inhibitors were introduced with the prospect of treating pain/inflammation without gastrointestinal and renal toxicity but have also been rocked with disturbing reports of adverse cardiovascular side effects (Mukherjee *et al.*, 2001; Graham, 2006). The search for new therapeutic agents with fewer side effects, therefore, continues. New agents under investigation include nitric oxide (NO)-NSAIDs (conventional NSAIDs that have NO-donating groups attached to them by ester linkages), leukotriene receptor antagonists, inhibitors of leucocyte trafficking, cytokine suppressive anti-inflammatory drugs (CSAIDs), lipoxins and resolvins (products of 15-lipoxygenase).

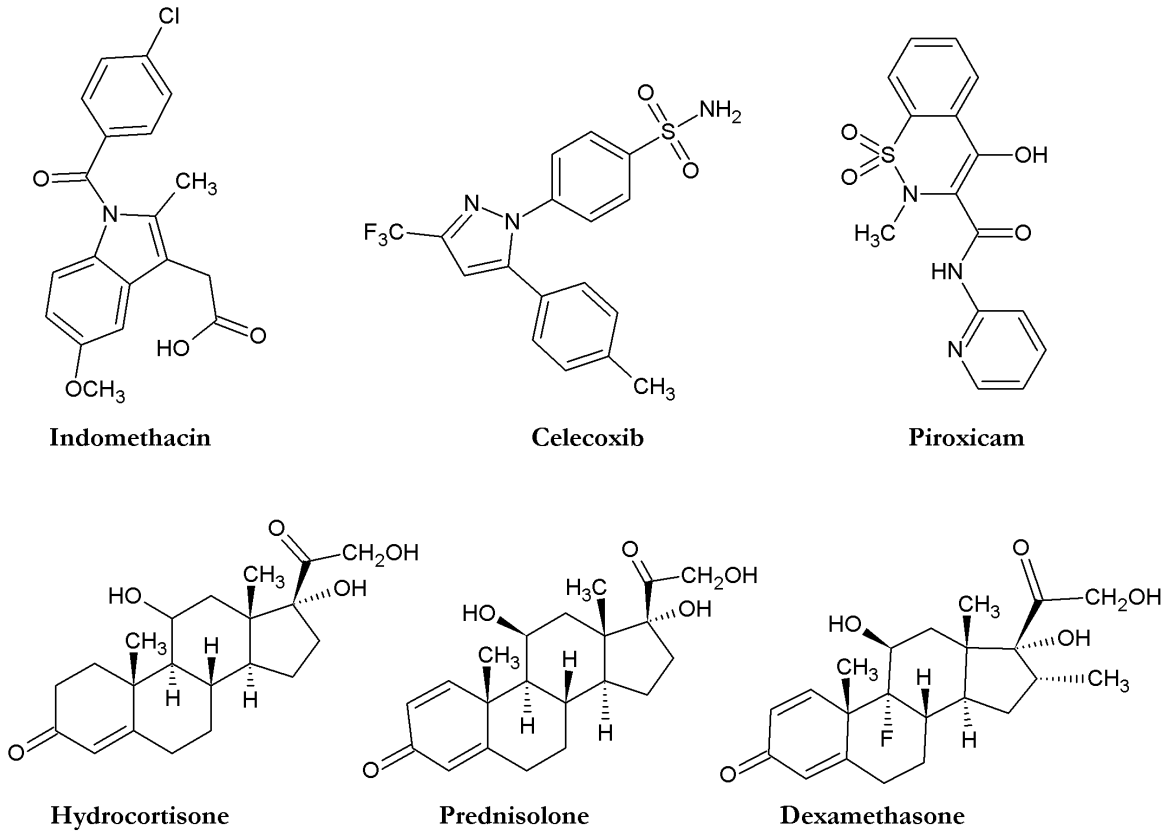

Figure 1.14 Examples of some commonly used anti-inflammatory drugs

1.4.6 Experimental Models of Inflammation

A number of animal models are available for both acute and chronic inflammation.

1.1.3.1 Models of Acute Inflammation

Acute inflammatory responses (swelling, redness, heat, pain) may be induced by injection of inflammatory agents such as killed bacteria (e.g. *E. coli*), polymorphonuclear leucocytes, chemotactic factors (e.g. LTB_4), vasoactive agents (e.g. PAF and histamine), arachidonic acid (in acetone) (Issekutz and Issekutz, 1989), carrageenan, zymosan, formalin and monosodium urate crystals (Higgs, 1989) into various parts of the body. The effect can be monitored by responses such as increase in foot volume produced by oedema (e.g. in rat's paw), detection of plasma markers in skin, local rise in temperature, measurement of inflammatory mediators in plasma exudates, hyperaemia, polymorphonuclear leucocyte accumulation, lymphocyte accumulation, monocytes infiltration, quantisation of haemorrhage, platelet deposition and thrombosis using diverse techniques (Issekutz and Issekutz, 1989).

Of all the animal models of acute inflammation, the carrageenan-induced acute foot oedema in laboratory animals (Winter *et al.*, 1962) is widely used to screen new anti-inflammatory drugs and remains an acceptable preliminary screening model. 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, 1972; 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 (Winter *et al.*, 1962; Kaur *et al.*, 2004).

1.1.3.2 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) (Whicher *et al.*, 1989). Other models have been developed including the polyarthritis induced by type II collagen in rats and mice, and the arthritis induced by streptococcal cell walls in the rat. Chronic inflammation induced by implantation of cotton wool pellets subcutaneously (cotton

pellet-induced granuloma) (Winter and Porter, 1957) and injection of turpentine oil (into pleural cavity/subcutaneous pouch) (Selye, 1953, Robert and Nezamis, 1957) have also been used.

Although the experimental arthritis in animals is not entirely similar in terms of all the clinical and biochemical features of patients with polyarthritic disease, it has been widely used as a model of rodent polyarthritis (Weichman *et al.*, 1987). It is employed extensively in research because of its reproducible predictiveness regarding the activity of NSAIDs although not yet useful in finding anti-arthritic agents with disease modifying properties (Weichman, 1989). Animal arthritic models closely resemble chronic inflammatory disease in man, although these diseases are distinct.

1.5 OXIDANTS AND ANTIOXIDANTS

In recent years, free radicals have received a lot of attention especially in experimental or clinical medicine and biology (Schaller, 2005). This can be attributed to the role of these oxidant chemical species in the aetiology of several human chronic diseases (Fig. 1.15) including atherosclerosis and cardiovascular diseases, mutagenesis and cancer, inflammatory lesions, several neurodegenerative disorders, and the aging process. The search for compounds, that can protect the human body from oxidative damage and retard the progress of many chronic diseases, has greatly focused on plant sources as they produce significant amount of antioxidants and represent a potential source of new compounds with antioxidant activity.

1.5.1.1 Free Radicals and Reactive Oxygen Species (ROS)

Free radicals represent a class of highly reactive intermediate chemical entities whose reactivity derives from the presence of unpaired electrons in their atomic structure, but which are capable of independent existence for very brief intervals of time (Halliwell, 1997; Cui *et al.*, 2004).

ROS is a collective term for all reactive forms of oxygen, including both the radical and non-radical species that participate in the initiation and/or propagation of radical chain reactions. Ozone (O_3), singlet oxygen ($^1O_2$) and molecular oxygen represents some of the prominent ROS.

Figure 1.15 Spectrum of human diseases where excessive free radical production is thought to play a significant role.

Ozone: Ozone (O_3) is a toxic form of oxygen that oxidizes proteins, nucleic acids, and lipids.

Singlet oxygen: Singlet oxygen ($^1O_2$), which is largely involved in photochemical reactions, is very reactive, although not a free radical. Singlet oxygen induces various genotoxic, carcinogenic, and mutagenic effects through its action on polyunsaturated fatty acids (PUFAs) and DNA (Cui *et al.*, 2004).

Molecular Oxygen: Molecular oxygen may be considered a diradical (dioxygen) because of its two unpaired electrons. The reduction of molecular oxygen to water in the electron transport chain by a stepwise addition of four electrons, results in the formation of several hydrogen-containing ROS including hydroperoxyl radical, superoxide radical, hydrogen peroxide, and the hydroxyl radical (Fig. 1.16).

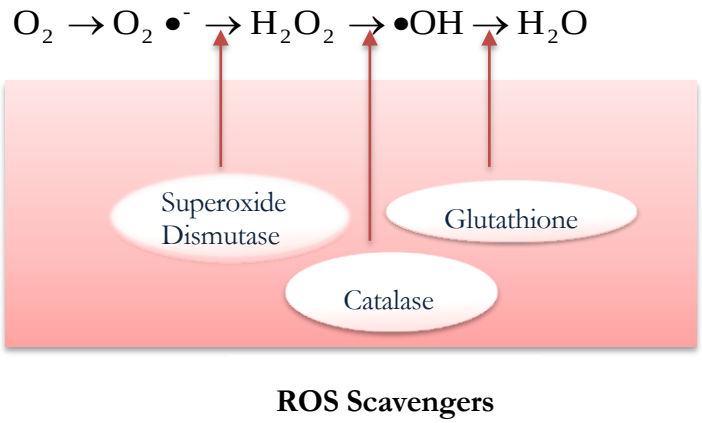

Figure 1.16 The process of formation of reactive oxygen species (ROS).

The superoxide is an anionic radical formed by the reduction of molecular oxygen through the acceptance of a single electron. The hydroperoxyl radical, which is unstable at physiological pH, dissociates to superoxide. Dismutation of superoxide yields hydrogen peroxide. Hydrogen peroxide and superoxide may undergo further transformations in the presence of transition metals (particularly Fe and Cu) to give rise to the highly reactive hydroxyl radicals, by the Haber-Weiss or Fenton reactions (Halliwell and Gutteridge, 1990).

$$\bullet O_2^- + H_2O_2 \rightarrow OH^- + \bullet OH + O_2 \text{ (Haber-Weiss reaction)}$$

$$Fe^{2+} + H_2O_2 \rightarrow OH^- + \bullet OH + Fe^{3+} \text{ (Fenton reaction)}$$

This property, combined with the membrane permeability of hydrogen peroxide, gives superoxide and hydrogen peroxide the ability to affect the integrity of distant molecules within the cell (Halliwell and Gutteridge, 1990; Cui *et al.*, 2004).

The hydroxyl radical, being the most aggressive member of the ROS family, can bring about extensive damage to different types of molecules, including proteins, nucleic acids and lipids. The most extensive studies on the oxidative properties of OH radicals have been carried out on membrane lipids in which the polyunsaturated fatty acids (PUFAs) are particularly vulnerable to

oxidation. The peroxidation of PUFAs by hydroxyl radicals constitutes one of the most severe attacks on cellular integrity (Gutteridge, 1995; Cui *et al.*, 2004).

Peroxyl Radical and Lipid Peroxidation: The peroxyl radicals may be formed *in vivo* from proteins and DNA by the interaction of sulfhydryl radicals with oxygen (Dean *et al.*, 1993). Peroxyl radicals are, however, believed to be produced primarily during lipid peroxidation, which is initiated by abstraction of a hydrogen atom from unsaturated lipids. Although lipid peroxidation has been found to play a useful role in some biological processes, peroxidation of membrane PUFAs may adversely affect many functionally important parameters, such as membrane fluidity, permeability, electrical potential, and controlled transport of metabolites across the membrane (Cui *et al.*, 2004).

The oxidation of lipids by ROS generally consists of three steps (Niki *et al.*, 1993):

a) **initiation**, in which the free radical attacks a methylene group in the PUFAs, leading to a rearrangement of the double bonds to the conjugated diene form, and simultaneously producing a carbon-centred alkyl radical. The alkyl radical reacts with molecular oxygen to give rise to a peroxyl radical;
b) **propagation**, in which the peroxyl radical, in its turn, starts a self-perpetuating chain reaction in which most of the membrane lipids are converted to a variety of hydroperoxides and cyclic peroxides. The hydroperoxides can be further degraded to hydrocarbons, alcohols, ether, epoxides, and aldehydes. Of these products, malondialdehyde and 4-hydroxynonenal have the additional ability to inactivate phospholipids, proteins, and DNA by bringing about cross-linking between these molecules; and
c) **termination**, in which the chain reaction is stopped by interactions between the radicals themselves, or between the radicals and antioxidants, giving rise to non-radical products or unreactive radicals.

There are also several forms of ROS that contain nitrogen or chlorine, in addition to oxygen. Nitrogen dioxide and nitric oxide (NO) are free radicals with odd numbers of electrons. Nitrogen dioxide is a strong oxidant, while NO acts as a weak reducing agent. Hypochlorous acid, which is a powerful oxidant, is produced by activated neutrophils from hydrogen peroxide by the haeme-containing enzyme, myeloperoxidase. There also exist others, such as the sulfhydryl free radicals, which do not contain the oxygen atom, but which undergo electron transfer and hydrogen transfer reactions with a variety of biological molecules (Cui *et al.*, 2004).

1.5.1.2 ROS and Oxidative Stress

Because of their high chemical reactivity, levels of ROS, in excess of normal needs of the cell, may indiscriminately damage the latter's structural and functional integrity. This occurs either by directly modifying cellular DNA, proteins, and lipids, or by initiating chain reactions that can bring about extensive oxidative damage to these critical molecules. Although cells possess a variety of defence mechanisms and repair systems against ROS, this can sometimes be inadequate, leading to oxidative stress in which the production of ROS overwhelms the antioxidant defences of the organism(Cui *et al.*, 2004).

Oxidative stress can be regarded as an imbalance between prooxidant/free radical production and opposing antioxidant defences. Acute oxidative stress as well as chronic oxidative stress have been implicated in a large number of human degenerative diseases (Fig. 1.15) affecting a wide variety of physiological functions, such as atherosclerosis, diabetes, ischaemia/reperfusion injury, inflammatory diseases (rheumatoid arthritis, inflammatory bowel disease, and pancreatitis), cancer, neurological diseases, hypertension, ocular diseases (cataract, senile muscular degeneration, and retrolental fibroplasia), pulmonary diseases, and haematological diseases(Cui *et al.*, 2004) . Even aging and age-related loss of physiological fitness have been attributed to the chronic effects of ROS on various biological macromolecules (Cui *et al.*, 2004).

1.1.3.3 ROS and Inflammation

It is well established that ROS play a significant role in inflammation. Local and systemic inflammatory response is associated with the production of large amounts of ROS such as superoxide anions, hydroxyl ions, hydrogen peroxide and peroxynitrite (Cuzzocrea *et al.*, 1998; Choi and Hwang, 2004). These excessively produced ROS can injure cellular biomolecules such as nucleic acids, proteins, carbohydrates and lipids, causing cellular and tissue damage, which in turn augments the state of inflammation (Surh and Packer, 2005). In a number of pathophysiological conditions associated with inflammation or oxidative stress, free radicals and ROS have been proposed to mediate cell damage via a number of independent mechanisms including the initiation of lipid peroxidation, the inactivation of a variety of enzymes and depletion of glutathione (Cuzzocrea *et al.*, 2001).

In addition to promoting cytotoxicity, ROS may also initiate and/or amplify inflammation via the upregulation of several different genes involved in the inflammatory response, such as those that code for pro-inflammatory cytokines (IL-1, TNF-α, IFNγ) and adhesion molecules (Fig. 1.17). This may occur by the activation of certain transcription factors, such as NF-kB which is a ubiquitous transcription factor and pleiotropic regulator of numerous genes involved in the immune and inflammatory response (Conner and Grisham, 1996; Wang *et al.*, 2007).

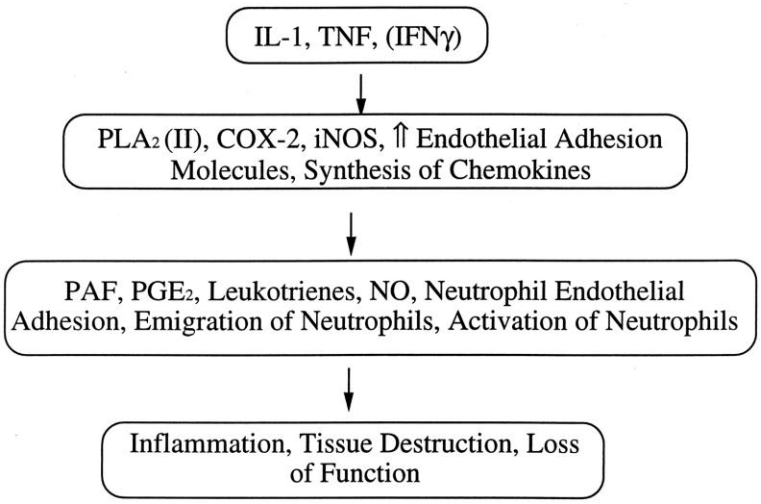

Figure 1.17 The inflammatory cascade triggered by IL-1 and TNF (Wang *et al.*, 2007).

1.5.2 Antioxidants

Antioxidants can be defined as endogenous or exogenous substances that have the ability, even at low concentrations, to delay or inhibit the oxidation of a substrate (Halliwell and Gutteridge, 1990; Gutteridge, 1995).

1.5.2.1 Modes of Action

Antioxidants may intervene at any of the three major steps: initiation, propagation, or termination of the oxidative process. They may act by:

a) removing or lowering the local concentrations of one or more of the participants in this reaction, such as oxygen, ROS, or metal ions, which catalyse oxidation (Fe^{3+}, Cu^{2+}, etc.).

b) interfering with the chain reaction that spreads oxidation to neighbouring molecules or
c) enhancing the endogenous antioxidant defences of the cell.

1.5.2.2 Classification of Antioxidants

Antioxidants may be classified according to their chemical nature and mode of action.

Enzyme antioxidants: They act on specific ROS after they are formed and degrade them to less harmful products. Examples are superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx). SODs convert the superoxide radical to hydrogen peroxide. Detoxification of hydrogen peroxide is carried out by CAT, which decomposes hydrogen peroxide to water and oxygen, or by GPx, which reduces hydrogen peroxide to water in the presence of GSH (Cui *et al.*, 2004).

Preventive antioxidants: They act by binding to and sequestering oxidation promoters and transition metal ions, such as iron and copper, which contain unpaired electrons and strongly accelerate free radical formation. Examples include transferrin and lactoferrin (which bind ferric ions), ceruloplasmin (which binds Cu, catalyses the oxidation of ferrous ions to ferric due to its ferroxidase activity, and increases the binding of iron to transferrin), haptoglobins (which bind haemoglobin), haemopexin (which binds haeme), and albumin (which binds copper and haeme) (Cui *et al.*, 2004).

Scavenging or chain-breaking antioxidants: They act by presenting themselves for oxidation at an early stage in the free radical chain reaction and giving rise to low energy products that are unable to propagate the chain further. Lipid-soluble and water-soluble scavengers act in cellular environments that are either hydrophobic or hydrophilic, respectively. The major lipid-soluble scavengers are vitamin E (α-tocopherol), β-carotene, and coenzyme Q (CoQ), while ascorbic acid, various thiols, uric acid, and bilirubin function in the aqueous milieu. Melatonin (N-acetyl-5-methoxytryptamine), an endogenous substance produced in the pineal gland, is a powerful antioxidant, which is believed to be of particular interest to the nervous system (Cui *et al.*, 2004).

1.5.3 In Vitro Assay Methods of Antioxidant Activity

Various *in vitro* methods are used to assay antioxidants. Based on the Halliwell and Gutteridge definition for antioxidants (Halliwell and Gutteridge, 1990), *in vitro* antioxidant assay methods can be classified into two—indirect and direct methods (Laguerre *et al.*, 2007).

Indirect methods: These do not involve an oxidisable substrate. They generally measure the capacity of a molecule (potential antioxidant) to reduce a stable artificial free radical (by hydrogen or electron transfer), or a transition metal (simply by electron transfer). Examples include 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) scavenging assay, total antioxidant capacity test and ferric-reducing antioxidant power assays.

Direct methods: Direct evaluation methods involve an oxidisable substrate. They are based on assessing the inhibitory effect of a potentially antioxidant substance on the oxidative degradation of a substrate in a test system subjected to natural or accelerated oxidation conditions. The oxidisable substrate usually consists of individual or mixed lipids, plant proteins, fluorophores, chromophores, DNA, or fluids containing biologically active chemical species such as low-density lipoproteins (LDLs) and biological membranes. An example is the linoleic acid autoxidation method.

The *in vitro* antioxidant assay methods are useful in exploring the mechanism of antioxidant activity of test antioxidant substances.

1.5.3.1 Total Phenol Assay

The principle behind the assay of total phenols is the reduction of phosphomolybdate-phosphotungstate salts of Folin-Ciocalteau reagent in alkaline medium by phenolic compounds (Slinkard and Singleton, 1977). The reduced Folin-Ciocalteau reagent is blue and can be spectrophotometrically quantified at 760 nm. Thus, the higher the concentration of phenolic compounds, the greater the degree of reduction and the higher the absorbance.

1.5.3.2 Total Antioxidant Capacity

This is a spectrophotometric method developed for the quantitative determination of antioxidant capacity (Prieto *et al.*, 1999). The assay is based on the reduction of Mo (VI) to Mo (V) by the antioxidant compound and the subsequent formation of a green phosphate/Mo (V) complex at acidic pH that has a maximal absorption at 695 nm. The higher the antioxidant

activity, the higher the absorbance of the green complex. This method, like the total phenol assay method, is quantitative, since the antioxidant activity is expressed as the number of equivalents of ascorbic acid or α-tocopherol.

1.5.3.3 Reducing Power Test

Reducing power is one of the parameters for assessing antioxidant activity of a compound. The method by Oyaizu (1986) 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 that is spectrophotometrically assessed at 700 nm.

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

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

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

1.5.3.4 DPPH Scavenging Assay

DPPH (2, 2-diphenyl-1-picrylhydrazyl hydrate) scavenging assay, as described by Blois (1958), is a simple, efficient and fast method for analysis of potential free radical scavenging of antioxidant compounds in natural extract from plants. DPPH is a stable radical with a characteristic violet colour. This is modified to yellow in the presence of a free radical quencher by the appearance of reduced 2, 2-diphenyl-1-picrylhydrazine (Fig. 1.18).

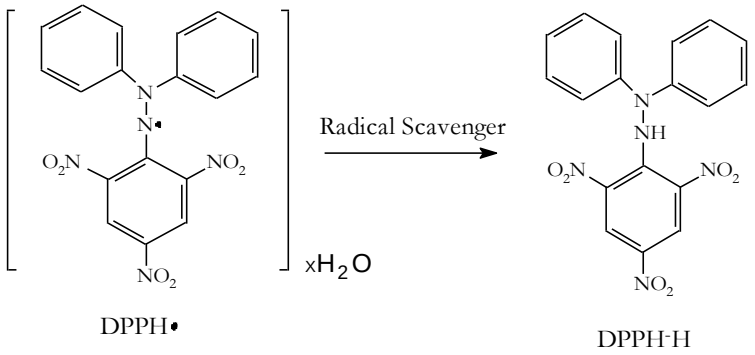

Figure 1.18 DPPH molecular structure and its reduced form.

An extract with free radical scavenging activity is able to mop up the free radicals and any excess free radical is measured spectrophotometrically. The percentage scavenging can be calculated as follows:

$$\%\,\text{Scavenging} = \frac{\text{ABSORBANCE}_{\text{CONTROL}} - \text{ABSORBANCE}_{\text{TEST}}}{\text{ABSORBANCE}_{\text{CONTROL}}} \times 100$$

1.5.3.5 Inhibition of Lipid Peroxidation (Linoleic Acid Autoxidation)

Lipid peroxidation is a chain reaction initiated by hydrogen abstraction or addition of oxygen radical resulting in the oxidative deterioration of polyunsaturated fatty acids. The resulting fatty acid radical is stabilized by rearrangement into a conjugated diene that retains the more stable products including hydroperoxides, alcohols, aldehydes, and alkanes. Among a great variety of aldehydes that are produced in the lipid peroxidation, malondialdehyde (MDA) is the widely oxidised derivative used as indicator of free radicals damage in the assay with thiobarbituric acid (TBA). The incorporation of any antioxidant in the reaction results in reduction of the extent of lipid peroxidation.

Linoleic acid is a polyunsaturated fatty acid that oxidises slowly at room temperature. Incubation at a higher temperature (usually 40°C) accelerates the peroxidation. Early degradation products formed (primary stage) are a variety of hydroperoxides and cyclic peroxides. The hydroperoxides can be further degraded to hydrocarbons, alcohols, ether, epoxides, and aldehydes (secondary stage). The amount of peroxides at the primary stage of linoleic acid peroxidation are measured by the Ferric thiocyanate (FTC) method, whereas thiobarbituric acid (TBA) method measures the production of carbonyl compounds degraded from the peroxides at the secondary stage (Inatani *et al.*, 1983).

In the FTC method, peroxides formed during linoleic acid oxidation; oxidize blue Fe^{2+} (in $FeCl_2$) to reddish brown Fe^{3+} (in $FeCl_3$). The Fe^{3+} ions formed are quantified by complexing with SCN^- ions (from ammonium thiocyanate) and measuring the absorbance at 500 nm. The higher the antioxidant activity, the lower the amount of peroxides generated from linoleic acid and the lower the Fe^{3+} formed. Hence, lower absorbance indicates a higher level of antioxidant activity.

The formation of MDA is the basis for the well-known TBA method used for evaluating the extent of lipid peroxidation. At low pH and high temperature (100 °C), malondialdehyde binds TBA to form a pink complex (TBA-MDA adduct) that can be measured at 532 nm (Fig. 1.19). The amount of the pink colour formed correlates with the extent of peroxidation of the linoleic acid. Antioxidant activity results in lower yield of chromogenic product (TBA-MDA).

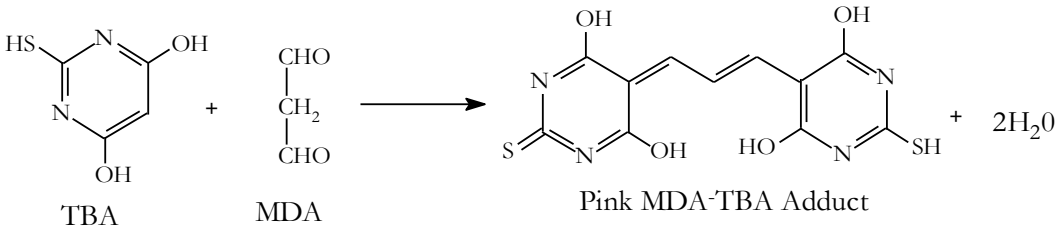

Figure 1.19 The reaction between TBA and MDA that gives a pink coloured TBA-MDA adduct.

The percentage inhibition of lipid peroxidation can be determined by:

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

Where

- **FRM** determines the degree of linoleic acid autoxidation in the absence of an antioxidant.
- **L** determines the underlying peroxidation of the linoleic acid before the initiation of accelerated autoxidation by incubation at 40°C.
- **Drug/Extract Alone** determines the absorbance of Drug/Extract solutions being tested.

1.5.4 Plants as Source of Antioxidants

Plants produce significant amounts of antioxidants to prevent oxidative stress by photons and oxygen during photosynthesis (Auddy *et al.*, 2003). Phenolic compounds (flavonoids and phenolic acids), nitrogen compounds (alkaloids, chlorophyll derivatives, amino acids and amines), carotenoids, lignans and terpenes are well known to possess antioxidant activity in suppressing the initiation or propagation of the chain reactions. Flavonoids and phenolic compounds are the main antioxidant compounds of fruits and vegetables (Wu and Ng, 2008).

1.6 AIMS AND OBJECTIVES OF STUDY

Current therapies for pain and inflammation, despite proven efficacy in alleviating symptoms and providing relief, all have considerable side effects. Anti-inflammatory agents including NSAIDs and glucocorticoids cause considerable gastrointestinal and renal damage among other side effects. Analgesics, aside NSAIDs, such as opioids also cause significant adverse effects like respiratory depression, emesis, tolerance and addiction. Due to the numerous and life-threatening side effects associated with the use of most of these agents, together with the general dissatisfaction among many sufferers of pain and inflammatory disorders about care, means that there is still the need to search for more effective anti-inflammatory and analgesic agents with minimal side effects at therapeutic doses.

Medicinal plants are important sources of new chemical substances with potential therapeutic effects and therefore research into plants with alleged traditional use as pain and inflammation relievers is a useful research strategy in the search for new analgesic and anti-inflammatory drugs. In Ghana and other parts of Africa, various parts especially the leaves of *Hilleria latifolia* are widely used in the treatment of pain and inflammation but with little scientific evidence for its efficacy. Hence, this study aimed at providing substantial pharmacological evidence for the traditional use of the leaves in the management of pain and inflammation.

The objective of the present study was to evaluate the anti-nociceptive and anti-inflammatory effects of the ethanolic extract of the aerial parts of *Hilleria latifolia* using animal models. Specific objectives included evaluating the extract for its:

1) Analgesic activity and possible mechanisms using various models for different types of pain

2) Anti-inflammatory activity in both acute and chronic inflammation

3) Antioxidant properties

4) Neurobehavioural effects including effects on anxiety and depression

5) Acute and sub-acute toxicity in rats

Chapter 2

PLANT COLLECTION, EXTRACTION AND PHYTOCHEMICAL TESTS

2.1 PLANT COLLECTION AND EXTRACTION

2.1.1 Plant Collection

The aerial parts of *H. latifolia* were collected from the campus of Kwame Nkrumah University of Science and Technology (KNUST), Kumasi near the Botanical Gardens (06°41'12.89"N; 01°33'59.51"W) during the month of July, 2007 and authenticated at the Department of Pharmacognosy, Faculty of Pharmacy and Pharmaceutical Sciences, College of Health Sciences, KNUST, Kumasi, Ghana. A voucher specimen (KNUST/HM1/09/L029) was kept at the herbarium of the Faculty.

2.1.2 Plant Extraction

The plant was room-dried for seven days and pulverised into fine powder. The powder was extracted by cold percolation with 70 % (v/v) ethanol and then concentrated into a green syrupy mass under reduced pressure at 60 °C using a rotary evaporator (Model: Rotavapor R-215, BÜCHI Labortechnik AG, Flawil, Switzerland). It was further dried in a hot air oven at 50 °C for a week and kept in a refrigerator for use. The yield was 19.67 % (w/w). This crude extract was subsequently referred to as HLE or extract in this study.

2.2 PHYTOCHEMICAL TESTS

Phytochemical tests were performed on HLE to determine the presence of tannins, saponins, glycosides, alkaloids, flavonoids, steroids and terpenoids.

2.2.1 Methods

2.2.1.1 Tannins

An amount of 0.5 g of HLE was boiled with 25 ml of water for 5 minutes, cooled and filtered. The volume of the filtrate was adjusted to 25 ml with water. To 1ml of the filtrate was added 10 ml of water and 5 drops of 1 % ferric chloride and observed for a blue-black or green

precipitate formation. The procedure was repeated using 5 drops of 1 % lead acetate and observed for any change in colour or formation of precipitate (Evans, 2002; Usman *et al.*, 2009).

2.2.1.2 Saponins

A small amount (0.2 g) of HLE was shaken with a few millilitres of water in a test tube and the mixture observed for the presence of a froth which does not break readily upon standing (Sofowora, 1993; Usman *et al.*, 2009).

2.2.1.3 Glycosides (General test)

An amount of 0.2 g of HLE was boiled in 5 ml dilute H_2SO_4 on a water bath for 2 minutes. The mixture was cooled, filtered and rendered distinctly alkaline with 2 to 5 drops of 20 % NaOH. 1 ml each of Fehling's A and B solutions were added to the filtrate, heated on a water bath for 2 minutes and observed for a red-brown precipitate (Houghton and Raman, 1998; Evans, 2002).

2.2.1.4 Alkaloids

An amount of 0.5 g of HLE was boiled with 10 ml of dilute hydrochloric acid (alcohol) in a test tube for 5 minutes. The supernatant liquid was filtered into another test tube and 1ml of the filtrate was taken into which three drops of Dragendorff's reagent (potassium bismuth iodide solution) was added, shaken and observed for the appearance of an orange-red spot and precipitate formation (Sofowora, 1993; Usman *et al.*, 2009).

2.2.1.5 Flavonoids (Ammonia test)

Five millilitres of dilute ammonia solution were added to a portion of the aqueous filtrate HLE followed by addition of concentrated H_2SO_4 and observed for yellow coloration (Ayoola *et al.*, 2008).

2.2.1.6 Steroids (Liebermann-Burchard's test)

An amount of 0.5 g of HLE was extracted with 2 ml of chloroform in a test tube. 2 ml acetic anhydride was added to the extract. Concentrated sulphuric acid was carefully added at the side of the test tube. A blue colour that appeared at the interface suggested the presence of steroids (Sofowora, 1993; Jana and Shekhawat, 2010).

2.2.1.7 Terpenoids (Salkowski test)

An amount of 0.5 g of HLE was extracted with 2 ml of chloroform in a test tube followed by addition of 1ml of concentrated sulphuric acid. The reddish-brown coloration at interface shows the presence of terpenoids (Sofowora, 1993; Jana and Shekhawat, 2010).

2.2.2 RESULTS

The phytochemical screening revealed the presence of alkaloids, flavonoids, saponins, tannins, glycosides, steroids and terpenoids (Table 2.1). Alkaloids and flavonoids were, however, detected in little amounts.

Table 2.1 Phytochemical constituents of the ethanolic extract of the aerial parts of *H. latifolia*.

CONSTITUENT	TESTS	INFERENCE
Tannins	Ferric chloride test	++
	Lead acetate test	++
Glycosides	General (Fehling's)test	++
Saponins	Frothing test	++
Alkaloids	Dragendorff's test	+
Flavonoids	Ammonia test	+
Steroids	Lieberman-Burchard's test	++
Terpenoids	Salkowski test	++

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

2.2.3 DISCUSSION

Among the phytochemicals produced by plants are secondary metabolites, which are defined as a group of natural compounds that, in contrast to primary metabolites, are not directly involved in growth, development or reproduction of organisms (Jenke-Kodama *et al.*, 2008). Although the presence of secondary metabolites in the biochemistry of plants is very often difficult to justify, they have formed the basis of medicines (Gurib-Fakim, 2006; Jenke-Kodama *et al.*, 2008). As have been reported by several authors, the presence of many biologically active

secondary metabolites such as alkaloids, flavonoids, tannins, glycosides, terpenoids and steroids in various plant extracts are responsible for their respective pharmacological properties (Barbosa-Filho *et al.*, 2006; Zulak *et al.*, 2006; Gomes *et al.*, 2009; Maganha *et al.*, 2010).

The presence of flavonoids, alkaloids, glycosides and saponins in HLE has confirmed preliminary phytochemical tests done elsewhere on *H. latifolia* (Iwu, 1993; Schmelzer and Gurib-Fakim, 2008).

Recently, flavonoids have attracted interest due to the discovery of their pharmacological activities as anti-inflammatory, analgesic, anti-tumour, anti-HIV, anti-infective (anti-diarrhoeal, anti-fungal), anti-hepatotoxic, anti-lipolytic, anti-oxidant, vasodilator, immunostimulant and anti-ulcerogenic (Gurib-Fakim, 2006). Tannins, aside their usefulness as astringents, have anti-inflammatory, antioxidant and anti-nociceptive, antiulcer, antimicrobial, antiviral and antitumor properties (Mota *et al.*, 1985; Lin *et al.*, 2001; Chen *et al.*, 2003; Souza *et al.*, 2007; Buzzini *et al.*, 2008; Koleckar *et al.*, 2008). Triterpene saponins also exhibit various pharmacological activities: anti-inflammatory, molluscicidal, anti-tussive, expectorant, analgesic and cytotoxic (Gurib-Fakim, 2006). The Alkaloids, the largest single class of secondary plant substances, have a remarkable range of pharmacological activity. These include CNS, analgesic, anti-inflammatory and anti-cancer activities (Barbosa-Filho *et al.*, 2006; Zulak *et al.*, 2006; Gomes *et al.*, 2009).

Even though the individual secondary metabolites in HLE have not been isolated and tested pharmacologically, it is believed that one or more of them may be responsible for the medicinal properties that *H. latifolia* has in traditional medical usage.

2.2.4 CONCLUSION

Preliminary phytochemical screening of the ethanolic extract of the aerial parts of *Hilleria latifolia* showed the presence of saponins, tannins, glycosides, steroids, terpenoids as well as small amounts of flavonoids and alkaloids.

Chapter 3

ANTI-NOCICEPTIVE EFFECTS

3.1 INTRODUCTION

Pain is the most common reason why patients seek advice from health professionals. Current analgesic therapies, despite their proven efficacy in alleviating symptoms and providing pain relief, all have considerable side effects including gastrointestinal ulceration, renal damage, respiratory depression, emesis, and tolerance and/or addiction (Jagerovic *et al.*, 2002; Rang *et al.*, 2007). This together with the fact that many pain sufferers are not satisfied with their pain care, makes the search for new analgesics, that can more effectively treat pain an important challenge to drug research. Natural products in general, and medicinal plants in particular, are believed to be important sources of new chemical substances with potential therapeutic efficacy. Taking into account that the most important analgesic prototypes (e.g. salicylic acid and morphine) were originally derived from the plant sources, the study of plant species traditionally used as pain killers should still be seen as a fruitful research strategy in the search of new analgesics.

Since *H. latifolia* is used traditionally in the treatment of painful illnesses, it became worthwhile to evaluate its anti-nociceptive property in animals. The anti-nociceptive effect of *H. latifolia* extract was assessed using animal models that predict both peripherally- and centrally-mediated pain including formalin test, writhing assay and tail immersion test. Some receptors and neurotransmitters involved in the pain mediation were also investigated using various antagonists and agonists to help predict the possible mechanism of action of the extract. Tolerance to the anti-nociceptive effects of *H. latifolia* extract was also investigated. The current study will help to substantiate the traditional uses of *H. latifolia* as well as provide an alternative to current analgesics.

3.2 METHODS

3.2.1 Animals

Male ICR mice (15–25 g) and male Sprague-Dawley rats (100-195 g) were purchased from the Noguchi Memorial Institute for Medical Research, Accra, Ghana and kept in the animal house of the Department of Pharmacology, Kwame Nkrumah University of Science and Technology,

Kumasi, Ghana. They were housed in groups of 5 in stainless steel cages (34 cm × 47 cm × 18 cm) with soft wood shavings as bedding. Water and a normal commercial pellet diet (GAFCO, Tema, Ghana) were made freely available to the animals. The studies were conducted in accordance with accepted principles for laboratory animal use and care (EU directive of 1986: 86/609/EEC). Approval for this study was obtained from the Departmental Ethics Committee.

3.2.2 Drugs and Chemicals

The following drugs and chemicals were used: formalin, acetic acid, theophylline (BDH, Poole, England); diclofenac (KRKA, Slovenia); morphine (PhytoRiker, Accra, Ghana); cyproheptadine (LETAP Pharmaceuticals Ltd., Accra, Ghana); ondansetron (GlaxoSmithKline, Uxbridge, U.K.); glibenclamide (Daonil®, Sanofi-Aventis, Guildford, UK); nifedipine (Denk Pharma, Germany); capsaicin, yohimbine, atropine, naloxone, N^G-L-nitro-arginine methyl ester/ L-NAME, L-glutamic acid (Sigma-Aldrich Inc., St. Louis, MO, USA); hexamethonium (Mayer and Baker, Dagenham, England), bisacodyl (Dulcolax®, Boehringer Ingelheim, Germany) and loperamide (Imodium®, Janssen-Cilag, USA).

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3.2.3 Acetic Acid-Induced Writhing Assay

This test was carried out as described by Koster *et al.* (1959) and Gonzalez-Trujano *et al.* (2007) with modifications. Mice were treated with HLE (30, 100 or 300 mg kg^{-1}, *p.o.*), diclofenac (10, 30 or 100 mg kg^{-1}, i.p.), or vehicle (1 ml 100 g^{-1}, *p.o.*) 30 min (i.p.) or 1 h (*p.o.*) before administration of the acetic acid and placed individually in a testing chamber (a Perspex chamber 15 cm × 15 cm × 15 cm). A mirror inclined at 45° below the floor of the chamber allowed a complete view of the mice.

Each animal was administered with acetic acid (0.6 %, 10 ml kg^{-1}) intraperitoneally. Injection of acetic acid induced a nociceptive behaviour, writhing, an exaggerated extension of the abdomen combined with the outstretching of the hind limbs. Responses were captured (30 min) for analysis by a camcorder (Everio™, model GZ-MG1300, JVC, Tokyo) placed directly opposite the mirror and attached to a computer. Tracking of the behaviour was done using a public domain software JWatcher™ Version 1.0 (University of California, Los Angeles, USA and Macquarie University, Sidney, Australia available at http://www.jwatcher.ucla.edu/.) to obtain

the total number of writhes per 5 min, starting 5 min after acetic acid administration. These data expressed in a time course helped to observe changes in the maximal number of writhing induced. A dose–response curve was also plotted to determine the significant anti-nociceptive dose.

3.2.4 Tail Immersion Test

Tail-immersion test was carried out as described by Sewell and Spencer (1976) with modifications.

This involved immersing the extreme 3.5 cm of the rat's tail in a water bath containing water at a temperature of 48 ± 0.5 ºC. The rat reacts by withdrawing the tail. The reaction time was recorded with a stop watch and a cut-off time of 15 s imposed on this measure.

Rats were randomly divided into one of the following study groups (five per group): control, diclofenac (10, 30 and 100 mg kg^{-1}, i.p.), morphine (1, 3 and 10 mg kg^{-1}, i.p.) and HLE (30, 100 and 300 mg kg^{-1}, *p.o.*). The reaction time (*T*) for the study groups was taken at 0.5, 1, 2, 3, 4 and 5 h intervals after a latency period of 30 min (i.p.) or 1 h (*p.o.*) following the administration of the drugs and extract.

The percentage maximal possible effect (% MPE) was calculated from the reaction times using the following formula:

$$\%\,\mathrm{MPE} = \frac{T_2 - T_1}{T_0 - T_1} \times 100$$

where $\mathbf{T_1}$ and $\mathbf{T_2}$ are the pre- and post- drug reaction times, and $\mathbf{T_0}$ is the cut-off time.

3.2.5 Formalin Test

The formalin test was carried out as described (Dubuisson and Dennis, 1977; Hunskaar and Hole, 1987) with a few modifications.

Each animal was assigned and acclimatized to one of 20 formalin test chambers (a Perspex chamber 15 cm × 15 cm × 15 cm) for one hour prior to formalin injection. Mice were then pre-treated with the test drugs [HLE (30, 100, 300 mg kg^{-1}, *p.o.*) and Morphine (1, 3, 10 mg kg^{-1}, i.p.)] 30 min for i.p. route and 1 h for oral route before intraplantar injection of 10 µl of 5 % formalin. The animals were immediately returned individually into the testing chamber. A

mirror inclined at 45° below the floor of the chamber allowed a complete view of the paws. The behaviour of the animals were then captured (1 h) for analysis by a camcorder (Everio™, model GZ-MG1300, JVC, Tokyo) placed directly opposite to the mirror and attached to a computer.

Pain response was scored for 1 h, starting immediately after formalin injection. A nociceptive score was determined for each 5-min time block by measuring the amount of time spent biting/licking of the injected paw (Hayashida *et al.*, 2003). Tracking of the behaviour was done using a public domain software JWatcher™ Version 1.0 (University of California, Los Angeles, USA and Macquarie University, Sidney, Australia available at http://www.jwatcher.ucla.edu/.). Average nociceptive score for each time block was calculated by multiplying the frequency and time spent in biting/licking. Data were expressed as the mean ± SEM of scores between 0–10 (first phase) and 10–60 min (second phase) after formalin injection.

3.2.6 Capsaicin-Induced Nociception

The procedure used was similar to that described previously (Beirith *et al.*, 1998) but with modifications.

Before testing, the animals were placed individually in one of 20 transparent Perspex chambers (15 cm × 15 cm × 15 cm). Following an hour adaptation period in the chamber, animals were pre-treated with HLE (10, 30, 100, 300 mg kg^{-1}, *p.o.*) and morphine (3 mg kg^{-1}, i.p.) 30 min for i.p. route and 1 h for oral route before intraplantar injection of 20 µl of capsaicin (1.6 µg/paw made in 10 % ethanol, 10 % Tween 80 and 80 % saline). Control animals received vehicle (normal saline, 10 ml kg^{-1}) systemically before intraplantar capsaicin. Pain response (biting/licking of the injected paw) was recorded (10 min) and scored (10 min) in the same way as that described previously in the formalin test above (section 3.2.5). Data were expressed as the mean ± SEM of scores between 0–10 min after capsaicin injection.

3.2.7 Glutamate-Induced Nociception

The procedure was carried out as described previously (Beirith *et al.*, 2002, Meotti *et al.*, 2006) with modifications.

Mice were acclimatised to test chambers and pre-treated with HLE, morphine or vehicle similar to that described above (section 3.2.6). Twenty microlitres of glutamate (10 µmol/paw prepared in saline) was injected into the ventral surface to the right hind paw of mice and immediately

returned individually into the testing chambers. The nociceptive behaviour (biting/licking of the injected paw) of the animals were then captured (15 min) and later scored (15 min) similarly to that described above (section 3.2.5). Data were expressed as the mean ± SEM of scores between 0–15 min after glutamate injection.

3.2.8 Assessment of Possible Mechanism of Action of HLE in the Formalin Test

To investigate the possible mechanisms by which HLE exerts its anti-nociceptive activity, mice were pre-treated with different drugs in the formalin test. The doses of antagonists, agonists and other drugs were selected based on data from literature and preliminary experiments in our laboratory.

3.2.8.1 The Opioid Pathway

Mice were pre-treated with naloxone (a non-selective opioid receptor antagonist; 2 mg kg^{-1}, i.p.) and after 15 min received HLE (30 mg kg^{-1}, *p.o.*), morphine (3 mg kg^{-1}, i.p.) or vehicle (10 ml kg^{-1}, *p.o.*). The nociceptive response to formalin injection was recorded 1 h after administration of HLE or vehicle and 30 min after administration of morphine.

Another group of mice was pre-treated with vehicle and after 15 min received HLE (30 mg kg^{-1}, *p.o.*), morphine (3 mg kg^{-1}, i.p.) or vehicle (10 ml kg^{-1}, *p.o.*), 1, 0.5 and 1 h before formalin injection, respectively.

3.2.8.2 The Nitric Oxide Pathway

Mice were pre-treated with L-NAME (N^G-L-nitro-arginine methyl ester, a NO synthase inhibitor, 10 mg kg^{-1}, i.p.) and after 15 min received HLE (30 mg kg^{-1}, *p.o.*), morphine (3 mg kg^{-1}, i.p.) or vehicle. The nociceptive response to formalin injection was recorded 1 h after administration of HLE or vehicle and 30 min after morphine administration.

3.2.8.3 ATP-Sensitive K^+ Channels

Mice were pre-treated with glibenclamide (an ATP-sensitive K^+ channel inhibitor, 8 mg kg^{-1}, *p.o.*) and after 30 min received HLE (30 mg kg^{-1}, *p.o.*), morphine (3 mg kg^{-1}, i.p.) or vehicle. The nociceptive response to formalin injection was recorded 1 h after administration of HLE or vehicle and 30 min after morphine administration.

3.2.8.4 *The Adenosinergic System*

Mice were pre-treated with theophylline (10 mg kg^{-1}, i.p., a non-selective adenosine receptor antagonist) and after 15 min received HLE (30 mg kg^{-1}, *p.o.*), morphine (3 mg kg^{-1}, i.p.) or vehicle. The nociceptive response to formalin injection was recorded 1 h after administration of HLE or vehicle and 30 min after morphine administration.

3.2.8.5 *The Serotonergic System*

To assess the possible contribution of serotonin 5-HT_2 and 5-HT_3 receptors to the anti-nociceptive effects caused by HLE, animals were pre-treated with cyproheptadine (8 mg kg^{-1}, *p.o.*, a 5-HT_2 receptor antagonist) and ondansetron (0.5 mg kg^{-1}, i.p., a 5-HT_3 receptor antagonist) and after 15 min (i.p.) or 30 min (*p.o.*) received HLE (30 mg kg^{-1}, *p.o.*), morphine (3 mg kg^{-1}) or vehicle (10 ml kg^{-1}). The nociceptive response to formalin injection was recorded 1 h after administration of HLE or vehicle and 30 min after morphine administration.

3.2.8.6 α_2-*adrenoceptors*

Mice were pre-treated with yohimbine (3 mg kg^{-1}, *p.o.*, a selective adrenoceptor antagonist) and after 30 min received HLE (30 mg kg^{-1}, *p.o.*), morphine (3 mg kg^{-1}, i.p.) or vehicle. The nociceptive response to formalin injection was recorded 1 h after administration of HLE or vehicle and 30 min after morphine administration.

3.2.8.7 *Voltage-Gated Calcium Channels*

Mice were pre-treated with nifedipine (10 mg kg^{-1}, *p.o.*, L-type VGCC blocker) and after 30 min received HLE (30 mg kg^{-1}, *p.o.*), morphine (3 mg kg^{-1}, i.p.) or vehicle. The nociceptive response to formalin injection was recorded 1 h after administration of HLE or vehicle and 30 min after morphine administration.

3.2.8.8 *The Muscarinic Cholinergic System*

Mice were pre-treated with atropine (5 mg kg^{-1}, i.p., a non-selective muscarinic receptor antagonist) and after 15 min received HLE (30 mg kg^{-1}, *p.o.*), morphine (3 mg kg^{-1}, i.p.) or vehicle. The nociceptive response to formalin injection was recorded 1 h after administration of HLE or vehicle and 30 min after morphine administration.

3.2.9 Tolerance Studies

The mouse paw formalin test was used to ascertain whether, after chronic treatment, tolerance develops to the anti-nociceptive activity of HLE and morphine. The procedure used was similar to that described previously (Villetti *et al.*, 2003). 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 HLE 60 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-pre-treated group was treated with saline i.p.; the other two saline-pre-treated groups were treated with either HLE 30 mg kg^{-1}, *p.o.* or morphine 3 mg kg^{-1}, i.p.; the group pre-treated with HLE 60 mg kg^{-1} was treated with HLE 30 mg kg^{-1}, *p.o.* and the group pre-treated with morphine 6 mg kg^{-1} was treated with morphine 3 mg kg^{-1}, i.p. HLE and morphine were administered 60 and 30 min before formalin injection, respectively.

In a separate experiment, HLE was administered to animals chronically treated with morphine to establish whether morphine-induced tolerance cross-generalizes to the anti-nociceptive effect of HLE. This second experiment also investigated whether chronic concurrent treatment of mice with morphine and HLE will abolish the development of morphine tolerance. Two groups of animals (n=5) were treated once daily for 8 days with morphine 6 mg kg^{-1}, i.p. Another group (n=5) received both morphine 6 mg kg^{-1}, i.p. and HLE (60 mg kg^{-1}, *p.o.*, 30 min before the morphine) for 8 days. Three other groups of animals (n = 5) received chronic dosing of saline i.p. also for 8 days. On day 9, the two groups of animals treated with chronic morphine received either morphine (3 mg kg^{-1}, i.p., 30 min before formalin) or HLE (30 mg kg^{-1}, *p.o.*, 60 min before formalin) respectively, whereas the three saline-treated groups received either a similar administration of saline, morphine (3 mg kg^{-1}, i.p.) or HLE (30 mg kg^{-1}, *p.o.*). Additionally, the group that was chronically treated with both morphine and HLE also received morphine (3 mg kg^{-1}, i.p.) 30 min before intraplantar formalin injection.

3.2.10 Effect of HLE on Gastrointestinal Transit

Gastrointestinal tract functions (contraction, relaxation and secretion) are controlled by neurohumoral systems, which in turn are regulated by various receptor systems, such as cholinergic, adrenergic, serotonergic, opioidergic and cell surface channels (Kamm, 2000). Many drugs affect gastrointestinal transit by acting as agonists or antagonists at specific cellular receptors (Peddyreddy *et al.*, 2006). To assess, therefore, the possible receptors systems that

HLE interacts with, its effect on gastrointestinal transit was examined. This was carried out using the charcoal meal test as previously described by Al-Qarawi *et al.* (2003).

Mice were randomly divided into 6 groups of 5 mice each and starved for 16 h prior to the experiment but were allowed free access to water. The mice were then dosed orally as follows:

Group I Distilled water

Group II HLE 30 mg kg^{-1}

Group III HLE 100 mg kg^{-1}

Group IV HLE 300 mg kg^{-1}

Group V Loperamide 5 mg kg^{-1}

Group VI Bisacodyl 50 mg kg^{-1}

One hour after oral treatment above, the animals were given a freshly prepared standard charcoal test meal (10 ml kg^{-1}, 10 % activated charcoal suspension in 5 % gum acacia, *p.o.*). After 20 min the animals were sacrificed by cervical dislocation. The small intestine was isolated without being stretched and the distance traversed by the charcoal meal from the pylorus to the ileocaecal junction was measured. The length of the entire small intestine was also measured. The percent transit was then calculated as:

$$\%\,\text{Transit} = \frac{C}{SI} \times 100$$

where **C** is the distance (cm) covered by the charcoal and **SI** the total length of the small intestine (cm).

3.2.11 Effect of HLE on Isolated Guinea Pig Ileum

To examine, *in vitro,* the receptors on which HLE may act directly *in vivo* to attenuate pain, the isolated guinea pig ileum preparation was used. Since preliminary qualitative assessment of HLE on the guinea pig ileum preparation (in my laboratory) revealed largely nicotinic effects, this experiment examined it in details.

Guinea pigs were killed by a sharp blow to the head and after the laparatomy, one or two pieces (2 cm) of ileum were dissected from 2 cm above the ileocaecal junction and intraluminal

content flushed out with cooled aerated Tyrode solution. The tissue was mounted in an organ bath (10 ml) containing Tyrode solution (32 ºC, pH 7.4) between two stainless steel hooks and subjected to continuous air bubbling. The lower hook was fixed at the bottom of the organ bath and the upper one was connected to a Harvard Apparatus isotonic transducer (Model: 50-6360, Havard Apparatus Ltd., Edenbridge, Kent, England). The ileum contractions were recorded (Universal Harvard Oscillograph; Model: 50-8622 2-Channel Modular Universal Oscillograph, Havard Apparatus Ltd., Edenbridge, Kent, England) under 1 g resting tension, following 60 min for equilibrium period. During this period, the organ bath solution was refreshed every 15 min. The preparation was challenged with a dose of 2 M KCl which was predetermined to be high enough to elicit maximal contraction of the preparation. After the challenge, the action was terminated by washing the preparation with Tyrode solution. The procedure was repeated until the elicited contractions were uniform. The composition of Tyrode solution (mM) was: NaCl (136), KCl (5), $CaCl_2$ (2), $NaHCO_3$ (11.9), NaH_2PO_4 (0.26), $MgCl_2$ (0.98) and glucose (5.6).

The effect of different concentrations of nicotine and HLE was then tested on the ileum in the presence or absence of graded concentrations (3×10^{-6} – 3×10^{-5} M) of the neuronal nicotinic receptor antagonist, hexamethonium.

3.2.12 Analysis of Data

A sample size of five animals (n = 5) was used in all experiments. 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 unit as the area under the curve (AUC). To determine the percentage inhibition for each treatment, the following equation was used:

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

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 non-linear regression (three-parameter logistic) equation:

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

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 and Christopoulos, 2003). GraphPad Prism for Windows version 5 (GraphPad Software, San Diego, CA, USA) was used for all statistical analyses and ED_{50} determination. $P<0.05$ was considered statistically significant in all analysis.

3.3 RESULTS

3.3.1 Acetic Acid-Induced Writhing Assay

Acetic acid injected intraperitoneally produced writhing, exhibited as an exaggerated extension of the abdomen combined with the outstretching of the hind limbs, in control mice pre-treated with physiological saline. HLE and diclofenac significantly suppressed the time-course of acetic acid-induced writhes (Figure 3.1a, c). Two-way ANOVA (*treatment* × *time*) revealed a significant effect of drug treatments on the acetic acid-induced abdominal constrictions (HLE: $F_{3,91}=57.98$; $P<0.0001$ and diclofenac: $F_{3,87}=42.20$; $P<0.0001$).

HLE (30-300 mg kg^{-1}, *p.o* 1 h before) significantly reduced ($F_{3, 15}=19.71$, $P<0.0001$) the number of abdominal writhes over 20 min with maximal inhibition of 70.60±6.48% (Figure 3.1b) at dose of 300 mg kg^{-1}. Similarly, the NSAID diclofenac (10-100 mg kg^{-1}, i.p.30 min before) profoundly inhibited ($F_{3, 14}=10.70$, $P=0.0006$) the acetic acid-induced writhes by a maximum of 98.10±1.90% (Figure 3.1d).

Figure 3.2 shows the dose-response curves for the inhibition of acetic acid-induced abdominal writhes in mice. HLE exhibited an inverted U-shaped dose response relationship with ED_{50} values of approximately 53.21 and 220.80 mg kg^{-1}. Generally, HLE was less potent than diclofenac (ED_{50}=13.81±6.83 mg kg^{-1}).

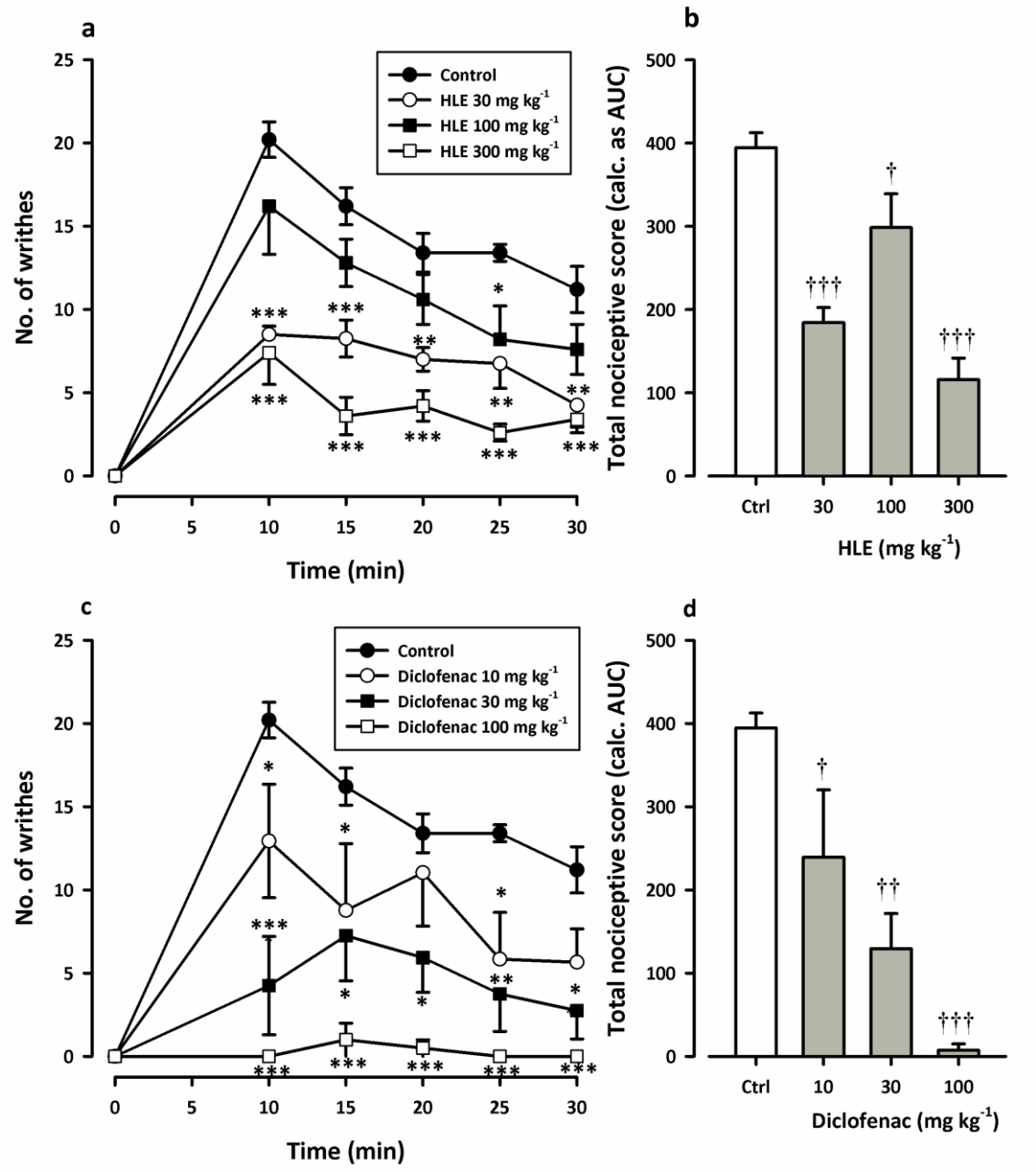

Figure 3.1 Effect of HLE (30-300 mg kg^{-1}, *p.o.*) and diclofenac (10-100 mg kg^{-1}, i.p.) on the time course curves of acetic acid-induced abdominal writhes(**a** and **c**) and the total nociceptive score (calculated as AUC) (**b** and **d**) in the mice. Data are expressed as mean ± S.E.M. (n=5). **P*<0.05; ***P*<0.01; ****P* <0.001 compared to vehicle-treated group (Two-way ANOVA followed by Bonferroni's *post hoc* test). †*P*<0.05; ††*P*<0.01; †††*P*<0.001 compared to vehicle-treated group (One-way ANOVA followed by Newman-Keuls *post hoc* test).

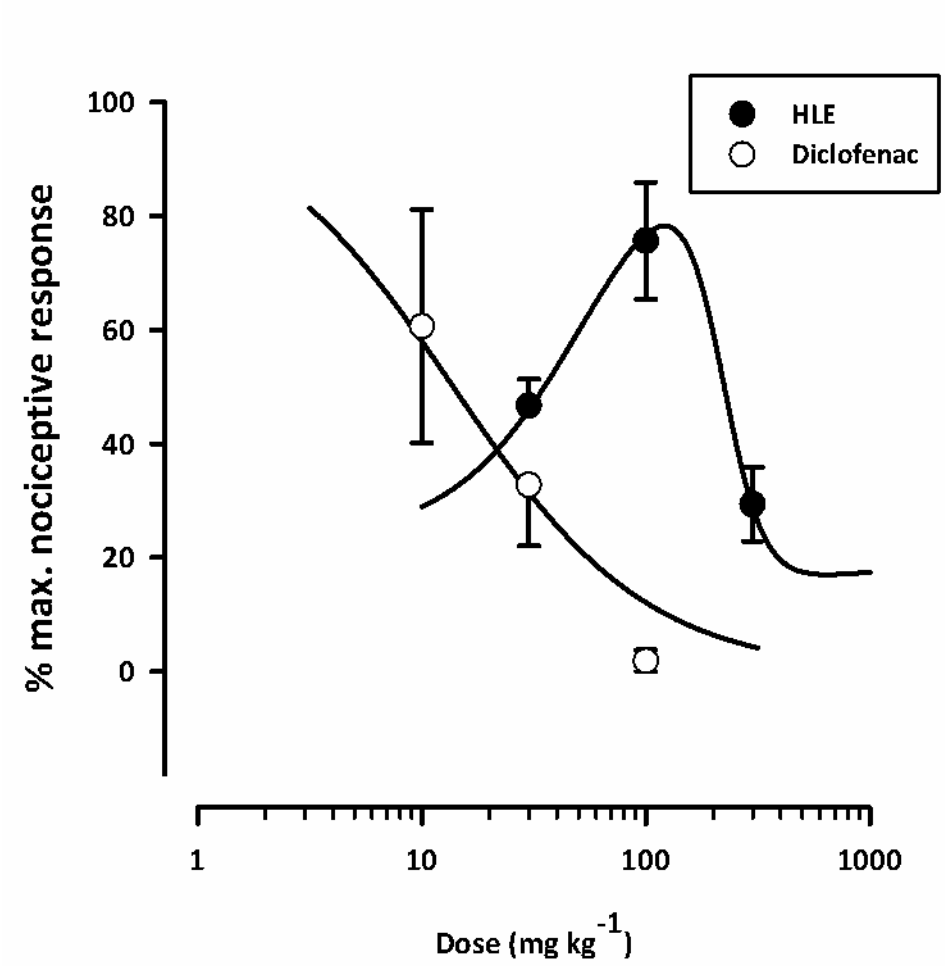

Figure 3.2 Dose response curves of HLE (30-300 mg kg^{-1}, *p.o.*) and diclofenac (10-100 mg kg^{-1}, i.p.) in the acetic acid-induced writhing test.

3.3.2 Tail Immersion Test

As shown by the time course curves in figure 3.3, all test drugs and extract caused an increase in the tail withdrawal latency, calculated as a percentage of the maximum possible effect (% MPE). Two-way ANOVA (*treatment* × *time*) revealed a significant effect of drug treatments on the tail withdrawal latencies (HLE: $F_{3,112}$=9.90; P<0.0001; diclofenac: $F_{3,112}$=26.47; P<0.0001 and morphine: $F_{3,112}$=25.09; P<0.0001; Fig. 3.3a, c, e).

HLE (30-300 mg kg^{-1}, *p.o.* 1 h before) increased the tail withdrawal latencies ($F_{3, 15}$=3.918, P=0.030; Fig. 3.3b) with a maximal effect at the dose of 30 mg kg^{-1}. Diclofenac (10–100 mg kg^{-1}, i.p.) elicited a significant anti-nociceptive activity by dose-dependently increasing the tail withdrawal latencies of animals pre-treated with it ($F_{3,16}$= 6.804, P=0.0036; Fig. 3.3d). Morphine (1-10 mg kg^{-1}, *i.p.*, fig 3.3f) also showed similar effects ($F_{3, 16}$ =9.43, P= 0.0008).

Dose-response curves for the anti-nociceptive effects of HLE, diclofenac and morphine in the tail immersion test are shown in Fig 3.4. HLE displayed a biphasic, U-shaped dose response relationship with approximate ED_{50} values of 56.86 and 156.68 mg kg^{-1}. By comparing the ED_{50} values from the curves, HLE was significantly less potent than diclofenac (ED_{50} 19.18±24.11 mg kg^{-1}) and morphine (ED_{50} 2.24±2.35 mg kg^{-1}).

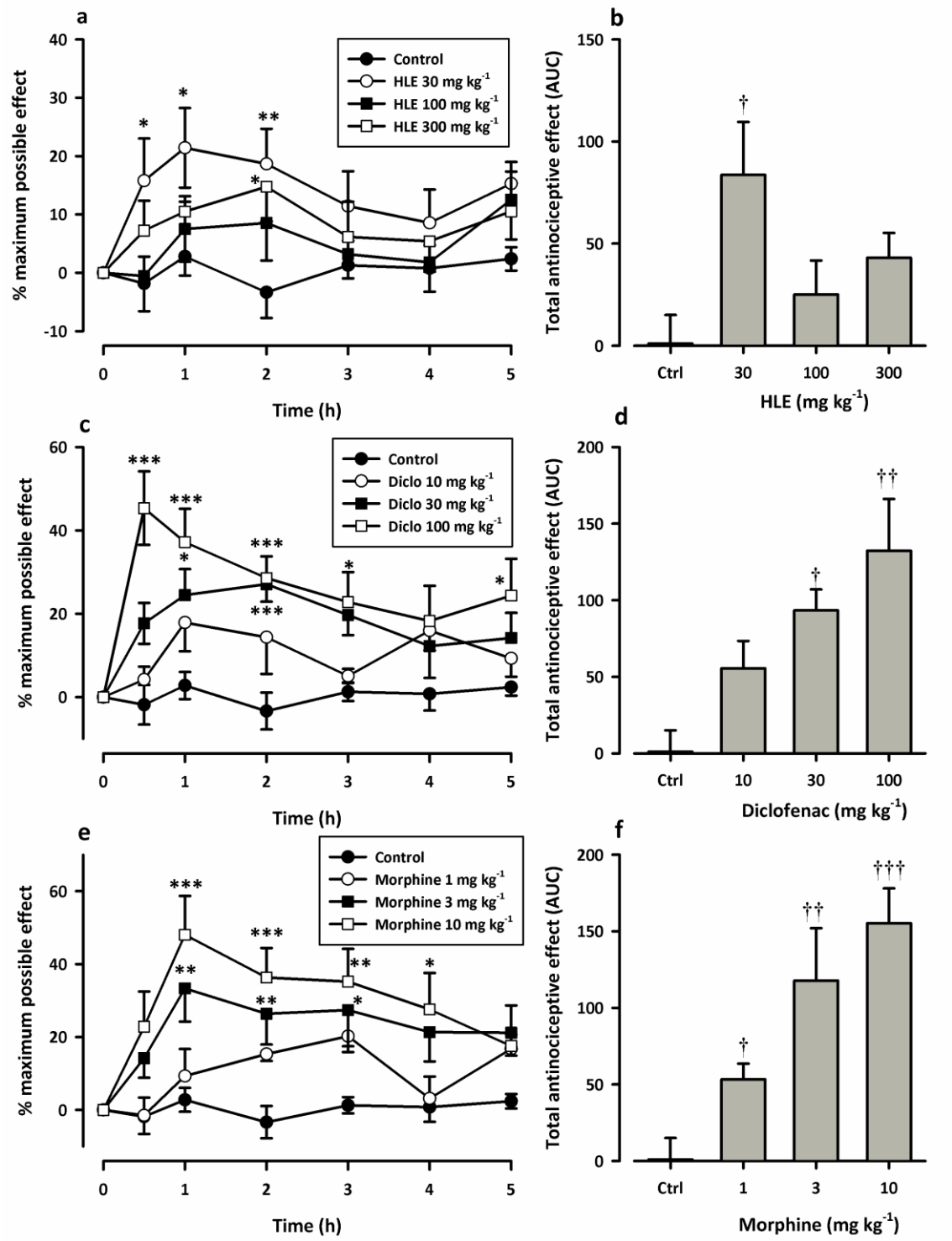

Figure 3.3 Effect of HLE (30-300 mg kg^{-1}, *p.o.*), diclofenac (Diclo; 10-100 mg kg^{-1}, i.p.) and morphine (1-10 mg kg^{-1}, i.p.) on the time course curve (**a**, **c**, **e**) of the tail immersion test and the AUC (**b**, **d**, **f**) in rats. Data are presented as mean±S.E.M. (n=5). **P*<0.05; ***P*<0.01; ****P*<0.001 compared to vehicle-treated group (Two-way ANOVA followed by Bonferroni's *post hoc* test). †P<0.05; ††P<0.01; †††P<0.001 compared to vehicle-treated group (One-way ANOVA followed by Newman-Keuls *post hoc* test).

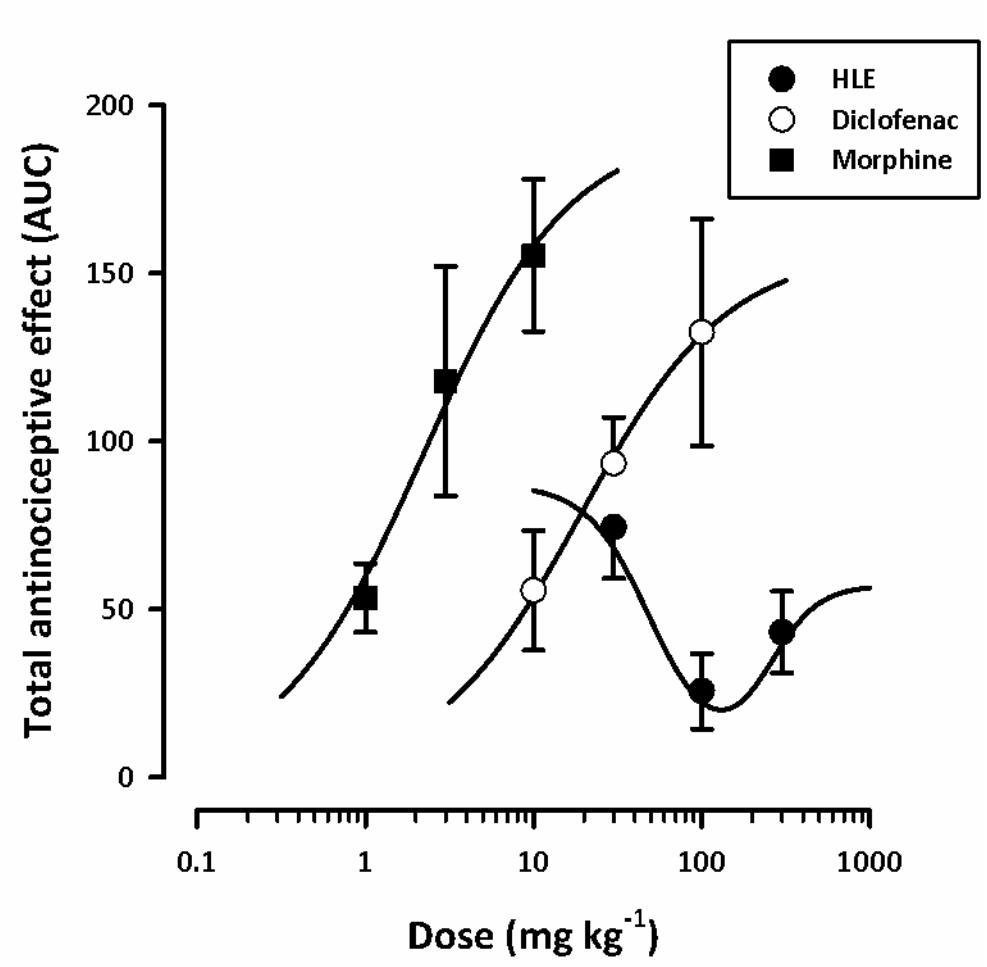

Figure 3.4 Dose response curves of HLE (30-300 mg kg^{-1}, *p.o.*), diclofenac (10-100 mg kg^{-1}, i.p.) and morphine (1-10 mg kg^{-1}, i.p.) in the tail immersion test.

3.3.3 Formalin Test

Injection of formalin (5 %, 10 µl) into the ventral surface of the right hind paw evoked a characteristic biphasic licking response in the mice as previously reported (Abbott *et al.*, 1995, Sakiyama *et al.*, 2008). This consisted of an initial intense response to pain beginning immediately after formalin injection and rapidly decaying within 10 min after formalin injection (first/neurogenic phase) and then followed by a slowly rising but longer-lasting response (second/inflammatory phase) from 10-60 min after formalin injection (Fig. 3.5) with maximum effect at approximately 20-30 min after formalin injection (Wang *et al.*, 1999, Hayashida *et al.*, 2003).

Figure 3.5 shows the effect of pre-treatment of HLE and morphine on formalin-induced pain in mice. All drug-treated groups displayed (Figure 3.5a, c) significant reduction in formalin-induced nociceptive behaviour when compared with the vehicle-treated group [(HLE: $F_{3,192}$=3.92; P<0.05; morphine: $F_{3,192}$=15.29; P<0.0001; Two-way ANOVA (*treatment* × *time*)]. Oral administration of HLE (30-300 mg kg^{-1}) 30 min before the injection of formalin inhibited both neurogenic ($F_{3,16}$=2.71; P=0.0797, fig 3.5b) and inflammatory ($F_{3,16}$=6.648; P=0.0051, fig 3.5b) phases of formalin-induced licking with maximal inhibition of 46.15±14.83 % and 49.14±12.74 % respectively. Morphine (1-10 mg kg^{-1}, i.p.), the positive analgesic control, similarly produced marked dose-related inhibition of both the neurogenic ($F_{3,16}$= 3.531, P=0.0390, fig 3.5d) and inflammatory ($F_{3,16}$= 15.54, P<0.0001, fig 3.5d) pain phases. Morphine reduced formalin-evoked nocifensive behaviours by 56.93±8.24 % in the early phase and 79.26±7.32 % in the late phase of the formalin test (Fig. 3.5d).

HLE (30-300 mg kg^{-1}) displayed an inverted U-shaped dose response relationship as shown in Figure 3.6. The ED_{50} values are approximately 35.80 and 310.46 mg kg^{-1} for the first phase and 37.15 and 123.03 mg kg^{-1} for the second phase. Comparison of ED_{50}s obtained by non-linear regression revealed that the extract was more potent in the second phase than the first. Likewise, morphine was four fold more potent in the second phase (ED_{50}=1.33±1.02 mg kg^{-1}) compared to the first phase (ED_{50}=4.72±2.45 mg kg^{-1}).

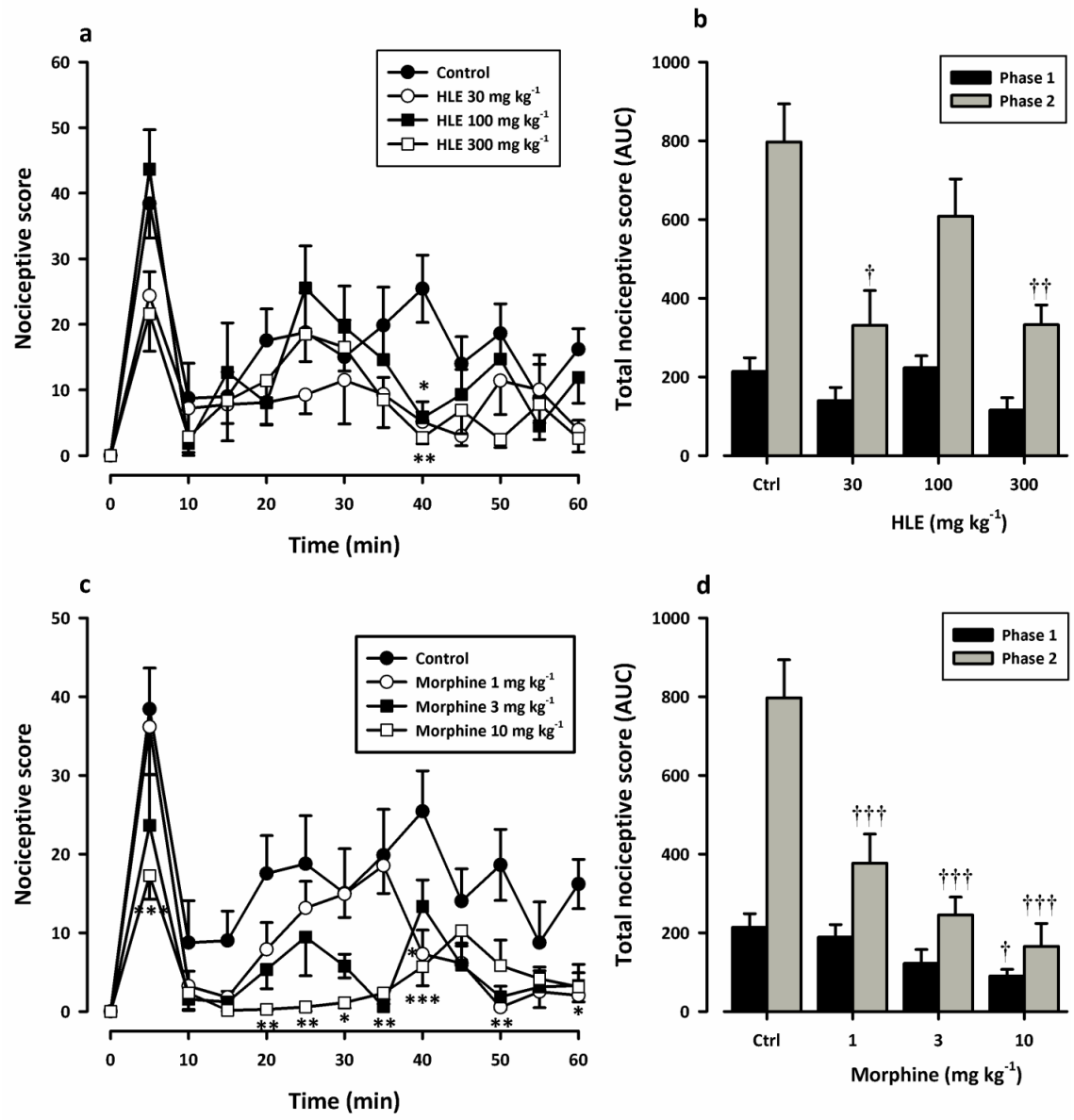

Figure 3.5 Effect of HLE (30-300 mg kg^{-1}, *p.o.*) and morphine (1-10 mg kg^{-1}, i.p.) on the time course curves (**a** and **c**) and the AUC (**b** and **d**) of formalin test in mice. Nociceptive scores are shown in 5 min time blocks up to 60 min post formalin injection for the time course curves. Values are means ± S.E.M. (n=5). **P*<0.05; ***P*<0.01; ****P*<0.001 compared to vehicle-treated group (Two-way ANOVA followed by Bonferroni's *post hoc* test). †P<0.05; ††P<0.01; †††P<0.001 compared to vehicle-treated group (One-way ANOVA followed by Newman-Keuls *post hoc* test).

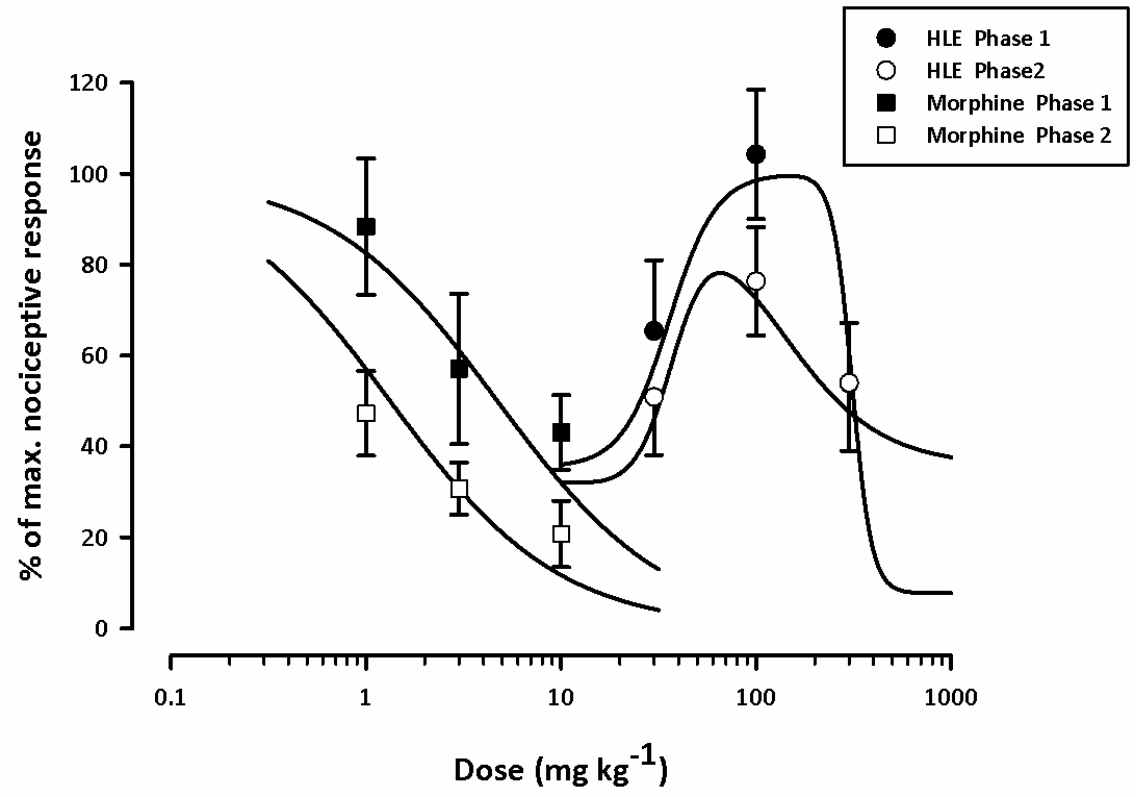

Figure 3.6 Dose response curves of HLE (30-300 mg kg^{-1}, *p.o.*) and morphine (1-10 mg kg^{-1}, i.p.) in the formalin test.

3.3.4 Glutamate-Induced Nociception

Figure 3.7a shows the effects of HLE and morphine on the time course curves of glutamate-induced nociception. HLE and morphine significantly suppressed the time-course of glutamate-induced licking [$F_{5, 66}$=8.03; P=0.0002, Two-way ANOVA (*treatment* × *time*); Figure 3.1a]

HLE (10-300 mg kg^{-1}, *p.o.* 1 h before i.pl. glutamate) produced significant inhibition of glutamate-induced pain ($F_{5, 22}$=8.00; P<0.0002, fig 3.7b) with a maximal inhibition of 53.41±8.25% at the dose of 100 mg kg^{-1}. Similarly, morphine (3 mg kg^{-1}, i.p. 30 min before) profoundly inhibited ($F_{5, 22}$=8.00; P=0.0002, fig 3.7b) the glutamate-evoked nocifensive behaviours by 92.22±4.66% (Figure 3.1d).

Figure 3.8 shows the dose-response curves for the inhibition of glutamate-induced pain by HLE in mice. HLE exhibited an inverted U-shaped dose-response relationship with ED_{50} values of 28.58 and 188.36 mg kg^{-1} from the non-linear regressional analysis.

3.3.5 Capsaicin-Induced Nociception

Capsaicin induced a clear nociceptive response exhibited by biting and licking of the injected paw. HLE and morphine (positive control) significantly suppressed the time-course of capsaicin-induced licking [$F_{5,48}$=8.63; P<0.0001, Two-way ANOVA (*treatment* × *time*), Figure 3.1c]

Oral administration of HLE (10-300 mg kg^{-1}) 60 min before the intraplantar injection of capsaicin produced dose-dependent attenuation of capsaicin-induced neurogenic pain ($F_{5,24}$=10.21; P<0.0001, fig 3.7c, d) with a significant inhibition of 59.49±7.89 % at the dose of 300 mg kg^{-1}. Similarly, morphine (3 mg kg^{-1}, i.p. 30 min before) profoundly inhibited ($F_{5,24}$=10.21; P<0.0001, fig 3.7c, d) the neurogenic pain by 84.07±4.88 % (Figure 3.1d).

Figure 3.8 shows the dose-response curves for the inhibition of capsaicin-induced neurogenic pain by HLE in mice. The ED_{50} from the non-linear regression was 90±116.76 mg kg^{-1}.

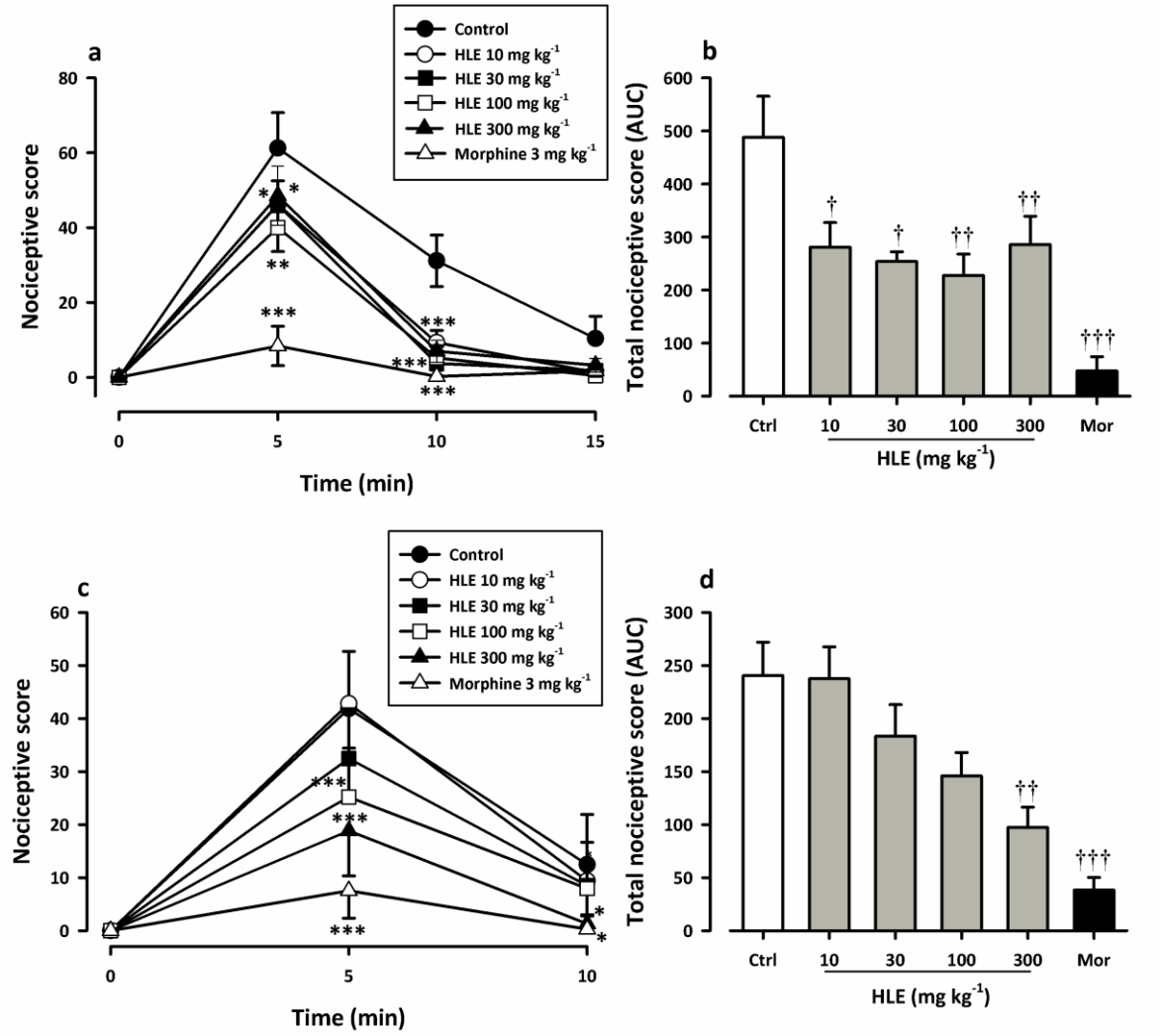

Figure 3.7 Effect of HLE (10-300 mg kg^{-1}, *p.o.*) and morphine (3 mg kg^{-1}, i.p.) on the time course curves and the AUC of glutamate- (**a** and **b**) and capsaicin- (**c** and **d**) induced nociception. Values are Means ± S.E.M. (n=5). **P*<0.05; ***P*<0.01; ****P*<0.001 compared to vehicle-treated group (Two-way ANOVA followed by Bonferroni's *post hoc* test). †*P*<0.05; ††*P*<0.01; †††*P*< 0.001 compared to vehicle-treated group (One-way ANOVA followed by Newman-Keuls *post hoc* test).

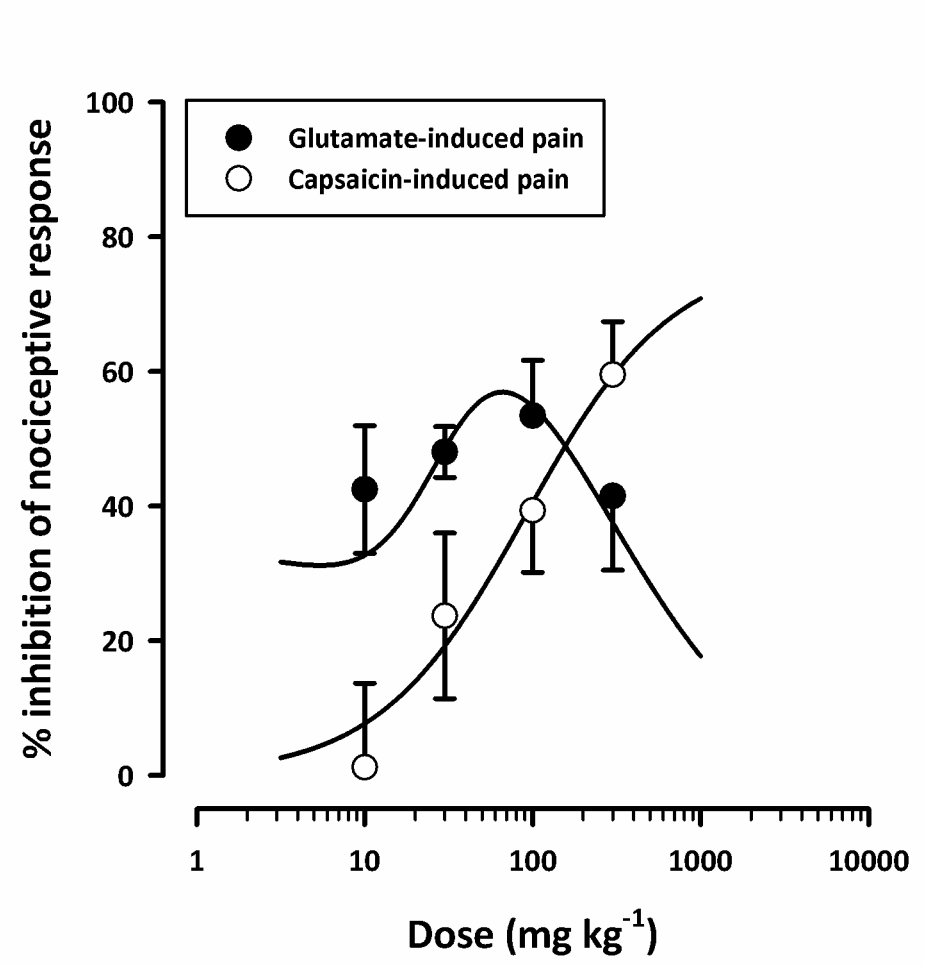

Figure 3.8 Dose response curves of HLE (10-300 mg kg^{-1}, *p.o.*) in the glutamate- and capsaicin-induced nociception.

3.3.6 Assessment of Possible Mechanism of Action of HLE

Figures 3.9-3.13 show the results of the effect of pre-treatment of mice with various antagonists on the anti-nociceptive activity of HLE and morphine. The results presented show that the pre-treatment of mice with naloxone (2 mg kg^{-1}, i.p.) partially reversed the anti-nociception by HLE (30 mg kg^{-1}, *p.o.*) in the inflammatory phase and had no effect on phase 1 (Figs. 3.9a & 3.12a). Naloxone, however, completely reversed the anti-nociception caused by morphine (3 mg kg^{-1} i.p.) against both phases of formalin-induced pain (Figs. 3.9b & 3.12b).

Previous treatment of the animals with theophylline (10 mg kg^{-1}, i.p.) abolished the anti-nociception caused by HLE (30 mg kg^{-1}, *p.o.*) in the second phase (Figs. 3.9c & 3.12a). Theophylline also completely reversed the anti-nociception caused by morphine (3 mg kg^{-1}, i.p.) in both phases of the formalin test (Figs. 3.9d & 3.12b).

Systemic pre-treatment of mice with L-NAME (10 mg kg^{-1}, i.p.) or glibenclamide (8 mg kg^{-1}, *p.o.*) did not prevent the anti-nociception caused by HLE (30 mg kg^{-1}, *p.o.*) in both phases of the formalin test (Figs. 3.9e, g & 3.12a). However, L-NAME and glibenclamide blocked morphine (3 mg kg^{-1} *i.p.*) anti-nociception in the first phase (Figs. 3.9f, h & 3.12b).

Yohimbine (3 mg kg^{-1}, *p.o.*) and nifedipine (10 mg kg^{-1}, *p.o.*) did not significantly inhibit the anti-nociception caused by either HLE (30 mg kg^{-1}, *p.o.*; Figs. 3.10a, c & 3.13a) or morphine (3 mg kg^{-1}, i.p.; Figs. 3.10b, d & 3.13b) in both phases of the formalin test.

Systemic pre-treatment of mice with atropine (5 mg kg^{-1}, i.p.) completely reversed the anti-nociception caused by HLE (30 mg kg^{-1}, *p.o.*) in both phases of the formalin test (Figs. 3.10e & 3.13a). Atropine abolished the anti-nociception caused by morphine (3 mg kg^{-1} *i.p.*), completely in the second phase but caused no significant change in the first phase (Figs. 3.10f & 3.13b).

Cyproheptadine (8 mg kg^{-1}, *p.o.*) did not inhibit the anti-nociception caused by either HLE (30 mg kg^{-1}, *p.o.*; Figs. 3.11a & 3.13a) or morphine (3 mg kg^{-1}, i.p.; Figs. 3.11b & 3.13b) in both phases of the formalin test.

Ondansetron (0.5 mg kg^{-1}, i.p.) did not significantly block anti-nociception caused by HLE (30 mg kg^{-1}, *p.o.*) in both phases of the formalin test (Figs. 3.11c & 3.13a). In contrast, ondansetron completely reversed the anti-nociception caused by morphine (3 mg kg^{-1} *i.p.*) in both phases (Figs. 3.11d & 3.13b).

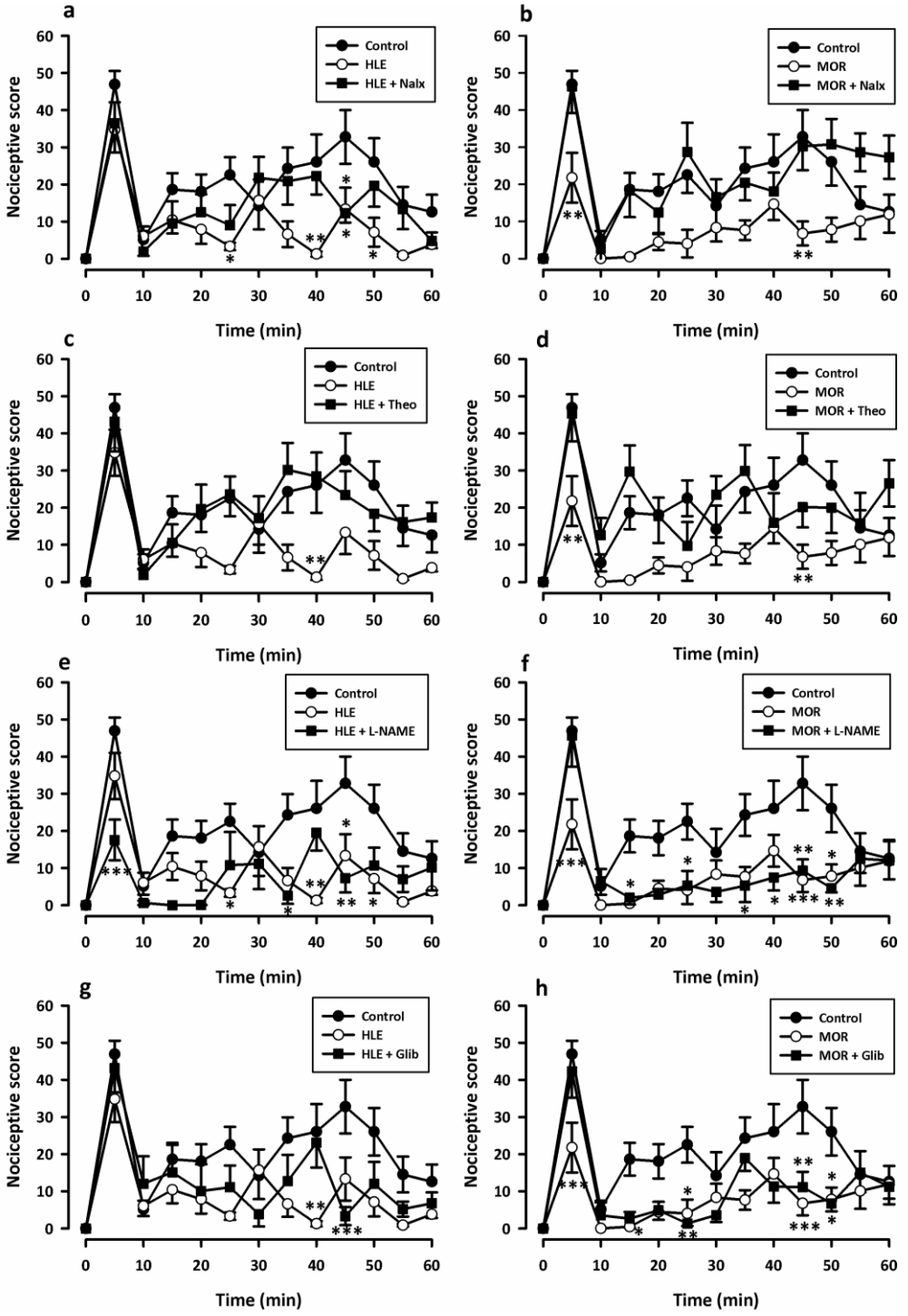

Figure 3.9 Effect of pre-treatment of mice with naloxone (Nalx; 2 mg kg^{-1}, i.p.), theophylline (Theo; 10 mg kg^{-1}, i.p), L-NAME (10 mg kg^{-1}, i.p) and glibenclamide (Glib; 8 mg kg^{-1}, *p.o.*) on the time course effects of HLE 30 mg kg^{-1} *p.o* (**a, c, e, g**) and morphine 3 mg kg^{-1} i.p. (**b, d, f, h**) in formalin-induced licking test in mice. Values are means ± S.E.M. (n=5). **P*<0.05; ***P*<0.01; ****P*<0.001 compared to vehicle-treated group (Two-way ANOVA followed by Bonferroni's *post hoc* test).

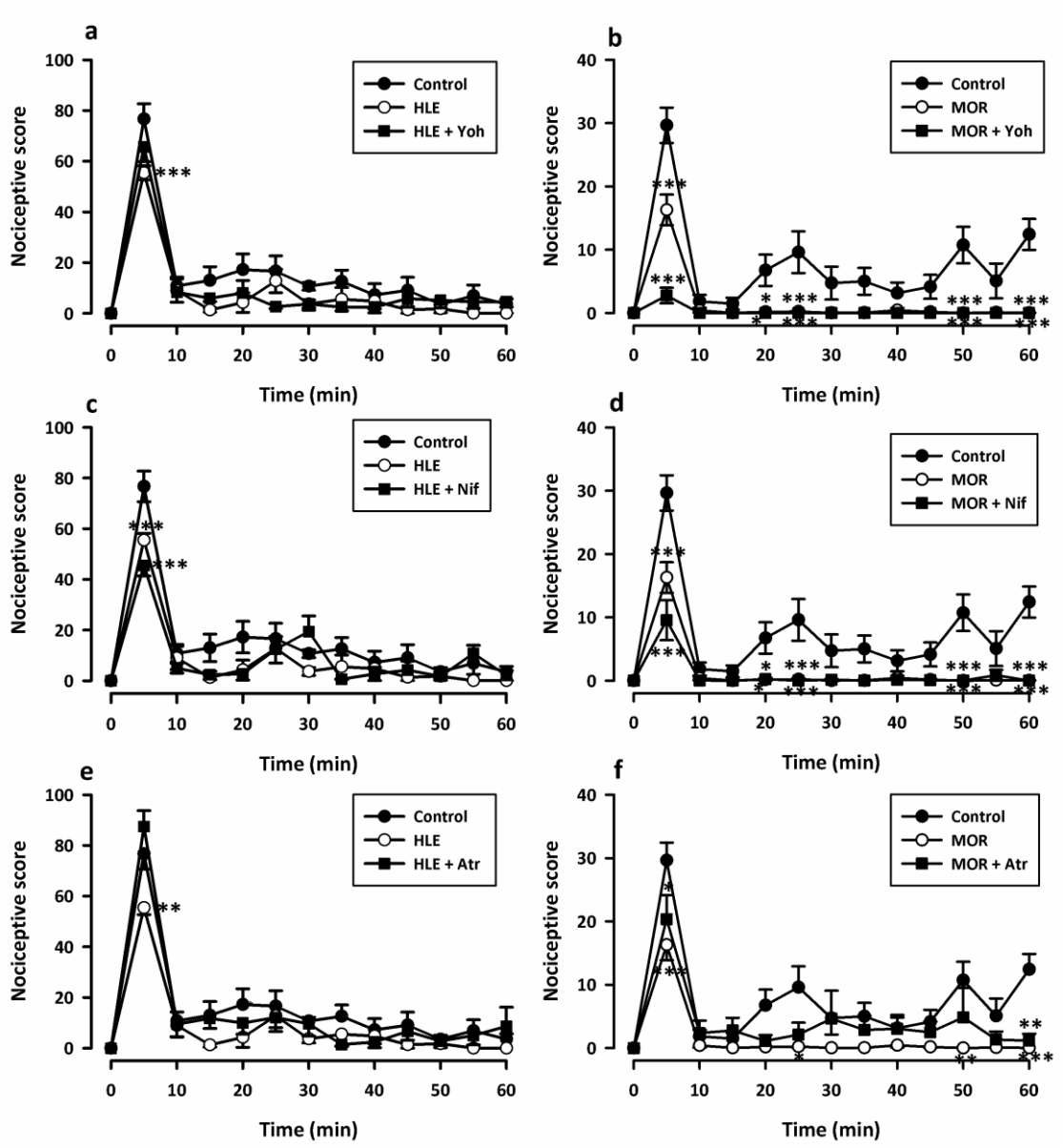

Figure 3.10 Effect of pre-treatment of mice with yohimbine (Yoh; 3 mg kg^{-1}, i.p), nifedipine (Nif; 10 mg kg^{-1}, i.p) and atropine (Atr; 5 mg kg^{-1}, *p.o.*) on the time course effects of HLE 30 mg kg^{-1} *p.o* (**a, c, e**) and morphine 3 mg kg^{-1} i.p. (**b, d, f**) in formalin-induced licking test in mice. Values are means ± S.E.M. (n=5). **P*<0.05; ***P*<0.01; ****P*<0.001 compared to vehicle-treated group (Two-way ANOVA followed by Bonferroni's *post hoc* test).

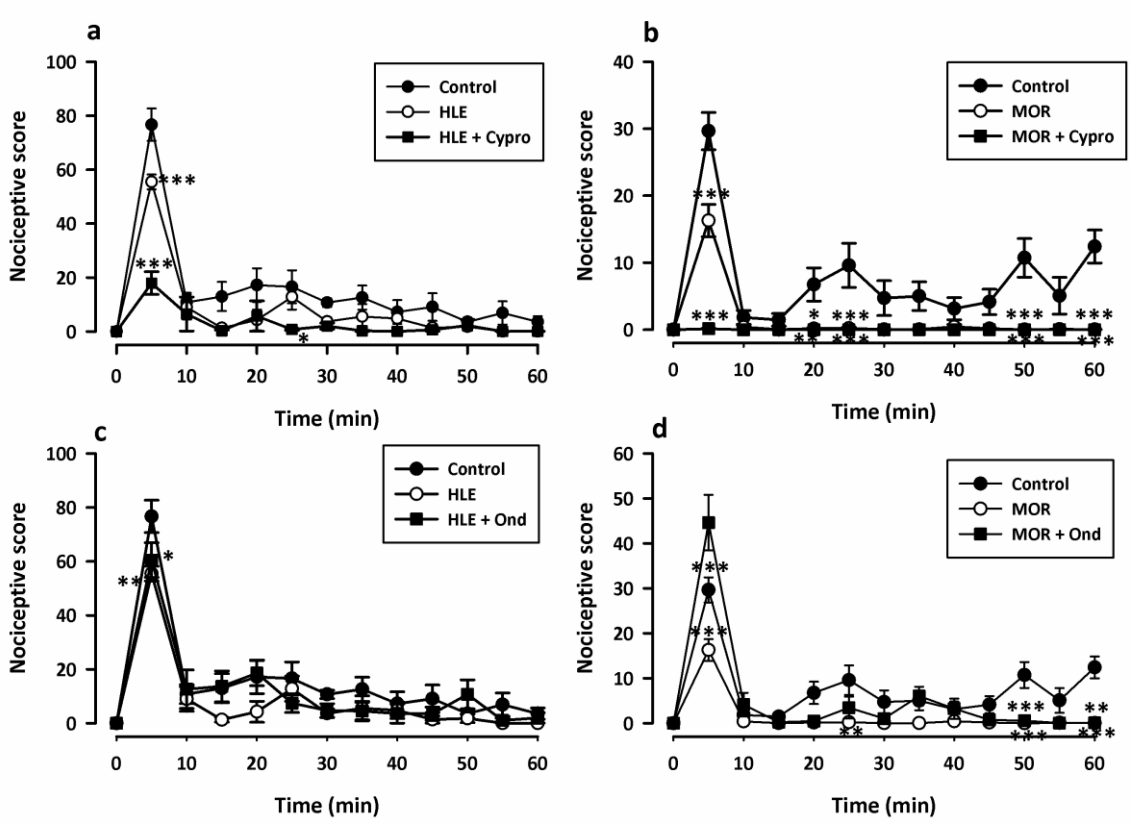

Figure 3.11 Effect of pre-treatment of mice with cyproheptadine (Cypro; 8 mg kg^{-1}, i.p) and ondansetron (Ond; 0.5 mg kg^{-1}, *p.o.*) on the time course effects of HLE 30 mg kg^{-1} *p.o* (**a, c**) and morphine 3 mg kg^{-1} i.p. (**b, d**) in formalin-induced licking test in mice. Values are means ± S.E.M. (n=5). **P*<0.05; ***P*<0.01; ****P*<0.001 compared to vehicle-treated group (Two-way ANOVA followed by Bonferroni's *post hoc* test).

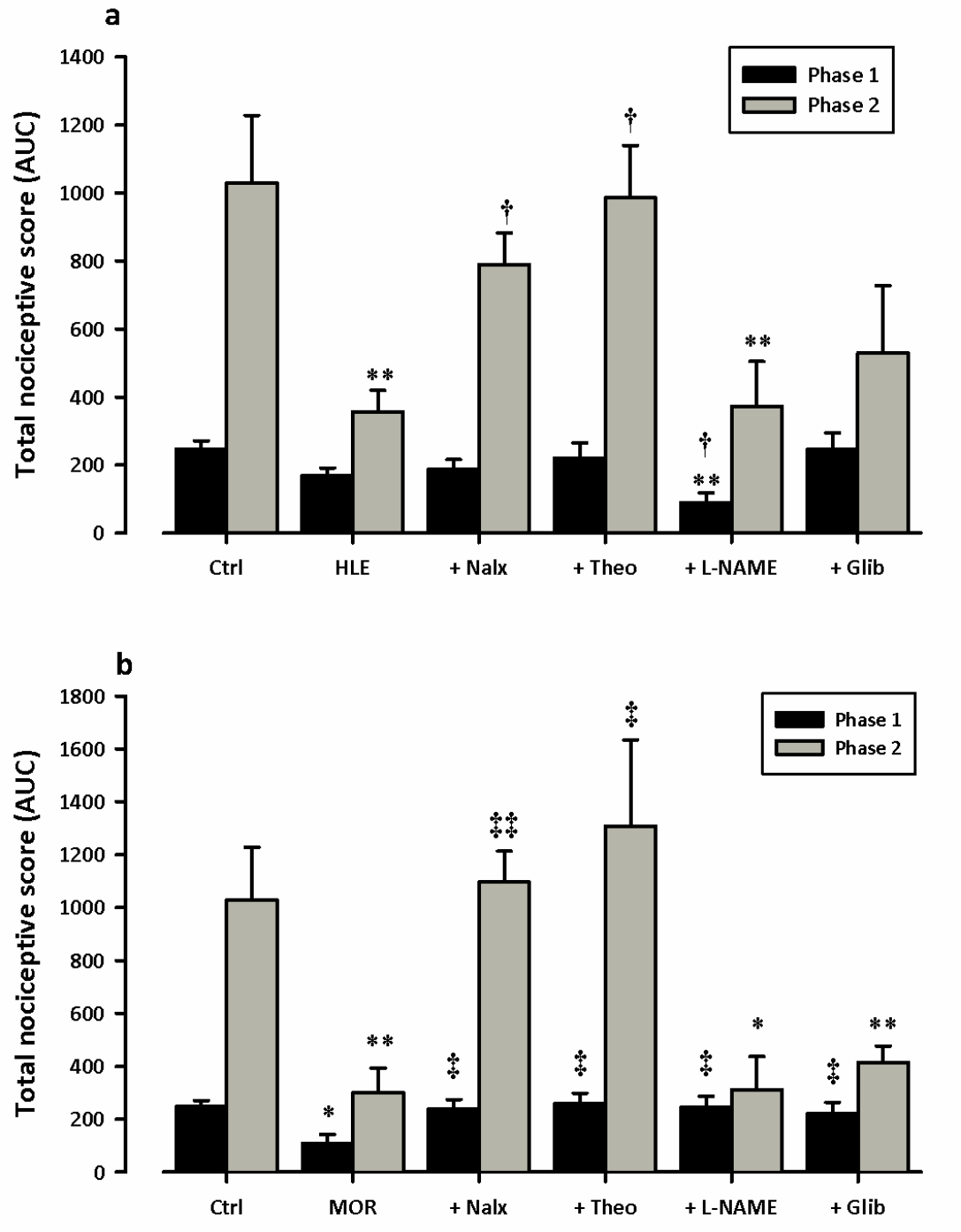

Figure 3.12 Effect of pre-treatment of mice with naloxone (Nalx; 2 mg kg^{-1}, i.p), theophylline (Theo; 10 mg kg^{-1}, i.p), L-NAME (10 mg kg^{-1}, i.p) and glibenclamide (Glib; 8 mg kg^{-1}, *p.o.*) on the anti-nociceptive effect of HLE 30 mg kg^{-1} *p.o* (**a**) and morphine 30 mg kg^{-1} i.p. (**b**) against the two phases of formalin-induced licking test in mice. Each column represents mean ± S.E.M (n = 5). **P*<0.05; ***P*<0.01 compared to respective vehicle-treated controls. †*P*<0.05 compared to HLE 30 mg kg^{-1}; ‡*P*<0.05; ⌗*P*<0.01 compared morphine 3 mg kg^{-1} (one-way ANOVA followed by Newman-Keuls *post hoc* test).

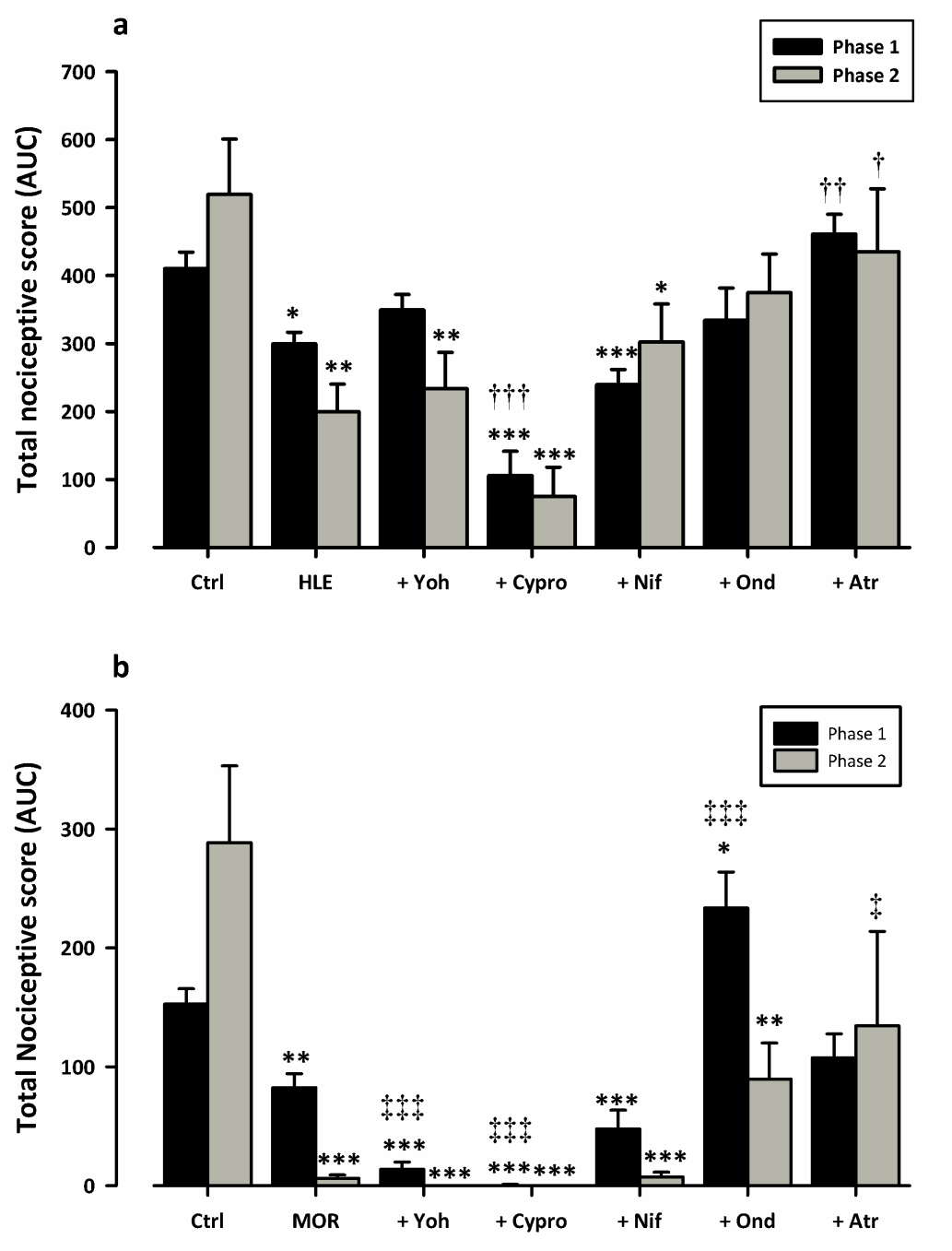

Figure 3.13 Effect of pre-treatment of mice with yohimbine (Yoh; 3 mg kg^{-1}, i.p.), cyproheptadine (Cypro; 8 mg kg^{-1}, i.p), nifedipine (Nif; 10 mg kg^{-1}, i.p.), ondansetron (Ond; 0.5 mg kg^{-1}, i.p.) and atropine (Atr; 5 mg kg^{-1}, *p.o.*) on the anti-nociceptive effect of HLE 30 mg kg^{-1} *p.o* (**a**) and morphine 3 mg kg^{-1} i.p. (**b**) against the two phases of formalin-induced licking test in mice. Each column represents mean ± S.E.M (n = 5). **P*<0.05; ***P*<0.01; ****P*<0.001 compared to respective vehicle-treated controls; †*P*<0.05; ††*P*<0.01; †††*P*<0.001 compared to HLE 30 mg kg^{-1}; ‡*P*<0.05; ‡‡‡*P*<0.001 compared to morphine 3 mg kg^{-1}(one-way ANOVA followed by Newman-Keuls *post hoc*).

3.3.7 Tolerance Studies

Figure 3.14 shows the results obtained from the tolerance studies. Morphine (3 mg kg^{-1}, i.p.) significantly attenuated nociceptive responses in both phases ($F_{3, 16}$= 27.87, P<0.0001 phase 1; $F_{3, 16}$= 5.41, P<0.01 phase 2) of formalin test in chronic vehicle-treated animals. However, the same dose of morphine administered at day 9 in animals chronically treated with morphine (6 mg kg^{-1}, i.p.) failed to show such effect indicating the development of tolerance (Fig. 3.14c, d). In contrast, oral administration of HLE (30 mg kg^{-1}) showed a comparable anti-nociceptive activity in mice given chronic treatment of either HLE (60 mg kg^{-1}, *p.o.*) or vehicle, indicating lack of tolerance development (Fig. 3.14a, b). Furthermore, HLE (30 mg kg^{-1}, *p.o.*) still demonstrated anti-nociceptive activity in mice chronically treated with morphine (6 mg kg^{-1}, i.p.), indicating that no cross-tolerance exists with morphine (Fig. 3.14). Additionally, the repeated administration of HLE (60 mg kg^{-1}, *p.o.*) 30 min prior to each morphine (3 mg kg^{-1}, i.p.) injection to mice during the 8-day protocol significantly attenuated the development of tolerance to morphine (Fig. 3.14c, d).

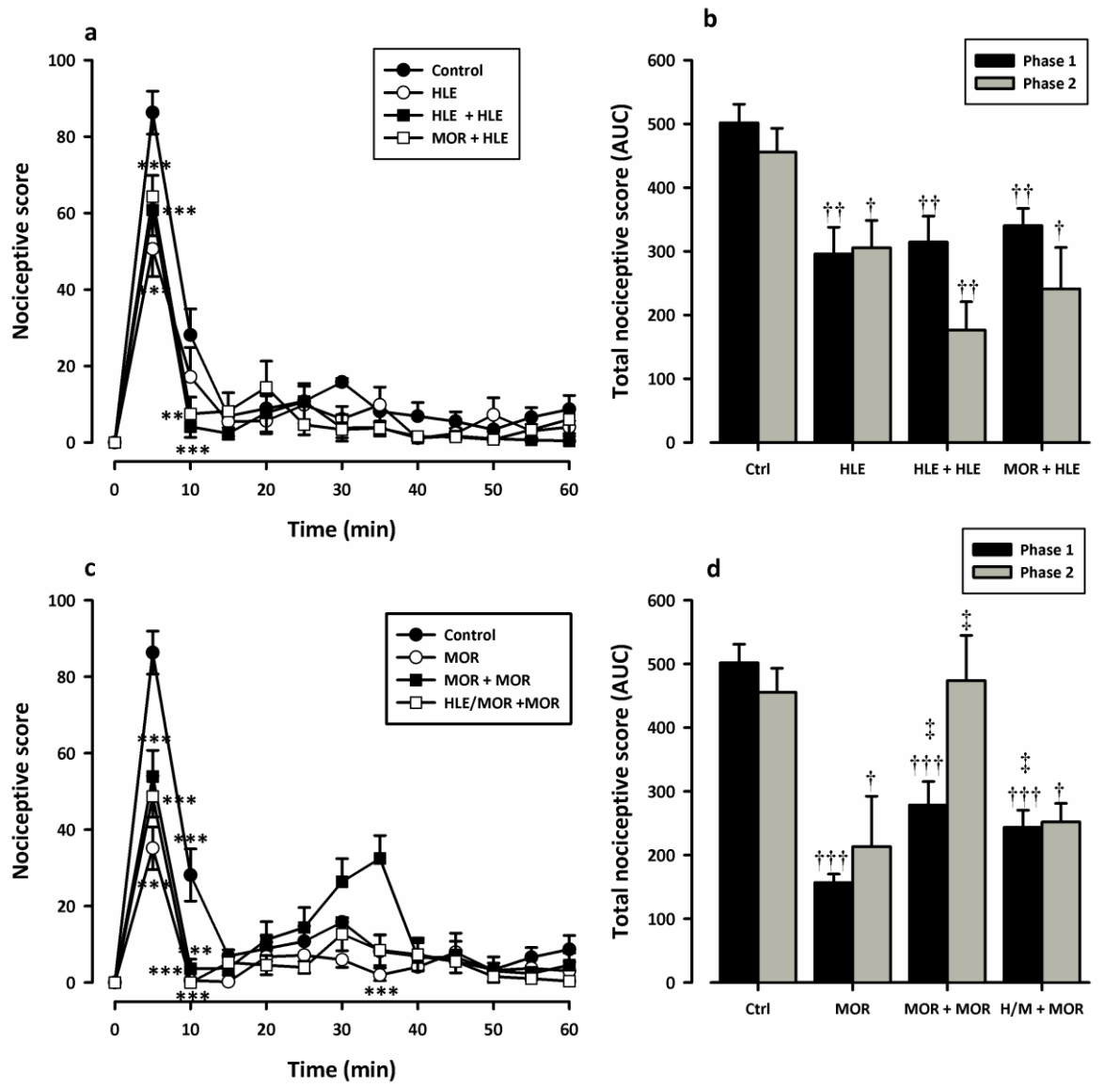

Figure 3.14 Effect of HLE (30 mg kg^{-1}, *p.o.*) and morphine (MOR; 3 mg kg^{-1}, i.p.) challenge on mice chronically treated with saline, HLE (60 mg kg^{-1}, *p.o.*), morphine (6 mg kg^{-1}, i.p.) or combination of HLE (60 mg kg^{-1}, *p.o.*) and morphine (6 mg kg^{-1}, i.p.) for 8 days on the time course (**a**, **c**) and total nociceptive score of both phases (**b**, **d**) of formalin-induced licking test in mice. Each value represents mean±S.E.M (n=5). **P*<0.05; ***P*<0.01; ****P*<0.001 compared to vehicle-treated group (Two-way ANOVA followed by Bonferroni's *post hoc* test). †*P*< 0.05; ††*P*<0.01; †††*P*<0.01 compared to respective vehicle-treated controls; ‡*P*<0.05 compared to morphine 3 mg kg^{-1} (one-way ANOVA followed by Newman-Keuls *post hoc*).

3.3.8 Effect of HLE on Gastrointestinal Transit

Figure 3.15 shows that HLE at 300 mg kg^{-1} produced a significant increase ($F_{5, 20}$=9.64, P<0.01) in gastrointestinal transit. The reference drug bisacodyl (50 mg kg^{-1}, *p.o.*) also increased (P<0.05) the gastrointestinal distance travelled by the charcoal plug in the mice. Loperamide (5 mg kg^{-1}, *p.o.*), however, significantly reduced (P<0.05) gastrointestinal transit in the mice.

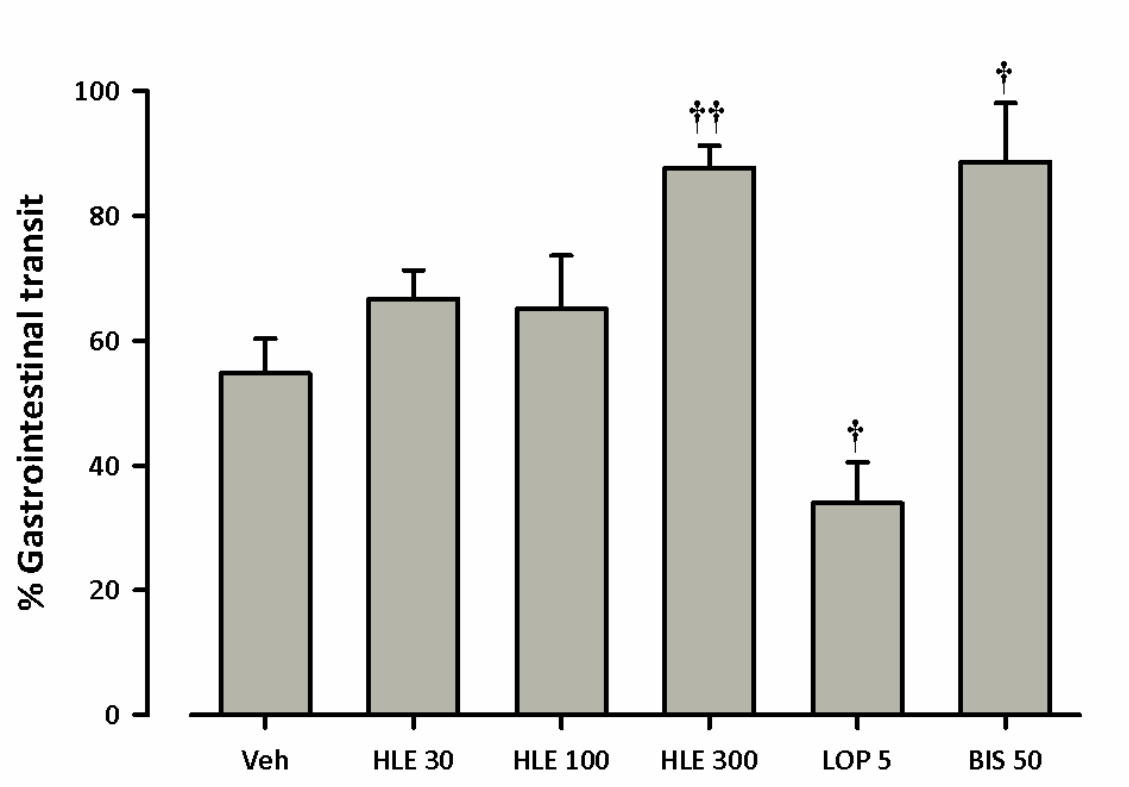

Figure 3.15 Effect of HLE (30-300 mg kg^{-1}, *p.o.*), bisacodyl (BIS; 50 mg kg^{-1}, *p.o.*) and loperamide (LOP; 5 mg kg^{-1}, *p.o.*) on gastrointestinal transit in mice. Each bar represents mean±S.E.M.(n=5). *P* values for group comparisons were obtained by one way ANOVA followed by Newman Keuls Multiple Comparison Test. †P<0.05; ††P<0.01 compared to the vehicle-treated group. Veh=vehicle

3.3.9 Effect of HLE on Isolated Guinea Pig Ileum

Hexamethonium caused a concentration-dependent rightward shift of the concentration-response curves of HLE ($F_{6, 14}$ =34.47, P<0.0001; Figure 3.16a) and nicotine ($F_{6, 14}$=55.5, P<0.0001; Figure 3.16b).

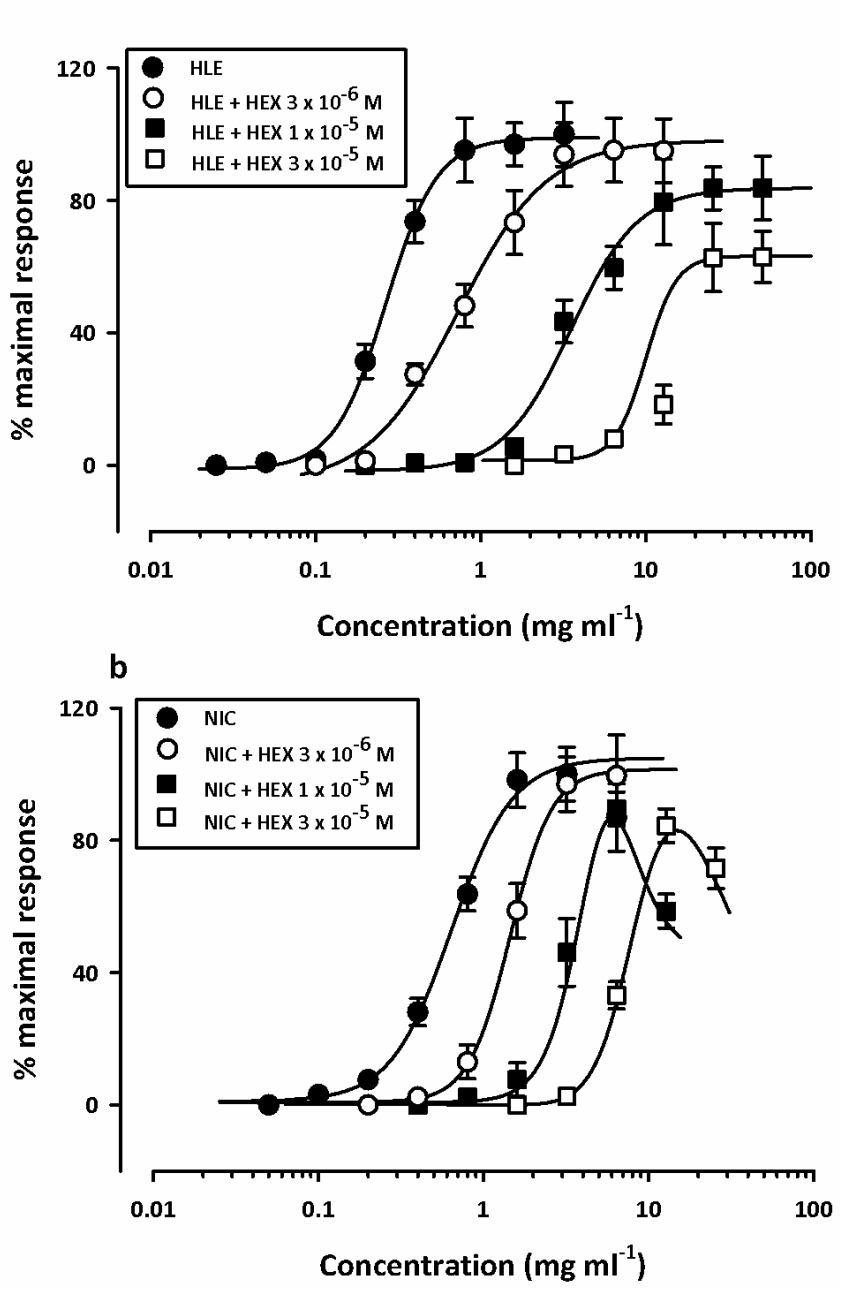

Figure 3.16 Effect of increasing concentrations of hexamethonium (3×10^{-6} – 3×10^{-5} M) on the concentration–response curves of HLE (**a**) and nicotine (**b**) on the isolated guinea-pig ileum preparation.Each point represents the mean of 2-3 experiments. HEX=hexamethonium, NIC=nicotine

3.4 DISCUSSION

This present study has demonstrated that the oral administration of the ethanolic extract of the aerial parts of *Hilleria latifolia* exerts significant anti-nociceptive activity against thermal- (tail immersion) as well as chemical- (acetic acid, glutamate, capsaicin and formalin) induced nociception in mice. This anti-nociceptive effect was partly or wholly reversed by the systemic administration of the naloxone, theophylline and atropine. Cyproheptadine, glibenclamide, ondansetron, yohimbine, nifedipine and L-NAME, however, did not significantly block the anti-nociceptive effect of the extract.

In order to obtain a full picture of the analgesic property of HLE, several behavioural animal models of nociception which differ with respect to stimulus quality, intensity and duration were employed. The nociceptive tests were selected such that both peripherally- and centrally-mediated effects were investigated; in all, the extract showed significant peripheral and central anti-nociceptive activity.

The abdominal writhing test, a peritoneovisceral inflammatory pain model, is a very sensitive and convenient method for screening the anti-nociceptive effect of compounds. Although in terms of specificity this method may have some insufficiencies (i.e. writhing may be suppressed by muscle relaxants and other non-analgesic drugs, leaving scope for the misinterpretation of results) (Le Bars *et al.*, 2001), it generally has a good correlation between the ED_{50} values obtained in animals using this test and analgesic doses administered in man (Collier *et al.*, 1968). The nociceptive effect induced in this model is easily prevented by non-steroidal anti-inflammatory drugs, as well as by opioids and analgesics with central actions (Dos Santos *et al.*, 2009). HLE significantly inhibited the abdominal constriction induced by acetic acid in mice. The actions of acetic acid are known to be the indirect cause of the release of nociceptive endogenous mediators such as bradykinin, substance P, serotonin, histamine, sympathomimetic amines, prostaglandins (PGE_2 and $PGF_{2\alpha}$) and pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and IL-8 (Deraedt *et al.*, 1980 ; Duarte *et al.*, 1988; Ribeiro *et al.*, 2000; Ikeda *et al.*, 2001; Dos Santos *et al.*, 2009). The inhibitory effects of HLE on inflammatory pain and abdominal constrictions produced after the administration of acetic acid in this study might therefore be said to be due to interference with the activation of nociceptors by one of these endogenous mediators or the suppression of sensitization of nociceptors to prostaglandins.

The anti-nociceptive effects of HLE, morphine and diclofenac were confirmed by the use of a thermal nociceptive stimulation (tail immersion in a 48°C water bath). The tail immersion test, a variant of the tail-flick pain model, is a sensitive and particularly useful test for demonstrating dose-related activity (Sewell and Spencer, 1976). The effectiveness of analgesics in this model is also highly correlated with relief of human pain (Grumbach, 1966); it has minimal liability to cause tissue damage and enables measurements of nociceptive sensitivity to be made on individual animals at frequent intervals and thus, allows the time course of a drug's effects to be obtained. HLE significantly attenuated thermal nociception in rats in this model. The tail immersion test gives a response that is believed to be a spinally-mediated reflex (Chapman *et al.*, 1985) but the mechanism of response could also involve higher neural structures (Jensen and Yaksh, 1986). It is therefore suggested that the anti-nociceptive effect of HLE, morphine and diclofenac in this model shows they act either partly or wholly by spinally-mediated central mechanisms.

The formalin-induced paw pain, an *in vivo* model of acute pain, has been well established as a valid model for analgesic study. It is undoubtedly the most predictive of acute pain (Dubuisson and Dennis, 1977) and very popular for the rapid and easy screening of pharmacological targets in drug evaluation (Saddi and Abbott, 2000; Vissers *et al.*, 2003). HLE showed significant anti-nociceptive effects in this model. The formalin test produces a distinct biphasic nociceptive response. A first phase (neurogenic pain) occurs within seconds of formalin injection as a direct consequence of chemical stimulation of peripherally localized TRPA-1 containing nociceptors (McNamara *et al.*, 2007). A second, later phase (inflammatory pain), occurs as a result of ongoing activity in primary afferents, the release of inflammatory mediators and a glutamate-dependent sensitization of nociceptive spinal neurones (Hunskaar and Hole, 1987; Tjolsen *et al.*, 1992; Munro, 2009). Therefore, the test can be used to clarify the possible mechanism of anti-nociceptive effect of a proposed analgesic (Tjolsen *et al.*, 1992). Centrally acting drugs, such as opioids, inhibit both phases equally (Shibata *et al.*, 1989); however, corticosteroids and most NSAIDs inhibit only the late phase (Hunskaar and Hole, 1987). NSAIDs, 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). HLE inhibited both phases of the formalin test but more effectively the second than the first. This implies that HLE is effective against both neurogenic and inflammatory pain. The inhibitory property on the second phase of formalin also suggests an anti-inflammatory action of HLE.

Hilleria latifolia extract, given orally, elicited a dose-dependent anti-nociceptive effect on the capsaicin-induced neurogenic paw licking response. Capsaicin (8-methyl-N-vanillyl-6-nonenamide), the pungent algesic substance obtained from hot red chilli peppers, is regarded a valuable pharmacological tool for studying a subset of mammalian primary sensory C-fibres and Aδ afferent neurons including polymodal nociceptors and warm thermoceptors (Jancso, 1992). It has been proposed that the capsaicin-induced nociception occurs as a result of the activation of the capsaicin (vanilloid) receptor, TRPV1, a ligand-gated non-selective cation channel present in primary sensory neurons (Caterina *et al.*, 1997; Tominaga *et al.*, 1998; Szallasi and Blumberg, 1999). The effect of HLE in this pain model suggests that HLE is effective against neurogenic pain in mice and its action may be due to an interaction with the capsaicin receptor (TPRV1).

Results obtained in this study also show that oral administration of HLE produced a significant inhibition of the nociceptive response caused by intraplantar injection of glutamate into the mouse hind paw. Glutamate, acting through a variety of receptors, plays an important role in peripheral and central pain transmission (Neugebauer, 2007). Its intraplantar injection evokes thermal and mechanical hyperalgesia (Carlton *et al.*, 1995; Jackson *et al.*, 1995; Carlton *et al.*, 1998) as well as spontaneous lifting and licking behaviours in mice (Beirith *et al.*, 2002). The nociceptive response induced by glutamate appears to involve peripheral, spinal and supraspinal sites of action and is largely mediated by both NMDA and non-NMDA receptors as well as by the release of nitric oxide or by some nitric oxide-related substance (Beirith *et al.*, 2002). The inhibitory capabilities of HLE by interference with the nociceptive response induced by glutamate, demonstrates, at least in part, an interaction of HLE with the glutamatergic system.

The intraplantar injection of formalin, capsaicin or glutamate is known to release endogenous chemical mediators such as neuropeptides, excitatory neurotransmitters, PGE_2, NO and kinins in periphery and spinal cord that contribute to the nociceptive process (Tjolsen *et al.*, 1992; Sakurada *et al.*, 1996; Santos and Calixto, 1997; Beirith *et al.*, 2002; Sakurada *et al.*, 2003). Therefore, the suppression of the capsaicin-, formalin- and glutamate-induced licking response caused by treatment with HLE, are complementary indications that the anti-nociceptive action of this extract could be associated with its ability to inhibit the production or action of some of the mediators.

With the exception of the capsaicin test (Fig. 3.8), HLE showed a typical biphasic dose-response pattern in all nociceptive tests used. The biochemical mechanism underlying this

pharmacological inversion is not yet clear and further studies will be necessary to establish it. Nonetheless, a possible explanation for the bell-shaped dose-response curves would be the presence of an inhibitory component, activated by high concentrations of HLE. Such a component may be a pro-nociceptive compound since a crude extract like HLE comprises several chemical constituents which could be acting via contradicting mechanisms. In the absence of such a compound, however, the inversion can be a consequence of the interaction of HLE with two functionally antagonistic receptors (Szabadi, 1977; Rovati and Nicosia, 1994) or; possibly, with single receptor coupled to two different signal transduction mechanisms with opposite effects (Pliska, 1994; Accomazzo *et al.*, 2002). The bell-shape dose response can also be explained by assuming HLE acts via a single receptor molecule with two different binding sites, which are responsible for evoking and inhibiting the receptor response (Jarv *et al.*, 1993; Jarv, 1994; Jarv *et al.*, 1995).

In an attempt to further characterise some of the mechanisms through which HLE exerts its activity, the anti-nociceptive effect of HLE was assessed in the presence of various antagonists of some mediators of the nociceptive pathway including naloxone, theophylline, L-NAME, glibenclamide, atropine, ondansetron, cyproheptadine, yohimbine and nifedipine. The formalin test was selected for this study, since it is more specific and with its biphasic control of pain, reflects different pathological processes and allows the elucidation of the possible mechanism involved in analgesia (Tjølsen *et al.*, 1992). The effect of HLE on receptors on isolated guinea pig ileum as well as on gastrointestinal transit was also assessed to determine other possible mechanisms of action of HLE.

Naloxone, a non-selective opioid antagonist partly reversed the anti-nociceptive effect of HLE in the second phase of the formalin test suggesting an opioidergic involvement in the anti-nociceptive effect of HLE.

The anti-nociceptive effects of HLE and morphine were reversed by theophylline implicating the involvement of adenosinergic pathway in their actions. Adenosine acts at several P1 receptors (A_1, A_{2A}, A_{2B}, and A_3) all of which are coupled to G proteins (Fredholm *et al.*, 2001). Activation of A_1 receptors produces anti-nociception while activation of A_2 and A_3 receptors produce pronociception (Sawynok, 1998). Since theophylline blocks adenosine A_1 and A_2 receptors, the anti-nociceptive effects may be due to the activation of A_1 receptors and/or an increment in endogenous adenosine either centrally or peripherally. The involvement of

adenosine in morphine anti-nociception is well known (Sawynok, 1998; Ribeiro *et al.*, 2002; Sawynok and Liu, 2003) and has been confirmed in this study.

The reversal of the anti-nociceptive effects of HLE by the non-selective muscarinic receptor antagonist, atropine implicates the muscarinic cholinergic system in the actions of the extract. It is well reported that the activation of muscarinic receptors (M_1-M_4) induces anti-nociception in various pain paradigms including thermal, inflammatory and neuropathic pain (Honda *et al.*, 2000; Jones and Dunlop, 2007; Sanders and Maze, 2007). Therefore, the anti-nociceptive effects of HLE may be due to activation of one or more of the muscarinic receptors.

In addition to the muscarinic cholinergic system mediating nociception, the nicotinic cholinergic system has also, in recent studies, been shown to be strongly involved in nociceptive processing, offering a number of tractable targets for the development of potent analgesics (Jones and Dunlop, 2007). In order to establish the possible involvement of the nicotinic cholinergic system in the anti-nociceptive activity of HLE, effect of HLE on neuronal nicotinic acetylcholine receptors (N_N AChR) was studied on the isolated guinea pig ileum preparation. HLE caused a concentration-dependent contraction of the ileum. This contraction was consistently blocked by hexamethonium, a N_N AChR antagonist, indicating that at least one constituent compound of HLE is a N_N AChR agonist. The N_N AChR agonist effect of HLE implicates the involvement of the nicotinic cholinergic system in the anti-nociceptive effects of HLE because many neuronal nicotinic agonists are anti-nociceptive (Damaj *et al.*, 1994; Decker *et al.*, 1998; Decker, 1999; Lawand *et al.*, 1999). The observed involvement of opioidergic and adenosinergic pathways in the anti-nociceptive effect of HLE is also consistent with the anti-nociceptive mechanisms of nicotine and other nicotinic agonists (Davenport *et al.*, 1990; Homayounfar *et al.*, 2005).

Serotonin is known to modulate nociceptive responses (Millan, 2002). However, its involvement in pain processing is complex, as 5-HT can inhibit or facilitate nociceptive transmission, depending on the nature of nociceptive stimuli applied. The activities of 5-HT receptors are complex and sometimes even contrasting, and can depend on the receptor subtype being activated, the relative contributions of pre- versus postsynaptic actions of receptors, the nociceptive paradigm in terms of quality and intensity of stimulus and the dose related effect, which can be pro- or anti- nociceptive, of agonists and antagonists of serotonergic receptor subtypes. Several pieces of evidence point to 5-HT_2 and 5-HT_3 receptors modulating nociceptive transmission, as activation of these receptors in the spinal cord

produces anti-nociception in the formalin test and other models (Jurgensen *et al.*, 2005, Lopes *et al.*, 2009). This study shows that neither 5-HT_{2A} nor 5-HT_3 receptors may play a dominant role in the anti-nociceptive effect of HLE.

Since yohimbine (a selective α_2-adrenoceptor antagonist), nifedipine (L-type VGCC blocker), glibenclamide (ATP-sensitive K^+ channel blocker) and L-NAME (a selective inhibitor of NO biosynthesis) did not block the anti-nociceptive effect of the HLE, it is suggested that extract's anti-nociceptive mechanism may not significantly involve α_2-adrenoceptors, L-type Voltage-gated calcium channels, ATP-sensitive K^+ channels or the nitric oxide pathway. However, other pharmacological and biochemical studies are further needed to characterize the precise mechanism(s) responsible for the anti-nociceptive action of HLE.

Gastrointestinal (GI) motility is controlled by neurohumoral systems through various receptor systems, such as cholinergic, adrenergic, serotonergic, opioidergic and cell surface channels (Kamm, 2000). Many drugs affect GI transit by acting as agonists or antagonists at specific cellular receptors (Peddyreddy *et al.*, 2006). Generally, stimulation of cholinergic and serotonergic (mainly 5-HT_3) systems accelerate GI transit while the adrenergic and opioidergic systems suppress it (Peddyreddy *et al.*, 2006). Since the evaluation of a drug's effect on GI transit can reveal information about the receptor systems it interacts with *in vivo*, the effect of HLE on GI transit was therefore examined in this study. HLE accelerated GI transit and reflects either direct stimulation of gastrointestinal muscle, activation of excitatory neural pathways or inhibition of inhibitory pathways resulting in increased tone and motor activity of the intestines. The results go in to further support, as discussed above, the involvement of the cholinergic system in the actions of HLE.

Opioids, such as morphine, are clinically used primarily as analgesics. But the development of tolerance that necessitates dose escalation regardless of disease progression, greatly limit their effectiveness and usage (Tang *et al.*, 2006; Chen *et al.*, 2008). Since the present study revealed the possible involvement of the opioidergic pathway in the anti-nociceptive activity of HLE, a study was carried out to determine if repeated administration of HLE could lead to the development of analgesic tolerance. The study further determines if morphine tolerance could cross-generalise to HLE and whether concurrent administration of morphine and HLE could abolish morphine tolerance. The results suggest that, unlike morphine, HLE does not induce tolerance to its anti-nociceptive effect after chronic administration in the formalin test. The lack of tolerance development after HLE treatment cannot be attributed to the use of a low dose,

because HLE was chronically administered at the dose maximally active in the late phase of formalin-induced pain. In view of the opioidergic activity of HLE without tolerance development, it is suggested that HLE might have components acting via pathways that interfere with the mechanism of opioid tolerance development. This view is supported by the fact that HLE attenuated the development of morphine tolerance in this current study. Another interesting finding is that morphine tolerance dose not cross-generalize to HLE, implying the extract can be used to treat pain in opioid-tolerant individuals.

3.5 CONCLUSION

The current study demonstrates that the ethanolic extract of the aerial parts of *Hilleria latifolia* has peripheral and central anti-nociceptive activity in chemical and thermal models of nociception without inducing tolerance. The anti-nociceptive effect involves an interaction with adenosinergic, nicotinic cholinergic, muscarinic cholinergic and opioidergic pathways.

Chapter 4

ANTI-INFLAMMATORY AND ANTIOXIDANT EFFECTS

4.1 INTRODUCTION

Inflammatory diseases continue to be one of the main health problems of the world's population. Although several agents are known to treat these types of disorders, prolonged use is undesirable due to the severe side effects. Consequently, there is a need to develop new anti-inflammatory agents with minimum side effects (Vane and Botting, 1995; Olajide *et al*., 1999; Gepdiremen *et al*., 2006).

Inflammatory response implicates macrophages and neutrophils, which secrete a number of mediators (eicosanoids, oxidants, cytokines and lytic enzymes) responsible for initiation, progression and persistence of acute or chronic state of inflammation (Lefkowitz *et al*., 1999). Polymorphonuclear leukocytes recruited to inflammatory sites are particularly adept to generating and releasing free radicals and reactive oxygen species (ROS) which, in excessive amounts, can injure cellular biomolecules such as nucleic acids, proteins, carbohydrates and lipids, causing cellular and tissue damage, which in turn augments the state of inflammation (Cochrane, 1991; Crockett-Torabi and Ward, 1996; Choi and Hwang, 2003). Inhibition of the release of deleterious mediators like the oxidants (free radicals and ROS) is, therefore, a potential strategy to control inflammation and is implicated in the mechanism of action of a number of anti-inflammatory drugs including diclofenac, indomethacin and tolfenamic acid (Kirkova *et al*., 1992; Maffei Facino *et al*., 1993; Takayama *et al*., 1994; Burguete *et al*., 2007; Rojo *et al*., 2009).

The ethanolic extract of the aerial parts of *H. latifolia*, a plant used in Ghana for various painful inflammatory conditions, was assessed for its effect on both acute and chronic inflammation. The carrageenan-induced oedema in the 7-day old chick (Roach and Sufka, 2003) and the adjuvant-induced arthritis in the rat (Pearson, 1956) were used as models to study the effect of the extract on acute and chronic inflammation respectively. Since antioxidant activity may be one of the mechanisms of anti-inflammatory action of the plant extract, this was also investigated in various *in vitro* models namely total phenol assay, antioxidant capacity by phosphomolybdate method, reducing power assay, 2, 2-diphenyl-1-picrylhydrazyl hydrate (DPPH) scavenging assay and lipid peroxidation assay.

4.2 METHODS

4.2.1 Animals

Cockerels (*Gallus gallus;* strain Shaver 579, Akropong Farms, Kumasi, Ghana) were obtained one day post-hatch and housed in stainless steel cages (34 cm × 57cm × 40 cm) at a population density of 12–13 chicks per cage. Food (Chick Mash, GAFCO, Tema, Ghana) and water were available *ad libitum* through 1-qt gravity-fed feeders and waterers. Overhead incandescent illumination was provided with room temperature at 29°C. Chicks were tested at 7 days of age.

Sprague-Dawley rats of both sexes (120–215 g) were also 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 cm × 47 cm × 18 cm) with soft wood shavings as bedding, fed with normal commercial pellet diet (GAFCO, Tema, Ghana), given water *ad libitum*.

All procedures and techniques used in these studies were in accordance with accepted principles for laboratory animal use and care (EU directive of 1986: 86/609/EEC). All protocols used were approved by the Departmental Ethics Committee.

4.2.2 Drugs and Chemicals

The following drugs and chemicals were used: diclofenac sodium (KRKA, Slovenia); dexamethasone sodium phosphate (Pharm-Inter, Brussels, Belgium); methotrexate sodium (Dabur Pharma, New Delhi, India); carrageenan sodium salt (Sigma-Aldrich Inc., St. Louis, MO, USA); ascorbic acid, ammonium molybdate, disodium hydrogen phosphate (Na_2HPO_4), sodium dihydrogen phosphate (NaH_2PO_4), ferric chloride, linoleic acid, methanol, ethanol, *n*-propyl gallate, potassium ferricyanide, sodium bicarbonate, sodium carbonate, sulphuric acid, tannic acid (BDH, Poole, England); thiobarbituric acid (TBA), 2, 2-diphenyl-1-picrylhydrazyl (DPPH), trichloroacetic acid (TCA), Folin-Ciocalteau reagent (Sigma-Aldrich Inc., St. Louis, MO, USA).

4.2.3 Anti-Inflammatory Activity

4.2.3.1 Carrageenan-induced Oedema in Chicks

The anti-inflammatory activity of HLE was assessed using the carrageenan-induced foot oedema in the chick (Roach and Sufka, 2003).

Oedema was induced by injecting carrageenan (10 µl of a 2 % solution in saline) into the sub-plantar tissue of the right footpads of the chicks. Foot volume was measured before carrageenan injection and at hourly intervals over 5 hours by water displacement (Fereidoni *et al.*, 2000). The foot oedema was quantified by measuring the percentage change in foot volume over the various time points.

Two sets of experiments were performed to assess the anti-inflammatory activity of HLE. The first was to study the effect of the drugs administered 1 h post carrageenan injection. The second examined the effects of the drugs given pre-emptively (30 min for i.p. route and 1 h for oral route) before the carrageenan challenge.

Groups of chicks (n=6) were treated with HLE suspended in 2 % tragacanth (10-300 mg kg^{-1}, *p.o.*). Dexamethasone (0.3-3.0 mg kg^{-1}, i.p) and diclofenac (10-100 mg kg^{-1}, i.p.) were used as standards. Drug vehicle (2 % tragacanth, 10 ml kg^{-1}, *p.o.*) served as a control. Drug effects were assessed by comparing either the peak oedema response attained during the 5 h or the total oedema (monitored as area under the time course curves) response developed during the period

4.2.3.2 Adjuvant – Induced Arthritis

Adjuvant arthritis was induced as previously described by Pearson (1956) and 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 4 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).

4.2.3.2.1 Drug Treatment

Drugs were administered on day 9 with the onset of arthritis. The initial weight of rats was taken on day 0 after grouping and subsequent weights were taken every 4 days. Drug doses were adjusted accordingly as the weights change.

Rats (n=6) were randomly selected into the following study groups for the experiment:

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-XII Treated with *H. latifolia* extract (10, 30, 100, 300 mg kg^{-1}, *p.o.*) from day 9 and administered every day.

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 drugs were administered.

4.2.3.2.2 Clinical Parameters

4.2.3.2.2.1 HIND LIMB SWELLING

Hind paw and ankle joint thicknesses were measured as previously described (Hoffmann *et al.*, 1997) using digital callipers. The paw and joint thicknesses were measured for both the ipsilateral and the contralateral hind limbs before intraplantar injection of CFA (day 0) and every other day up to the 28th day. The oedema component of inflammation was quantified by measuring the difference in paw/joint thickness between day 0 and the various time points.

Photographs of the affected hind limbs were taken on day 29.

4.2.3.2.2.2 BODY WEIGHT

The loss of body weight typical of this model of arthritis was monitored with a balance.

4.2.3.2.2.3 ARTHRITIC INDEX

On day 29, the severity of arthritis was graded according to the extent of erythema and oedema of the periarticular tissues (Kinne *et al.*, 1995; Zhao *et al.*, 2000), using a scale of 0-4 per limb, where 0 = no inflammation, 1 = unequivocal inflammation of 1 joint of the paw, 2 = unequivocal inflammation of at least 2 joints of the paw, or moderate inflammation of 1 joint; 3 = severe inflammation of 1 or more joints; and 4 = maximum inflammation of 1 or more joints in the paw. The scores for each paw were then added to get the total arthritis score (maximum possible score 16 per animal). The total score for each rat was designated as the arthritic index. The arthritis score of each rat on day 0 was determined to be 0.

The hind paw thickness, ankle joint thickness and arthritic index were used as the measurement parameters of inflammation and arthritis.

4.2.3.2.3 Radiographic Assessment

Measurement of paw or joint swelling only gives an indication of oedematous changes in these regions; however, the most obvious damage takes place in the tibiotarsals joint itself. Hence radiographs of the hind limbs are important. Radiographs of the hind limbs were obtained from selected animals on day 29. The animals were anaesthetized by intraperitoneal injection with pentobarbitone. 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. Using the radiographs, the severity of bone and joint destruction was scored for each hind limb according to the method described by Hoffmann *et al.* (1997). Briefly, radiographic scoring was performed by assessing soft tissue swelling, periosteal new bone formation, joint space narrowing, periarticular osteoporosis, and bone destruction on a scale of 0 (normal) to 3 (maximum) per hind limb. The maximum radiographic score was 6 per animal. The radiological score for normal control rats was determined to be 0. The radiological score was termed the radiological index.

4.2.3.2.4 Haematological Parameters

Plasma antioxidant enzymes which may contribute to the anti-inflammatory activity of HLE were assessed.

4.2.3.2.4.1 ANTIOXIDANT ENZYME ASSAYS

4.2.3.2.4.1.1 Superoxide Dismutase (SOD)

Superoxide dismutase (SOD) enzyme activity was determined in plasma according to the method of Misra and Fridovich (1972) with modifications for a microassay. Ten microlitres of diluted plasma samples were added to a 96-well plate followed by three hundred microlitres of carbonate buffer (0.05 M, pH 10.2, 0.1 mM EDTA). Ten microlitres of 9.6 mM epinephrine (0.3 mM final concentration in well) was then added to mixture in each well. The assay plate was then immediately inserted into a BioTek absorbance microplate reader (Model: ELx808, BioTek Instruments, Inc., Vermont, USA) and the change in absorbance at 490 nm was recorded for 4 min. The enzyme activity was expressed as the amount of enzyme that inhibits the oxidation of epinephrine by 50 % which is equal to 1 unit.

4.2.3.2.4.1.2 Catalase (CAT)

Catalase was measured by tracing the degradation of H_2O_2 spectrophotometrically, according to the method of Aebi (1974). Three millitres of reaction solution made up of 10 mM H_2O_2 in 50 mM potassium phosphate buffer (pH 7.0) was added to a quartz cuvette and pre-warmed at 25 °C for 5 min. Twenty microlitres of diluted plasma sample was added to the cuvette and the change in absorbance at 240 nm was monitored between 30 and 210 s in a Cecil UV/VIS spectrophotometer (Model: CE 2041, Milton, England). The enzyme activity was expressed in Units per millilitre (U/ml) using a micromolar extinction coefficient for H_2O_2 of 0.0436 cm^2 $\mu mole^{-1}$. One unit is defined as that amount of enzyme that causing the decomposition of one micromole of hydrogen peroxide per minute at 25 °C and pH 7.0.

4.2.4 Anti-Oxidant Activity

4.2.4.1 Total Phenol Assay

The antioxidant property of many plant sources is due to phenolic compounds in them. Therefore, total soluble phenolics present in HLE was determined by a colorimetric method using the Folin-Ciocalteau reagent (Slinkard and Singleton, 1977).

Various concentrations (0.1, 0.3, 1.0, 3.0 mg ml^{-1}) of HLE (1 ml) were mixed with the Folin-Ciocalteau phenol reagent (1 ml; diluted 1:10 with distilled water). One millilitre of 2 % w/v Na_2CO_3 was added to this mixture and incubated at room temperature (28 ^{0}C) for 2 hours. Samples were centrifuged at 650 *g* for 10 minutes and the absorbance of the supernatant read at 760 nm using a Cecil UV/VIS spectrophotometer (Model: CE 2041, Milton, England).

Distilled water (1 ml) was added to 1 ml Folin-Ciocalteau reagent processed in the same way as the test drugs and used as blank. All measurements were done in triplicates.

Tannic acid (0.01, 0.03, 0.1, 0.3 mg ml^{-1}) was used as a reference standard. The total phenolics were expressed as milligrams per millilitre of tannic acid equivalents (TAEs).

4.2.4.2 Antioxidant capacity by Phosphomolybdate method

The total antioxidant capacity assay was carried out as described (Prieto *et al.*, 1999).

Various aqueous concentrations of HLE (0.1, 0.3, 1, 3 mg ml^{-1}) were prepared and centrifuged at 650 *g*. An aliquot of 0.3 ml was combined with 3 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate) in an Eppendorf tube. The tubes were capped and incubated in a water bath at 95 °C for 90 min. After cooling to room temperature, the absorbance of the aqueous solution of each was measured at 695 nm using Cecil UV/VIS spectrophotometer (Model: CE 2041, Milton, England) against a blank.

The blank solution was obtained by adding 0.3 ml of reagent solution to 3 ml volume of water and incubated under the same conditions as the rest of the samples. All measurements were done in triplicates

Ascorbic acid (0.01, 0.03, 0.1, 0.3 mg ml^{-1}) was used as the standard and the total antioxidant capacity is expressed as milligrams per millilitre of ascorbic acid equivalents (AAE).

4.2.4.3 Reducing power

The antioxidant potential of the extract was determined using the Fe^{3+} reduction (reducing power) test described by Oyaizu (1986).

One millilitre of various concentrations of HLE (0.03, 0.1, 0.3, 1 mg ml^{-1}) were mixed with 2.5 ml of 0.2 M sodium phosphate buffer (pH 6.6) and 2.5 ml 1 % potassium ferricyanide solution [$K_3Fe(CN)_6$] in a test tube. The mixtures were incubated at 50 °C for 20 min. Trichloroacetic acid (10%; 2.5 ml) was then added to the mixtures and centrifuged at 650 *g* for 10 minutes.

Two and a half millilitres of the supernatant was mixed with 2.5 ml distilled water and 0.5 ml of 0.1% ferric chloride solution and the absorbance read at 700 nm using Cecil UV/VIS spectrophotometer (Model: CE 2041, Milton, England)

For blank samples, 1 ml of distilled water was added to 2.5 ml sodium phosphate buffer and 2.5 ml potassium ferricyanide and the mixture processed as above.

Propyl gallate (0.001, 0.003, 0.01, 0.03 mg ml^{-1}) was used as standard antioxidant. All measurements were done in triplicates.

4.2.4.4 Scavenging of 2, 2-diphenyl-1-picrylhydrazyl (DPPH)

The free radical scavenging activity was determined using method as described by Blois (1958).

Methanolic solutions of test drugs were prepared, centrifuged at 650 *g* for 10 minutes and the supernatant used for the experiment.

One millilitre methanolic solution of HLE (0.1, 0.3 and 1.0 mg ml^{-1}) was added to 3 ml methanolic solution of DPPH (20 mg/l) in a test tube. The reaction mixtures were kept at 25°C for 1 h. The absorbance of the residual DPPH was then determined at 517 nm using a Cecil UV/VIS spectrophotometer (Model: CE 2041, Milton, England).

The scavenging action of HLE (0.1, 0.3 and 1.0 mg ml^{-1}) in methanol was compared to the standard, *n*-propyl gallate (0.001, 0.03, 0.01mg ml^{-1} in methanol). All measurements were in triplicates.

One millilitre methanol (99.8%) added to 3.0 ml DPPH solution, incubated at 25°C for 1 h served as control and methanol (99.8%) was used as blank.

The percentage scavenging was calculated from the results as follows:

$$\%\,\text{Scavenging} = \frac{\text{ABSORBANCE}_{\text{CONTROL}} - \text{ABSORBANCE}_{\text{TEST}}}{\text{ABSORBANCE}_{\text{CONTROL}}} \times 100$$

The concentration of extracts required to cause a 50% scavenging was also calculated (IC_{50}).

4.2.4.5 Linoleic acid autoxidation

The inhibitory effect of HLE on lipid peroxidation was determined by the linoleic acid autoxidation method as carried out by Inatani *et al.*(1983) using thiobarbituric acid.

A mixture of 2 ml of HLE (0.1, 0.3, 1, 3 mg ml^{-1}) in 99.5 % ethanol, 2.05 ml of 2.51 % (v/v) linoleic acid in 99.5 % ethanol, 4 ml of phosphate buffer (0.05 M, pH 7.0), and 1.95 ml of water was placed in a vial with a screw cap and placed in an oven at 40 °C in the dark for 7 days.

To 2 ml of the mixture, which was prepared above, was added 2 ml of 20 % trichloroacetic acid aq. solution and 1 ml of 0.67 % thiobarbituric acid aq. solution. This mixture was placed in a boiling water bath for 10 min and, after cooling, was centrifuged at 650 *g* for 10 min. The absorbance of the supernatant was measured at 532 nm using a Cecil UV/VIS spectrophotometer (Model: CE 2041, Milton, England). Ethanol was used as blank throughout the experiment whilst *n*-propyl gallate (0.003, 0.01, 0.03, 0.1 mg ml^{-1}) was used as standard. Each test was carried out in duplicates.

Percentage inhibition of linoleic acid autoxidation by the test drugs was assessed, comparing the absorbance of the test drug with that of the control (mixture without any test drug). The following controls were prepared in order to calculate the percentage inhibition:

Linoleic acid alone (L): 2 ml of 99.5 % ethanol + 2.05 ml of 2.51 % (v/v) linoleic acid in 99.5 % ethanol + 4 ml phosphate buffer + 1.95 ml water with no incubation. This was assayed with TBA.

Full Reaction Mixture (FRM): 2 ml of 99.5 % ethanol + 2.05 ml of 2.51 % (v/v) linoleic acid in 99.5 % ethanol + 4 ml phosphate buffer + 1.95 ml water with incubation for 7 days and assayed with TBA.

Drug/Extract alone: 0.4 ml of the selected Drug/Extract + 4.6 ml 99.5 % ethanol and absorbance determined.

$$\%\,\text{INHIBITION} = \frac{(\text{FRM} - \text{L}) - (\text{Drug/Extract Test} - \text{Drug/Extract Alone} - \text{L})}{(\text{FRM} - \text{L})} \times 100$$

Where

- **FRM** determines the degree of linoleic acid autoxidation in the absence of an antioxidant.
- **L** determines the underlying peroxidation of the linoleic acid before the initiation of accelerated autoxidation by incubation at 40 °C.
- **Drug/Extract Alone** determines the absorbance of Drug/Extract solutions being tested.

Data was presented as percentage inhibition of linoleic acid autoxidation against concentration.

4.2.5 Analysis of Data

For the acute inflammation experiment, 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* test. Total foot volume for each treatment was calculated in arbitrary unit as the area under the curve (AUC) and to determine the percentage inhibition for each treatment, the following equation was used.

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

Data from the arthritis experiment was treated like that in the acute inflammation study. Raw scores for ipsilateral and contralateral paw/joint diameters (thicknesses) 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 oedema response of adjuvant-induced arthritis for the 28 days period. The time-course curves for paw/joint diameters were subjected to two-way (treatment × time) repeated measures analysis of variance with Bonferroni's *post hoc* test. Total paw/joint diameter (thickness) for each treatment was calculated in arbitrary unit as the AUC and to determine the percentage inhibition for each treatment.

Differences in AUCs were analysed by One-way ANOVA followed by Student-Newman-Keuls *post hoc* test. ED_{50}, EC_{50} and IC_{50} (dose/concentration responsible for 50 % of the maximal effect) values were determined by using an iterative computer least squares method, with the following nonlinear regression (three-parameter logistic) equation:

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

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 and Christopoulos *et al.*, 2003). GraphPad Prism for Windows version 5 (GraphPad Software, San Diego, CA, USA) was used for all statistical analyses and ED_{50} determinations. $P < 0.05$ was considered statistically significant.

4.3 RESULTS

4.3.1 Anti-Inflammatory Activity

4.3.1.1 Carrageenan-induced Oedema in Chicks

Administration of carrageenan (10 µl, 2 % suspension) induced moderate inflammation resulting in foot oedema in the 7-day old chicks peaking at 2-3 h as described by Roach and Sufka (2003)(Fig. 4.1).

Figures 4.1 (a, c, e) and 4.2 (a, c, e) show the time course curves for effects of HLE, diclofenac and dexamethasone on carrageenan-induced oedema in the pre-emptive and curative protocols respectively. Two-way ANOVA (*treatment* × *time*) revealed a significant effect of drug treatment for HLE (pre-emptive: $F_{3, 120}$= 28.59, P<0.0001; curative: $F_{4, 25}$= 2.87, P=0.044), diclofenac (pre-emptive: $F_{3, 120}$= 27.60, P<0.0001; curative: $F_{3, 20}$=4.96, P=0.0098) and dexamethasone (pre-emptive: $F_{3, 120}$=34.14, P<0.0001; curative: $F_{3, 20}$=3.74, P<0.028).

Total oedema produced by each treatment is expressed in arbitrary units as AUC of the time-course curves. HLE (10-300 mg kg^{-1}, *p.o.*) significantly reduced foot oedema with maximal inhibition of 38.11± 5.55 % (pre-emptive; fig. 4.1b) and 30.91±4.66 % (curative; fig. 4.2b) at doses 30 and 10 mg kg^{-1} respectively. Similarly, the NSAID diclofenac (10-100 mg kg^{-1}, i.p.)

dose dependently reduced the oedema by 59.33±10.82 % (pre-emptive; fig. 4.1d) and 42.87±7.46 % (curative; fig. 4.2d) at the dose of 100 mg kg^{-1}. Dexamethasone (0.3-3 mg kg^{-1}, i.p.), a steroidal anti-inflammatory agent inhibited the oedema with maximal effect of 42.77±7.64 % (pre-emptive; fig. 4.1f) and 36.60±6.76 % (curative; fig. 4.2f) at the dose of 3 mg kg^{-1}.

Dose-response curves for the inhibition of foot oedema are shown in figure 4.3. HLE displayed, to a large extent, a U-shaped dose response relationship with approximate ED_{50} values: 59.70 and 151.01 mg kg^{-1} (pre-emptive treatment); 72.95 and 213.80 mg kg^{-1} (curative treatment). By comparing the ED_{50} values from the curves, HLE was significantly less potent than diclofenac (ED_{50} pre-emptive: 11.58±17.83 mg kg^{-1}, curative: 25.65±32.48 mg kg^{-1}) and dexamethasone (ED_{50} pre-emptive: 0.77±0.85 mg kg^{-1}, curative: 2.93±1.02 mg kg^{-1}). Generally, all test drugs were more effective at inhibiting foot oedema when given pre-emptively than curatively (table 4.1).

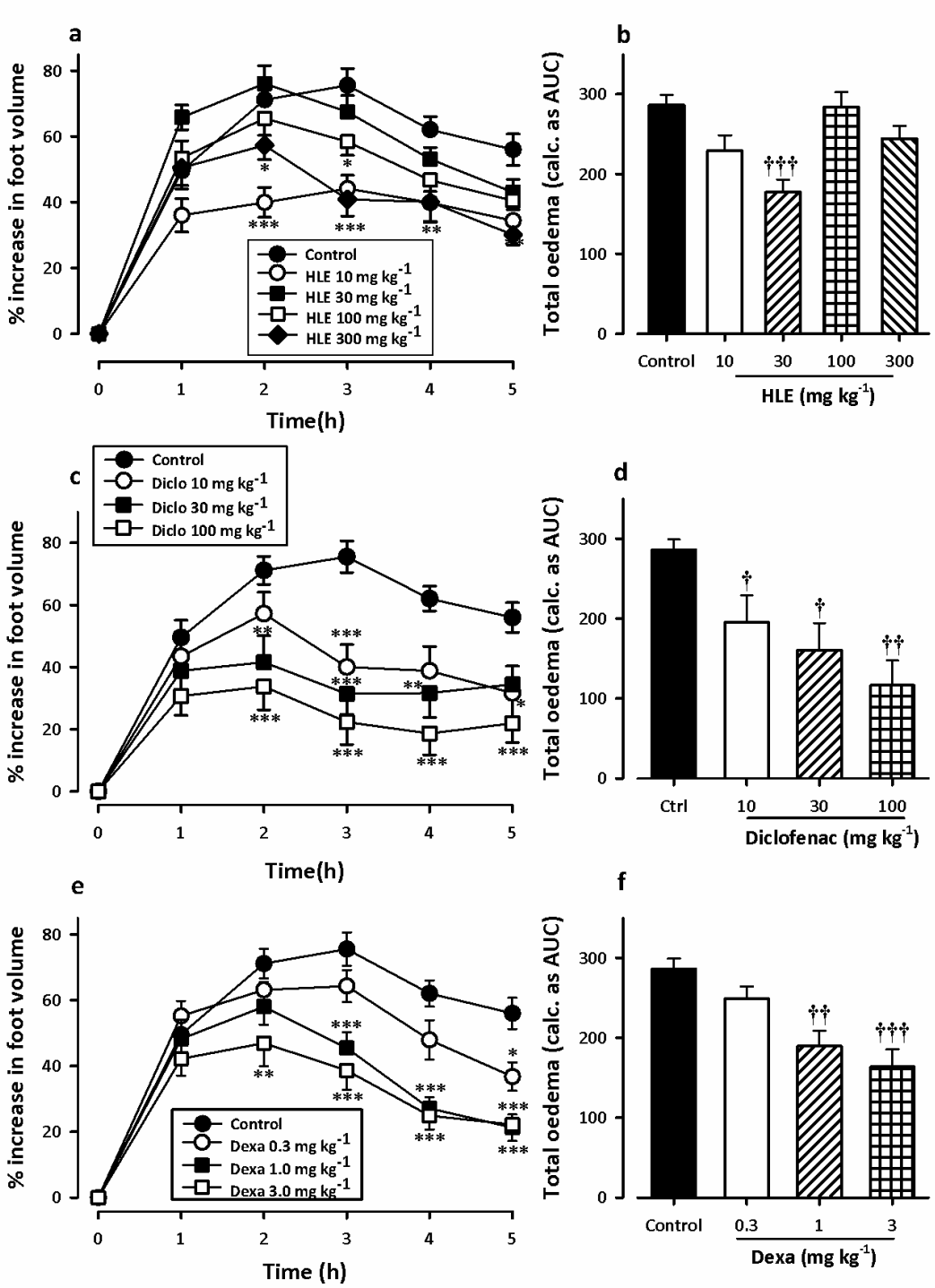

Figure 4.1 Effect of HLE (10-300 mg kg^{-1}; *p.o.*), diclofenac (10-100 mg kg^{-1}, i.p.) and dexamethasone (Dexa; 0.3-3 mg kg^{-1}, i.p.) on time-course curves (**a**, **c** and **e** respectively) and the total oedema response (**b**, **d** and **f** respectively) in the pre-emptive protocol of the carrageenan-induced foot oedema in chicks. Values are mean±S.E.M. (n=6). **P*<0.05; ***P*<0.01; ****P*<0.001 compared to vehicle-treated group (Two-way ANOVA followed by Bonferroni's *post hoc* test). †*P*<0.05; ††*P*<0.01; †††*P*<0.001 compared to vehicle-treated group (One-way ANOVA followed by Newman-Keuls *post hoc* test).

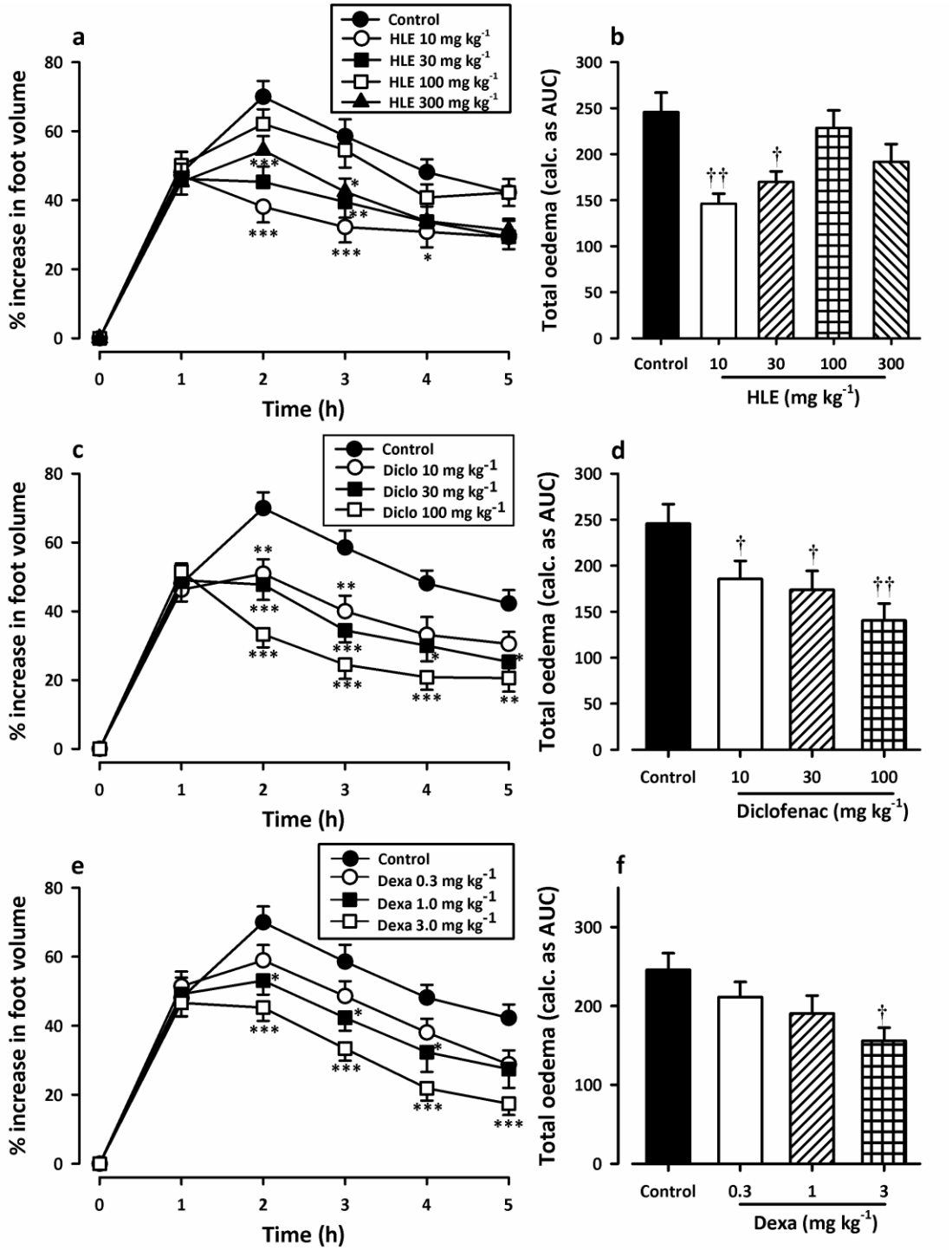

Figure 4.2 Effect of HLE (10-300 mg kg^{-1}; *p.o.*), diclofenac (10-100 mg kg^{-1}, i.p.) and dexamethasone (0.3-3 mg kg^{-1}, i.p.) on time-course curves(**a**, **c** and **e** respectively) and the total oedema response (**b**, **d** and **f** respectively) in the curative protocol of the carrageenan-induced foot oedema in chicks. Values are mean±S.E.M. (n=6). **P*<0.05; ***P*<0.01; ****P*<0.001 compared to vehicle-treated group (Two-way ANOVA followed by Bonferroni's *post hoc* test). †*P*<0.05; ††*P*<0.01; †††*P*<0.001 compared to vehicle-treated group (One-way ANOVA followed by Newman-Keuls *post hoc* test).

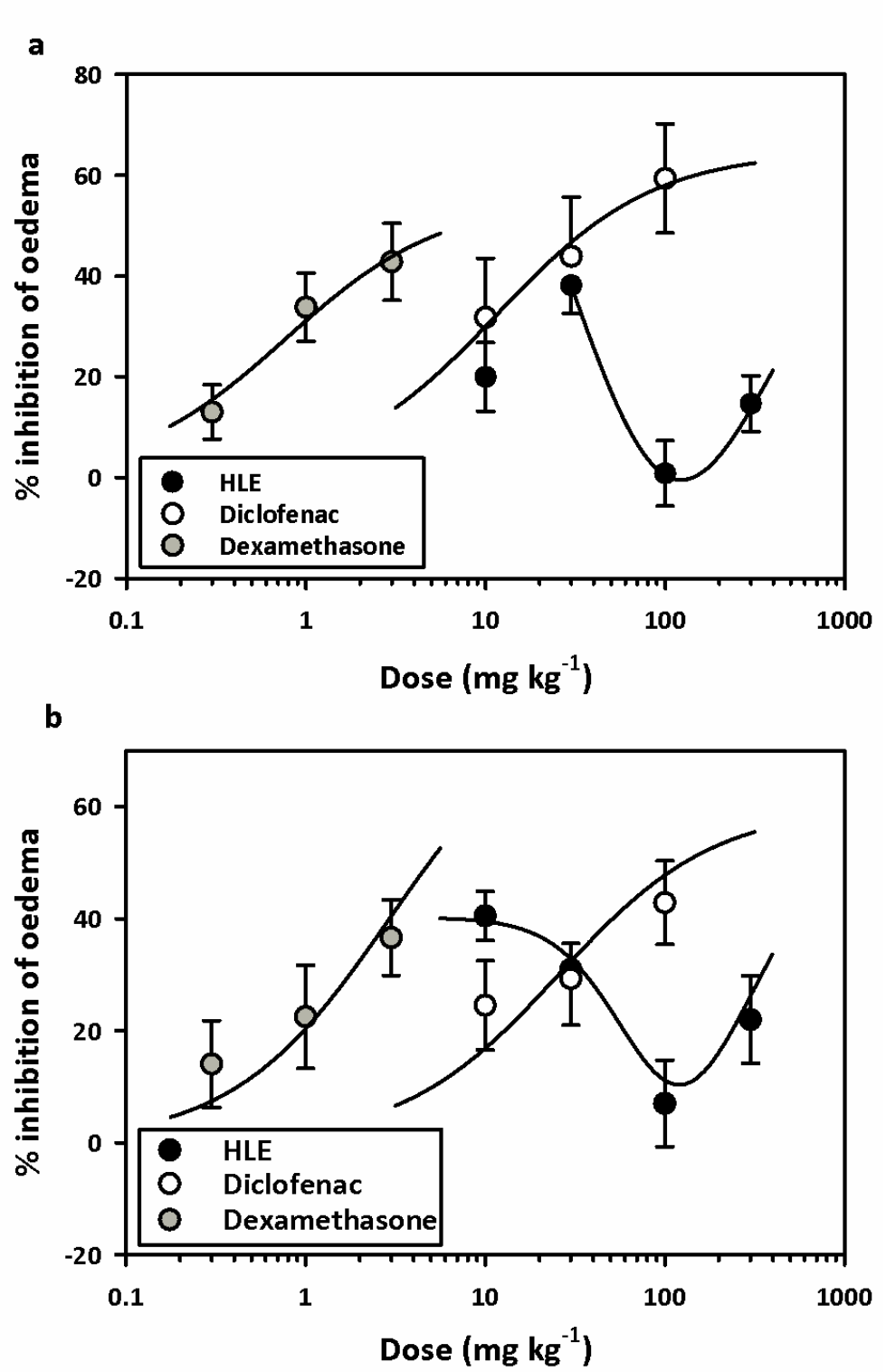

Figure 4.3 Dose response curves for HLE (10-300 mg kg^{-1} *p.o*), diclofenac (10-100 mg kg^{-1} i.p) and dexamethasone (0.3-3.0 mg kg^{-1} i.p) in the pre-emptive (**a**) and curative (**b**) protocols of carrageenan-induced foot oedema in the chick.

Table 4.1 ED_{50} values for chick carrageenan-induced oedema.

Drugs	ED_{50} (mg kg^{-1})	
	Pre-emptive	Curative
HLE	59.70, 151.01	72.95, 213.80
Diclofenac	11.58±17.83	25.65±32.48
Dexamethasone	0.77±0.85	2.93±1.02

4.3.1.2 Adjuvant – Induced Arthritis

Intraplantar injection of CFA into the right foot pad of rats induced an inflammatory response characterized by paw swelling in both the ipsilateral and the contralateral paw. The response on the injected paw was biphasic. It consisted of an acute phase (days 0-10 post CFA inoculation) characterized by unilateral inflammatory oedema of the ipsilateral paw peaking around days 4-6 and a subsequent polyarthritic/chronic phase (10-28 post CFA inoculation) characterised by inflammatory oedema of the contralateral paw. Throughout the 28-day experiment, there was no significant change in the paw volume of the non-inflamed control groups injected with IFA.

HLE, dexamethasone and methotrexate significantly suppressed the time-course of ipsilateral paw oedema in rats. Two-way ANOVA (*treatment* × *time*) revealed a significant effect of drug treatments on the paw oedema (HLE: $F_{5,30}$=78.44, P<0.0001; dexamethasone: $F_{4,25}$=144.88, P<0.0001 and methotrexate: $F_{4,25}$=80.05, P<0.0001; Figure 4.4a, c, e). Dexamethasone and methotrexate also significantly suppressed the time-course of ankle joint swelling in a dose-dependent manner (Dexamethasone: $F_{4,25}$=43.37, P<0.0001 and methotrexate: $F_{4,25}$=26.24, P<0.0001; two-way ANOVA; Figure 4.5b, c). HLE, however, could not significantly ($F_{4,25}$=1.10, P=0.3779; Figure 4.5a) suppress the time-course of ankle joint swelling in the rats.

HLE (10-300 mg kg^{-1}, *p.o*) significantly reduced the total ipsilateral paw oedema response over the 19 days of treatment with a maximal inhibition of 32.64±2.74 % (Figure 4.4b) at 10 mg kg^{-1}. The DMARD methotrexate (0.1-1 mg kg^{-1}, i.p.) and the steroidal anti-inflammatory agent dexamethasone (0.3-3 mg kg^{-1}, i.p) profoundly and dose-dependently reduced the total

ipsilateral paw oedema by 57.30±4.96 % (Figure 4.4f) and 64.51±2.30 % (Figure 4.4d) at 1 and 3 mg kg^{-1} respectively. HLE only reduced ankle joint swelling by 13.46±6.54 % at 10 mg kg^{-1} (Figure 4.5b) whilst methotrexate and dexamethasone reduced it by maxima of 38.44± 9.89 % and 46.00±8.20 % at 1 and 3 mg kg^{-1} respectively (Figure 4.5d, f),

HLE (10-300 mg kg^{-1}) could not significantly reduce ($F_{4,25}$=0.74, P=0.57) the extent of spread of oedema from the ipsilateral to the contralateral paw (Figure 4.4a, b). However, dexamethasone ($F_{4,25}$= 9.23, P=0.0001) and methotrexate ($F_{4,25}$=7.04, P=0.0006) dose-dependently and significantly prevented the spread of the arthritis from the ipsilateral to the contralateral paws of the treated animals (Figure 4.4c, d, e, f).

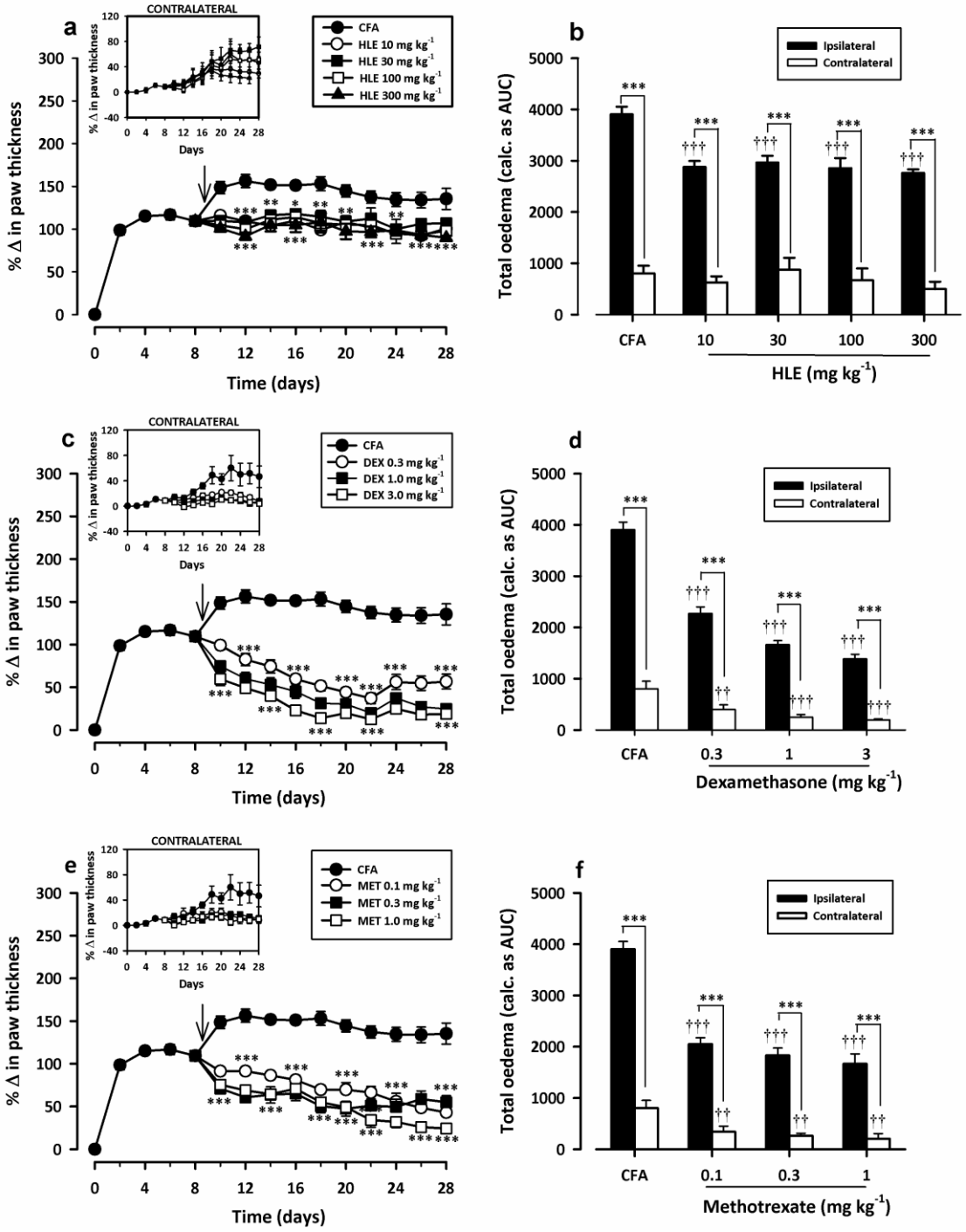

Figure 4.4 Effect of HLE (10 - 300 mg kg^{-1}; *p.o.*), dexamethasone (DEX; 0.3 - 3 mg kg^{-1}; i.p.) and methotrexate (MET; 0.1 - 1 mg kg^{-1}; i.p.) on time course curve (**a**, **c** and **e** respectively) and the total oedema response (**b**, **d** and **f** respectively) in adjuvant-induced arthritis in rats. The total oedema was calculated as AUCs over the 19 d period of drug treatment. Values are mean±S.E.M. (n=6). **P*<0.05; ***P*<0.01; ****P*<0.001 compared to vehicle-treated group (Two-way ANOVA followed by Bonferroni's *post hoc* test). †*P*<0.05; ††*P*<0.01; †††*P*<0.001 compared to vehicle-treated group (One-way ANOVA followed by Newman-Keuls *post hoc* test).

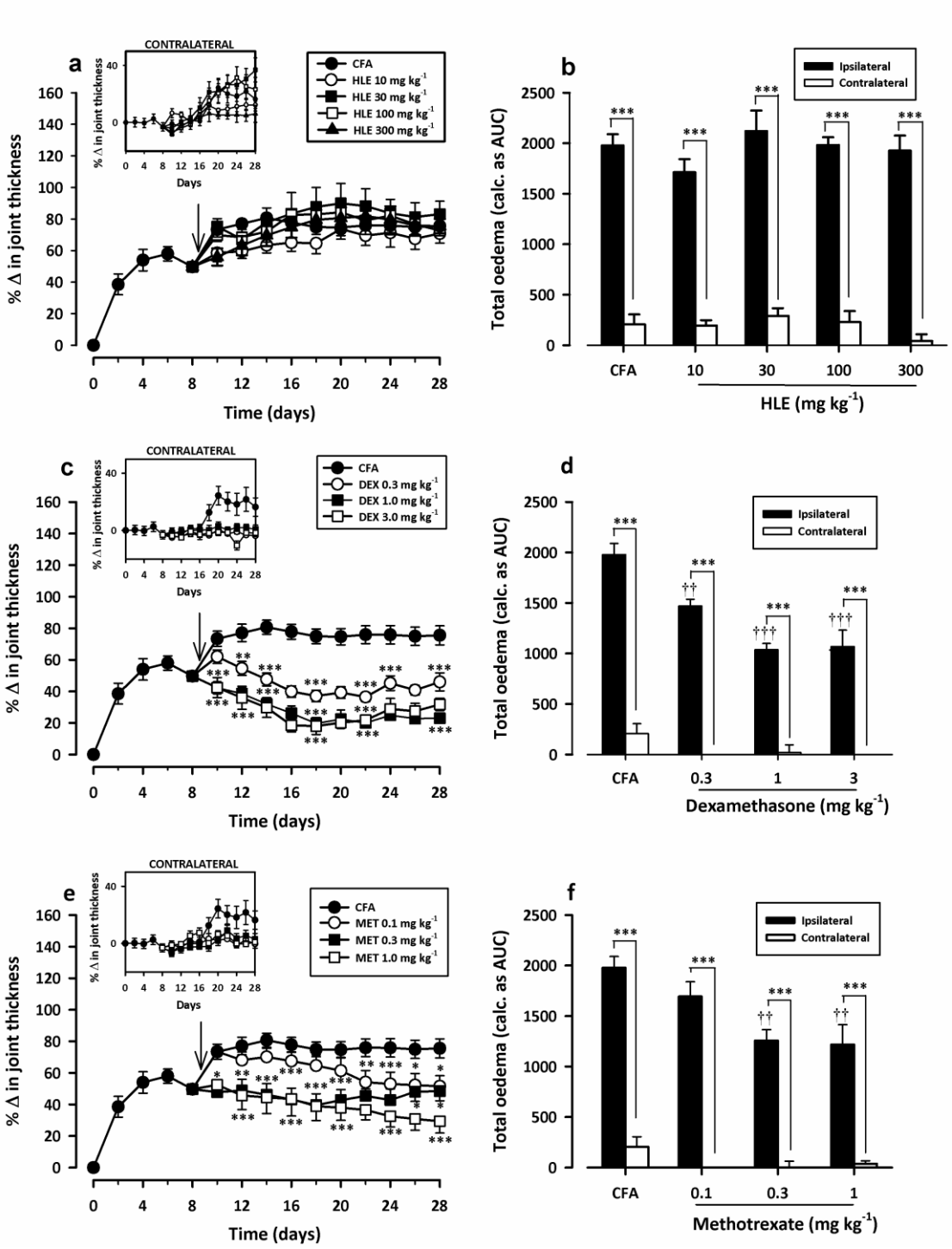

Figure 4.5 Effect of HLE (10 - 300 mg kg^{-1}; *p.o.*), dexamethasone (DEX; 0.3 - 3 mg kg^{-1}; i.p.) and methotrexate (MET; 0.1 - 1 mg kg^{-1}; i.p.) on time course curve (**a**, **c** and **e** respectively) and the total oedema response (**b**, **d** and **f** respectively) in adjuvant-induced arthritis in rats. The total oedema was calculated as AUCs over the 19 d period of drug treatment. Values are mean±S.E.M. (n=6). **P*<0.05; ***P*<0.01; ****P*<0.001 compared to vehicle-treated group (Two-way ANOVA followed by Bonferroni's *post hoc* test). †*P*<0.05; ††*P*<0.01; †††*P*<0.001 compared to vehicle-treated group (One-way ANOVA followed by Newman-Keuls *post hoc* test).

Dose-response curves for the inhibition of paw and ankle joint oedema are shown in Figure 4.6. HLE displayed, to a large extent, a U-shaped dose response relationship with approximate ED_{50} values: 19.91 and 47.64 mg kg^{-1} (effect on paw oedema); 17.42 and 45.81 mg kg^{-1} (effect on ankle joint oedema). HLE was the least potent compared to methotrexate and dexamethasone. Dexamethasone was the most potent (Table 4.2).

The body weight changes of rats in each treatment group over the 28 days period is shown in Figure 4.7. Rats in the IFA control group gained the most weight (43.75±8.31 %). HLE at 10 mg kg^{-1} showed weight gain comparable to the CFA group. However, there was a general dose-dependent reduction in weight gain in HLE-treated group. Dexamethasone (0.3-3 mg kg^{-1}) caused significant ($F_{3, 20}$=12.70, P<0.0001) weight loss compared to the CFA group. Rats in the methotrexate-treated group generally gained weight comparable to that of the CFA group except at the dose of 0.3 mg kg^{-1} (Fig. 4.7f).

The results from the assay of superoxide dismutase and catalase are shown in table 4.3. There was a decrease in superoxide dismutase (SOD) and catalase activity levels in the CFA-treated group compared to the IFA-treated group. Except for HLE at doses 10 and 300 mg kg^{-1}, all drug treatments did not significantly affect plasma SOD levels compared to the CFA group. In the case of catalase, all the drug treatments failed to reverse ($F_{11,24}$=1.32; P=0.276) the fall in the enzyme levels induced by the adjuvant arthritis.

Table 4.2 ED_{50} values for Freund's adjuvant-induced arthritis.

Drugs	**ED_{50} (mg kg^{-1})**	
	Paw	**Ankle joint**
HLE	19.91, 47.64	17.42, 45.81
Dexamethasone	0.19±0.05	0.32±0.17
Methotrexate	0.02±0.02	0.16±0.23

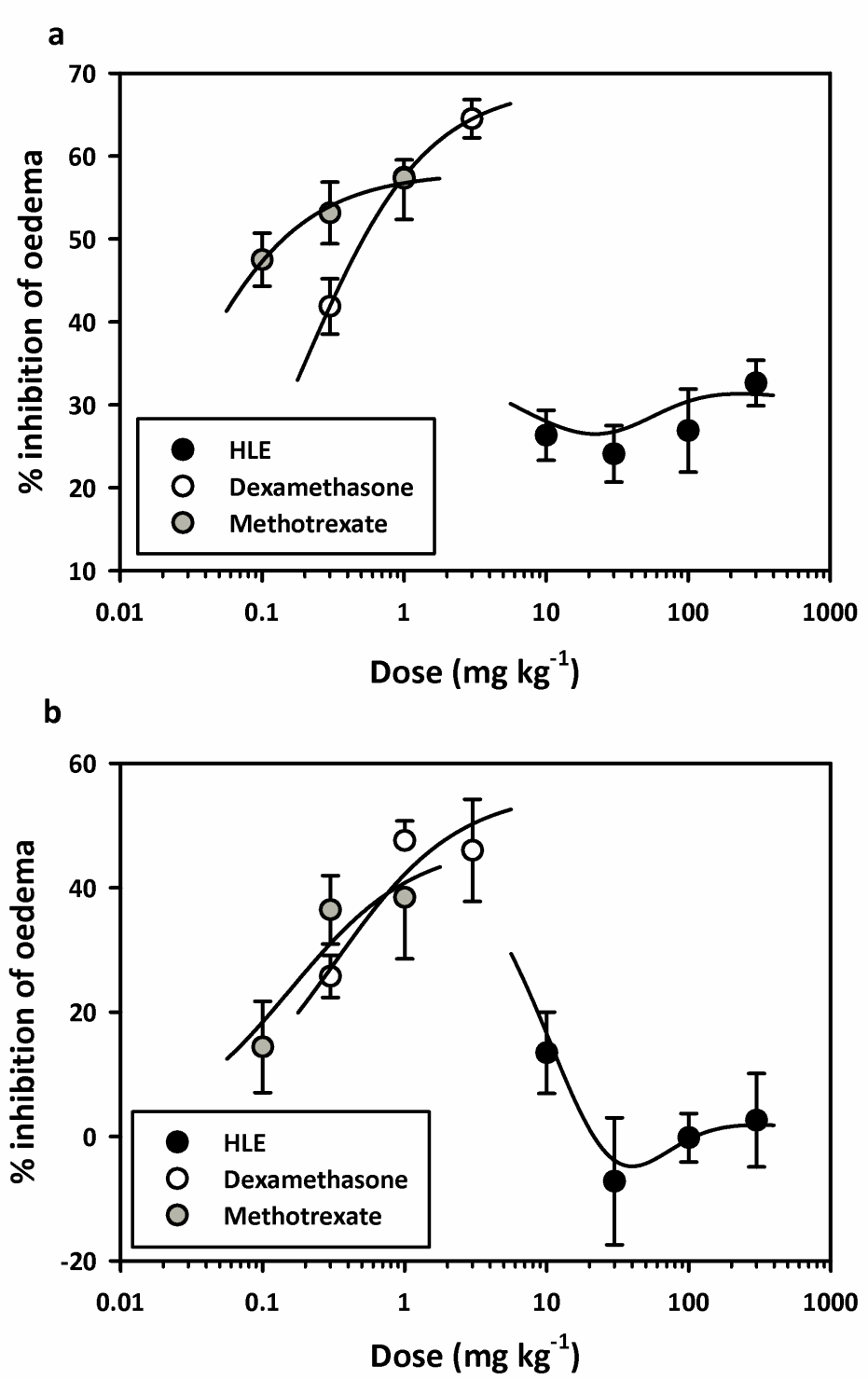

Figure 4.6 Dose response curves for HLE (10-300 mg kg^{-1} *p.o*), dexamethasone (0.3-3.0 mg kg^{-1} i.p.) and methotrexate (0.1-1 mg kg^{-1} i.p) with respect to paw swelling (**a**) and ankle joint swelling (**b**) in Freund's adjuvant-induced arthritis. Each point represents the mean±SEM (n=6).

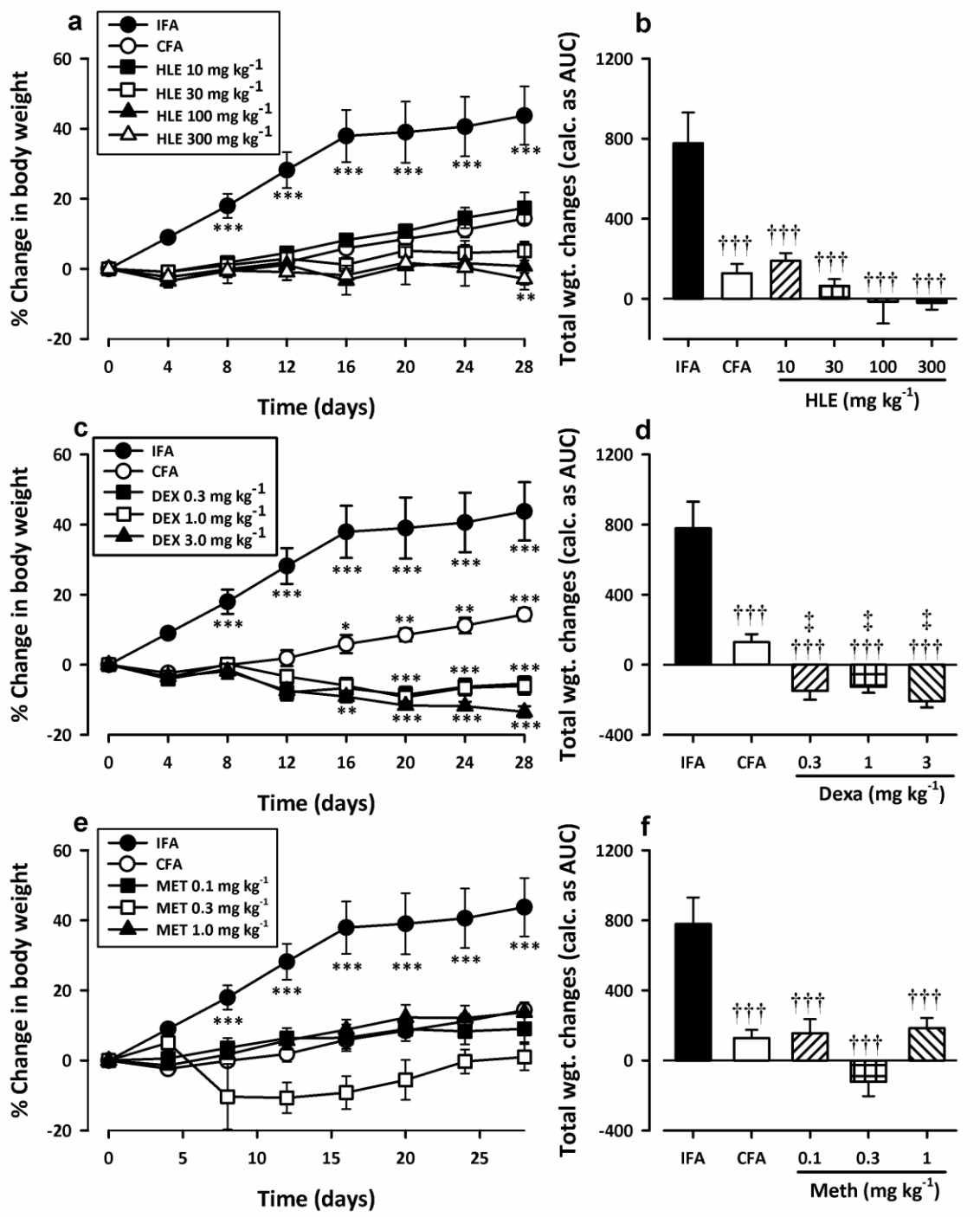

Figure 4.7 Effect of HLE (10 - 300 mg kg^{-1}; *p.o.*), dexamethasone (DEX; 0.3 - 3 mg kg^{-1}; i.p.) and methotrexate (MET; 0.1 - 1 mg kg^{-1}; i.p.) on time course curve (**a**, **c** and **e** respectively) and the total weight changes (**b**, **d** and **f** respectively) in adjuvant-induced arthritis in rats. The total weight change was calculated as AUCs over the 19 d period of drug treatment. Values are mean±S.E.M. (n=6). **P*<0.05; ***P*<0.01; ****P*<0.001 compared to vehicle-treated group (Two-way ANOVA followed by Bonferroni's *post hoc* test). †*P*<0.05; ††*P*<0.01; †††*P*<0.001 compared to vehicle-treated group (One-way ANOVA followed by Newman-Keuls *post hoc* test).

Table 4.3 Effect of various treatments on the plasma levels of antioxidant enzymes SOD and CAT in adjuvant-induced arthritis in rats.

Treatment	SOD (U/ml)	CAT(U/ml)
IFA Control	21.34±3.50*	122.40±8.48
CFA Control	11.10±0.98	90.09±4.34
HLE		
10 mg kg^{-1}	28.40±1.18*	77.42±2.71
30 mg kg^{-1}	7.66±1.73	80.46±19.12
100 mg kg^{-1}	11.73±1.34	86.29±22.38
300 mg kg^{-1}	16.02±6.04	66.22±21.10
Dexamethasone		
0.3 mg kg^{-1}	9.54±0.80	84.79±18.69
1.0 mg kg^{-1}	8.17±1.78	97.44±33.00
3.0 mg kg^{-1}	8.75±1.18	96.47±9.57
Methotrexate		
0.1 mg kg^{-1}	8.61±0.30	108.61±8.59
0.3 mg kg^{-1}	11.32±0.80	102.3±16.16
1.0 mg kg^{-1}	7.28±0.88	51.54±13.01

Values are mean±SEM (n=3). *P<0.05 compared to CFA-treated group (One-way ANOVA followed by Newman-Keuls *post hoc* test). SOD: Superoxide dismutase; CAT: Catalase.

From the arthritic indices (Table 4.4) and photographs (Plates 4.1-4.2), HLE (10-300 mg kg^{-1}), dexamethasone (0.3-3 mg kg^{-1}) and methotrexate (0.1-1 mg kg^{-1}) showed significant ($F_{11, 59}$=15.58, P<0.0001; Table 4.4) clinical improvement in arthritis. HLE reduced the arthritic index by a maximum of 60.00 % at the dose of 10 mg kg^{-1} whilst dexamethasone and methotrexate similarly inhibited by 87.50 % (at 3 mg kg^{-1}) and 77.50 % (at 1 mg kg^{-1}) respectively (Table 4.4). The IFA group showed no sign of erythema or swelling (Plate 4.1A). The CFA group, however, developed the severest arthritis showing immense erythema and swelling in both the ipsilateral and contralateral paws (Plate 4.1B). As regards the effectiveness of the doses of HLE used, 30 mg kg^{-1} was the least effective (Plate 4.2B).

Table 4.4 Arthritic and radiological indices of rats in the adjuvant-induced arthritis.

Groups	**Arthritic Index**	**Radiological Index**
IFA Control	0***	0***
CFA Control	6.67±0.42	3.33±0.21
H. latifolia extract		
10 mg kg^{-1}	2.67±0.33***	1.50±0.22***
30 mg kg^{-1}	6.18±0.65	3.00±0.26
100 mg kg^{-1}	4.50±1.03*	2.50±0.22*
300 mg kg^{-1}	4.00±0.93**	1.83±0.31***
Dexamethasone		
0.3 mg kg^{-1}	1.83±0.67***	0.17±0.17***
1 mg kg^{-1}	1.33±0.21***	0***
3 mg kg^{-1}	0.83±0.66***	0***
Methotrexate		
0.1 mg kg^{-1}	2.40±0.51***	0.33±0.21***
0.3 mg kg^{-1}	1.67±0.33***	0.20±0.20***
1 mg kg^{-1}	1.50±0.22***	0***

One-way ANOVA followed by Newman-Keuls *post hoc* test. *P<0.05; **P<0.01; ***P<0.001 compared to CFA-treated group.

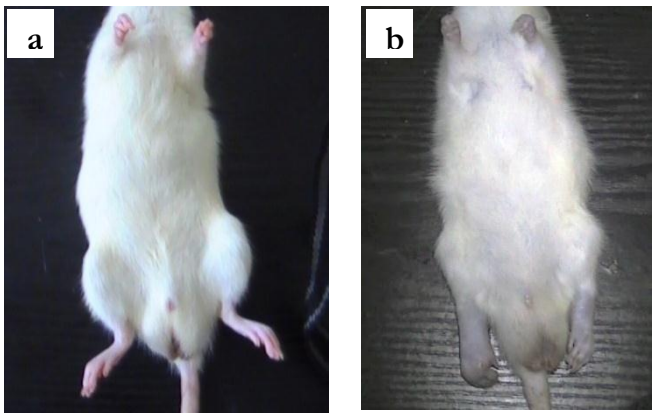

Plate 4.1 Photographs of rats pre-treated with (**a**) IFA (non-arthritic control) and (**b**) CFA (arthritic control) in the adjuvant-induced arthritis.

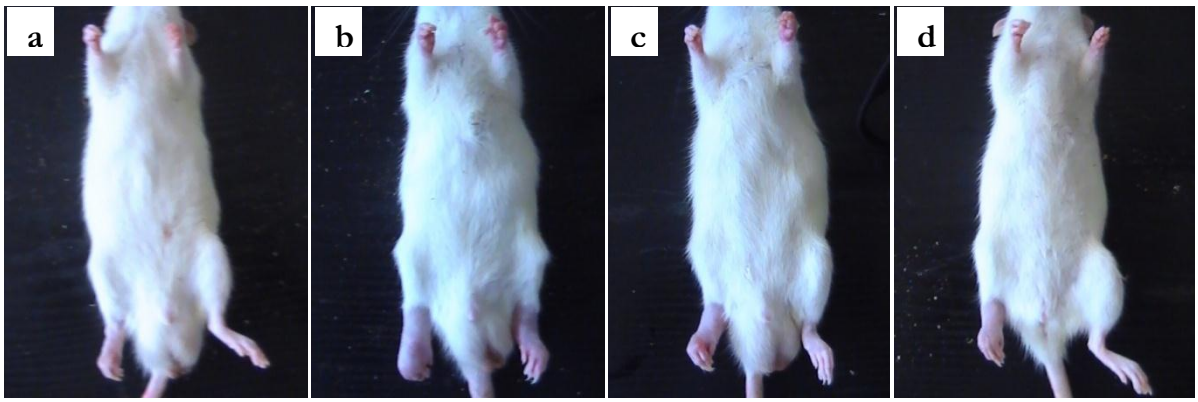

Plate 4.2 Photographs of rats treated with *H. latifolia* extract (10, 30, 100 and 300 mg kg^{-1}) (**a**, **b**, **c** & **d** respectively) in the adjuvant-induced arthritis.

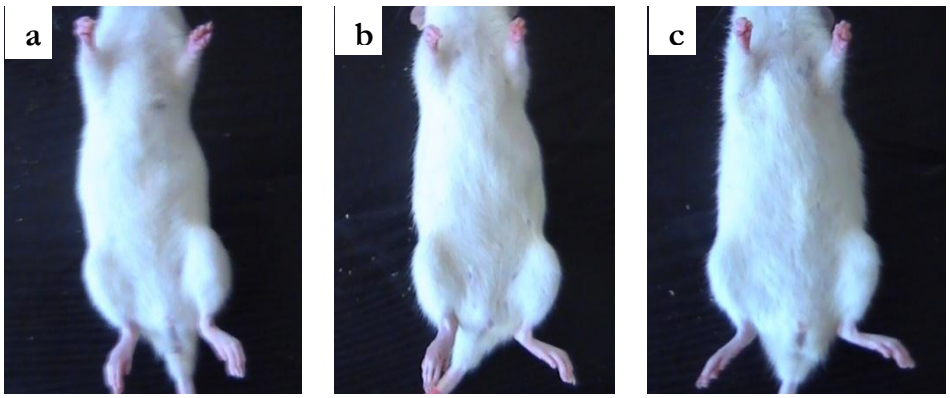

Plate 4.3 Photographs of rats treated with dexamethasone (0.3, 1.0 and 3.0 mg kg^{-1}) (**a**, **b** & **c** respectively) in the adjuvant-induced arthritis.

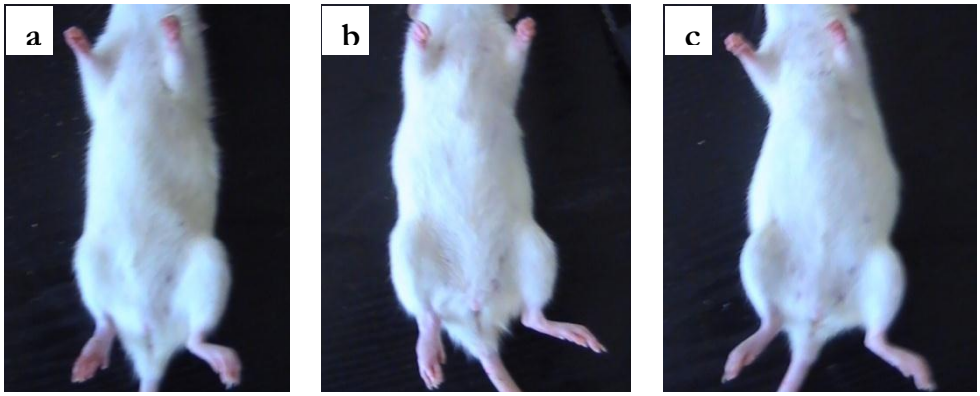

Plate 4.4 Photographs of rats treated with methotrexate (0.1, 0.3 and 1.0 mg kg^{-1}) (**a**, **b** & **c** respectively) in the adjuvant-induced arthritis.

Radiographs of the hind limbs for each group are shown in Plates 4.5-4.8. Comparing the radiographs of the hind limbs from each group, the CFA group demonstrated most severe bone destruction displaying reduced bone density and focal areas of excessive bone resorption. The bones were intact in the IFA/non-arthritic control which recorded the lowest radiographic score. HLE at doses 10 and 300 mg kg^{-1} suppressed the pathological changes in bone with maximal inhibition of radiological index of 54.95%, compared with that of the CFA group. Similarly, dexamethasone (0.3-3 mg kg^{-1}) and methotrexate (0.1-1 mg kg^{-1}) almost totally prevented bone destruction in AIA radiographically (Table 4.4, plate 4.5-4.8) both reducing the radiological index by maxima of 100%.

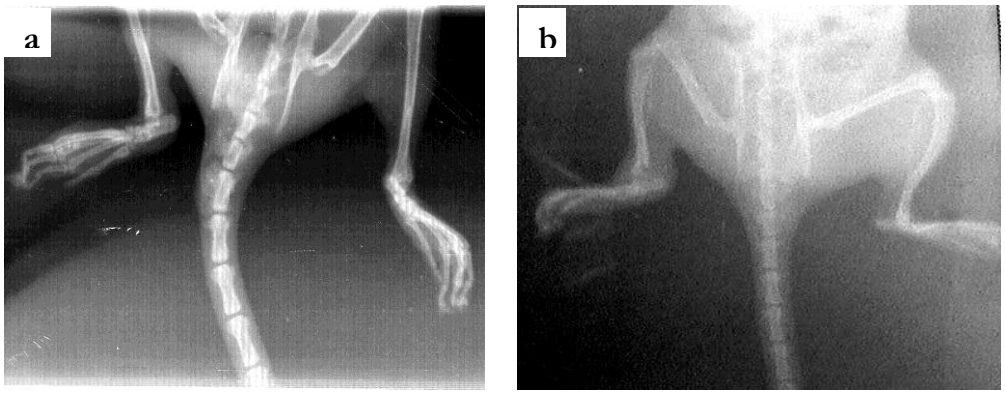

Plate 4.5 Radiographs of rats treated with IFA/ non arthritic control (**a**) and CFA/arthritic control (**b**) in the adjuvant-induced arthritis.

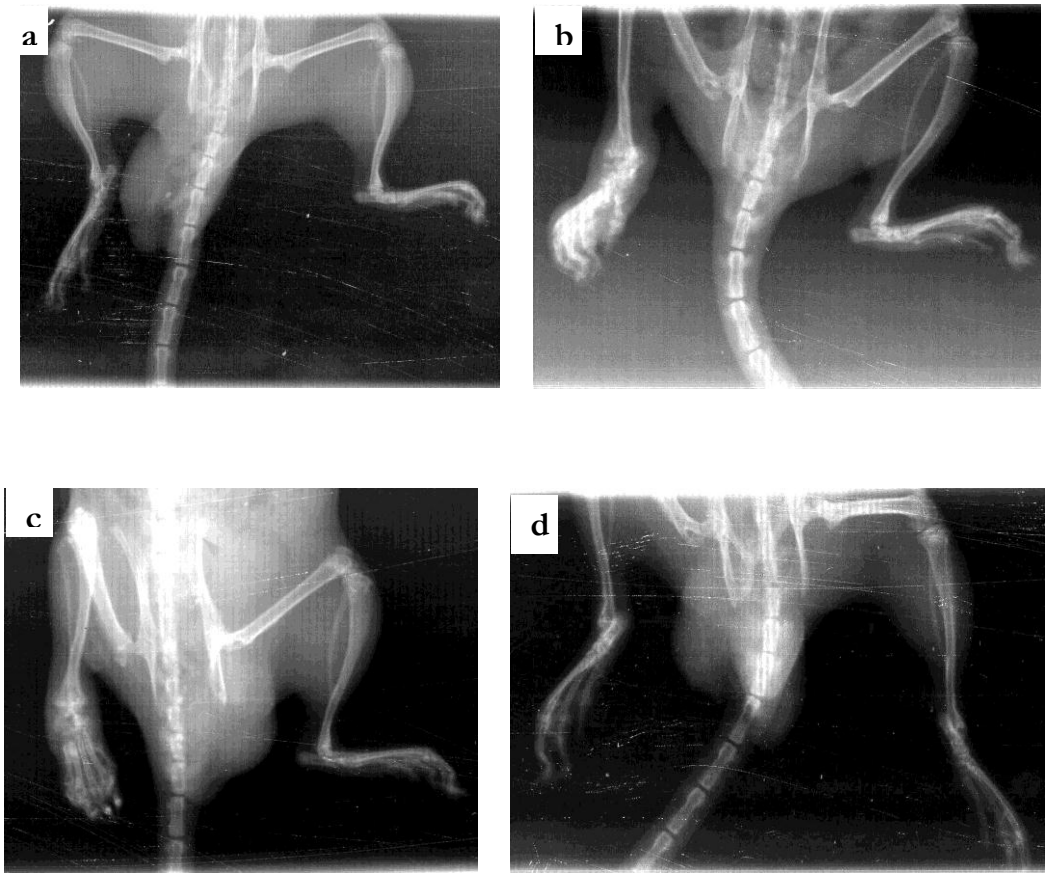

Plate 4.6 Radiographs of rats treated with *H. latifolia* extract (10, 30, 100 and 300 mg kg^{-1}) (**a**, **b**, **c** & **d** respectively) in the adjuvant-induced arthritis.

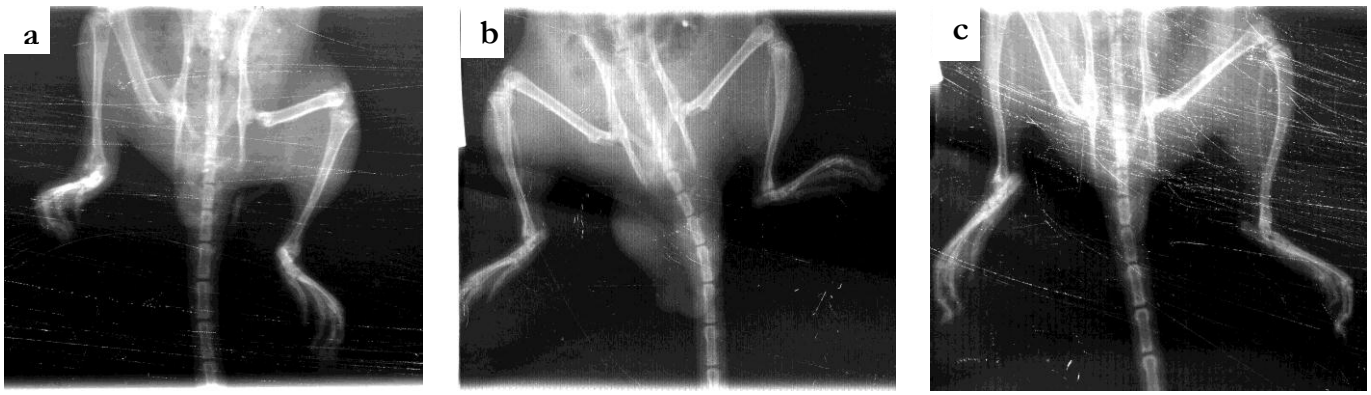

Plate 4.7 Radiographs of rats treated with dexamethasone (0.3, 1.0 and 3.0 mg kg^{1}) (**a**, **b** & **c** respectively) in the adjuvant-induced arthritis.

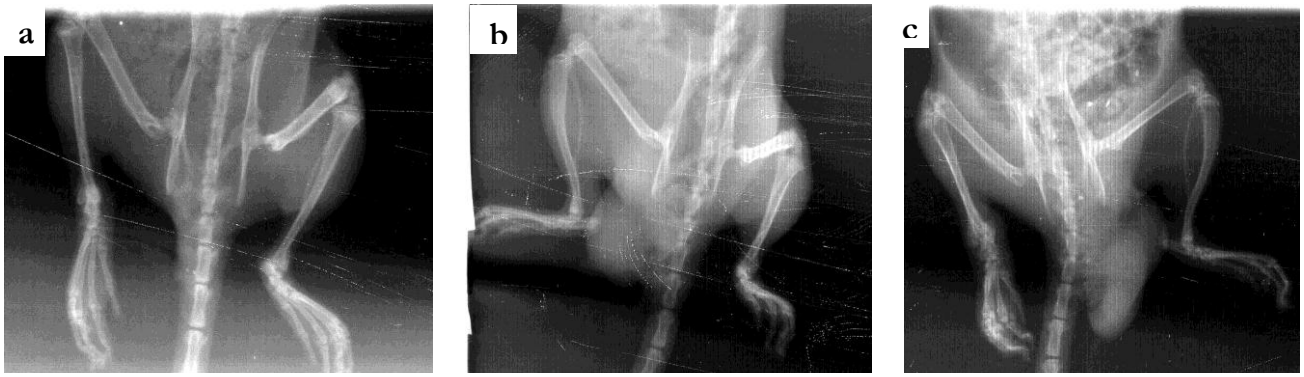

Plate 4.8 Radiographs of rats treated with methotrexate (0.1, 0.3 and 1.0 mg kg^{1}) (**a**, **b** & **c** respectively) in the adjuvant-induced arthritis.

4.3.2 Antioxidant Activity

4.3.2.1 Total Phenol Content

The total phenol content determination showed a concentration-dependent increase ($F_{3,6}$=427.9, P<0.0001) in the total phenolics in HLE (Figure 4.8a, b) when expressed in tannic acid equivalents. The total phenol content was estimated to be 29.40±1.09 mg tannic acid equivalent/g of HLE (Table 4.5).

4.3.2.2 Total Antioxidant Capacity

HLE (0.1-3 mg ml^{-1}) showed a concentration-dependent increase ($F_{3, 8}$=44.86, P<0.0001) in total antioxidant capacity when expressed as ascorbic acid equivalents (Fig. 4.8c, d). The total antioxidant capacity of the HLE was estimated to be 55.16±13.60 mg ascorbic acid equivalent/g of HLE (Table 4.5).

Table 4.5 Total phenol content and total antioxidant capacity.

Measurement	Value
Total phenol content (mg/g)†	29.40±1.09
Total antioxidant capacity (mg/g)‡	55.16±13.60

Data are mean±SEM (n=3).

†Total phenolic content was expressed as mg tannic acid equivalent/g of dried extract.

‡Total antioxidant capacity was expressed as mg ascorbic acid equivalent/g of dried extract.

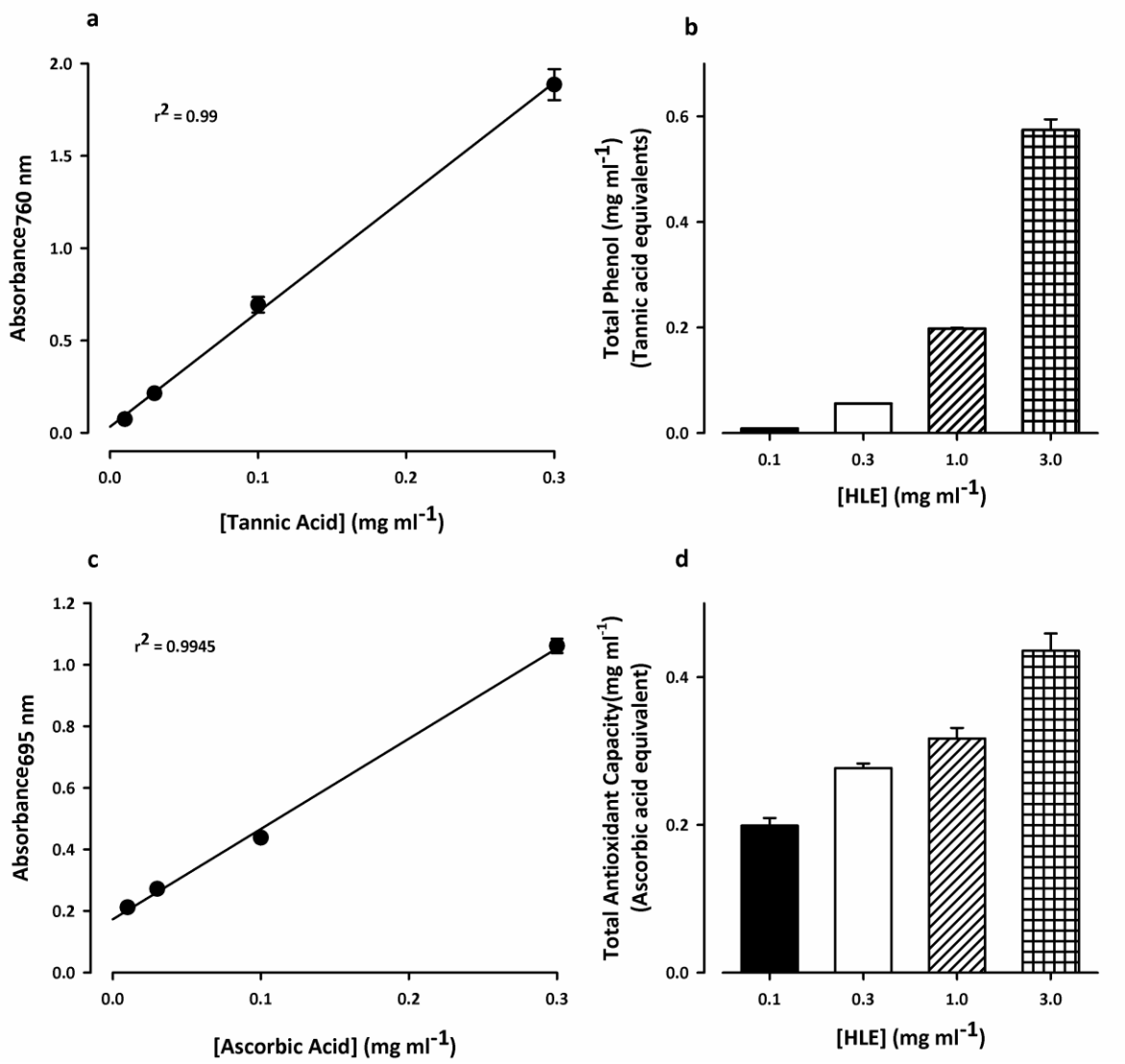

Figure 4.8 Absorbance of tannic acid (0.01-0.3 mg ml^{-1}) and ascorbic acid (0.01-0.3 mg ml^{-1})(**a** and **c** respectively), total phenols present in various concentrations of HLE (0.1-3 mg ml^{-1}), expressed as tannic acid equivalent(**b**) and total antioxidant capacity of various concentrations of HLE (0.1-3 mg ml^{-1}), expressed as ascorbic acid equivalents(**d**). Values are mean±S.E.M. (n=3).

4.3.2.3 *Scavenging of 2, 2-diphenyl-1-picrylhydrazyl (DPPH)*

The DPPH assay determines the ability of an agent to scavenge free radicals. HLE (0.1-1 mg ml^{-1}) showed a concentration-dependent scavenging activity in a similar manner to *n*-propyl gallate (Fig. 4.9a). The IC_{50} values (in mg ml^{-1}) of 0.2269±0.037 and 0.00323±0.001 for HLE and *n*-propyl gallate respectively (Table 4.6), suggests that HLE has lesser ability to scavenge free radicals compared to *n*-propyl gallate ($F_{1,14}$=114.7, P<0.0001).

4.3.2.4 *Reducing Power*

HLE (0.03-1 mg ml^{-1}) and *n*-propyl gallate (0.001-0.03 mg ml^{-1}) exerted a concentration-dependent Fe^{3+} reducing activity with EC_{50} values (in mg ml^{-1}) of 2.071±0.782 and 0.1071±0.049 respectively (Fig. 4.9b; Table 4.6). The *n*-propyl gallate was however more potent, exhibiting a 19-fold reducing power compared to the extract ($F_{1,20}$=7.76, P=0.0114; Table 4.6).

4.3.2.5 *Linoleic Acid Autoxidation*

The result of the linoleic acid autoxidation determination (Fig. 4.9c) showed a concentration-dependent inhibitory activity by both HLE and the standard, *n*-propyl gallate with IC_{50} values (in mg ml^{-1}) of 0.1122±0.010 and 0.03657±0.007 respectively (Table 4.6). *N*-propyl gallate was more potent when compared to HLE ($F_{1,14}$=27.29, P=0.0001).

Table 4.6 EC_{50}/IC_{50} values for HLE and *n*-propyl gallate in the antioxidant tests.

Drug	**EC_{50}/IC_{50} (mg ml^{-1})**		
	Reducing Power	**DPPH Scavenging**	**Linoleic acid autoxidation**
HLE	2.071±0.782	0.2269±0.037	0.1122±0.010
n-Propyl gallate	0.1071±0.049	0.00323±0.001	0.03657±0.007

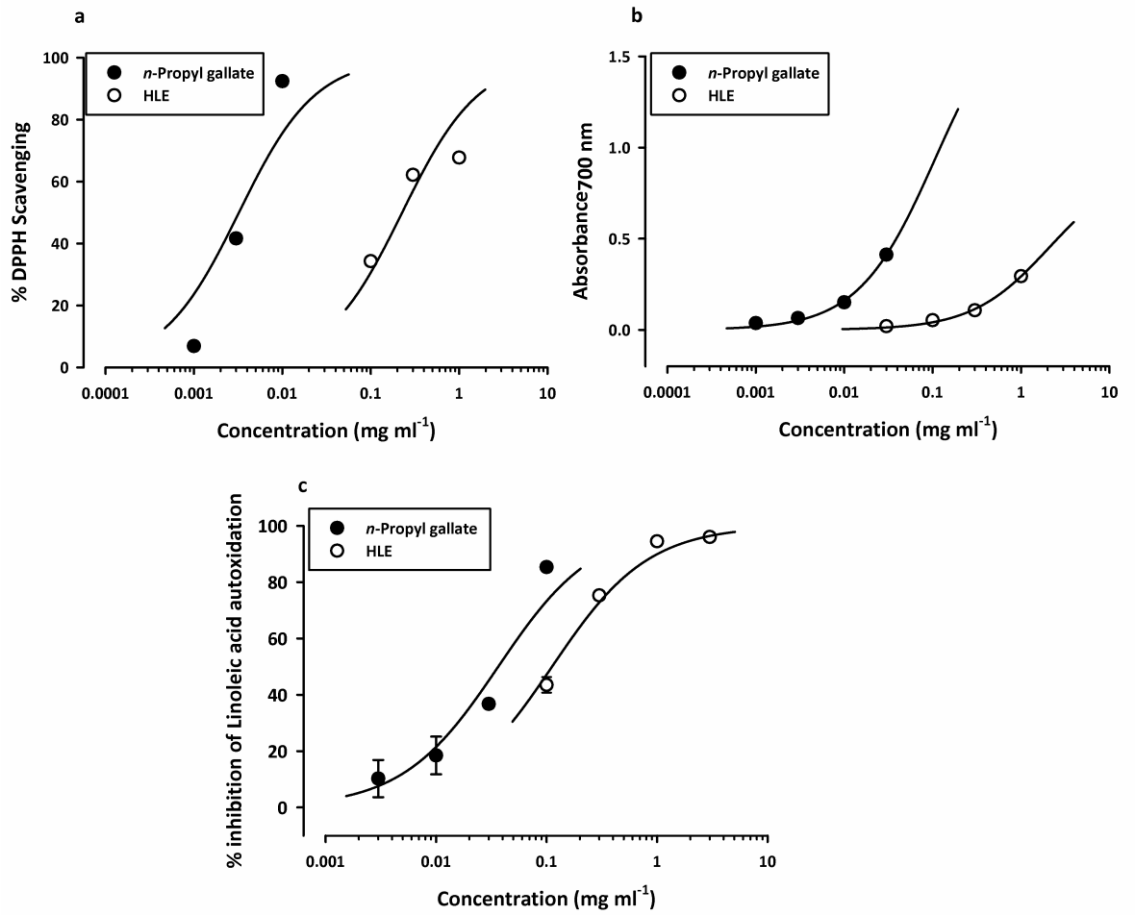

Figure 4.9 Free radical scavenging ability of HLE (0.1-1 mg ml^{-1}) compared to *n*-propyl gallate (0.001-0.01 mg ml^{-1}) in the DPPH radical assay(**a**), reducing power of HLE (0.03-1 mg ml^{-1}) compared to *n*-propyl gallate (0.001-0.03 mg ml^{-1})(**b**), and Percentage inhibition of lipid peroxidation (linoleic acid autoxidation) by HLE (0.1-3 mg ml^{-1}) compared to *n*-propyl gallate (0.003-0.1 mg ml^{-1})(**c**). Values are mean±S.E.M.

4.4 DISCUSSION

The present study establishes the anti-inflammatory activity of the aerial parts of *H. latifolia* in acute and chronic inflammatory animal models used. It also evaluated the *in vitro* antioxidant properties of HLE, since it may be one of the mechanisms of its anti-inflammatory action. HLE exhibited antioxidant activity in all the five assay models used: total phenol assay, reducing power test, total antioxidant capacity, DPPH scavenging activity and lipid peroxidation assay.

Carrageenan-induced oedema test (Winter *et al.*, 1962) is a classical model of acute inflammation that has been extensively used to screen new anti-inflammatory drugs (Di Rosa and Willoughby, 1971). In this study chicks were used instead of the commonly used rodents. Carrageenan-induced oedema has been validated in 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 oedema, that is differentially attenuated by the systemic administration of typical anti-inflammatory compounds and compares favourably with the more commonly used rodent models (rat and mice) in the screening of drugs with anti-inflammatory activities (Roach and Sufka, 2003).

The development of carrageenan-induced oedema is commonly correlated with the early exudative stage of inflammation, one of the important processes of inflammatory pathology (Winter *et al.*, 1962; Ozaki, 1990). The inflammatory response induced by carrageenan is biphasic (Vinegar *et al.*, 1969) characterised by marked oedema formation resulting from the rapid production of several inflammatory mediators such as histamine, serotonin and bradykinin (first-phase), which is subsequently sustained by the release of prostaglandins and nitric oxide (second-phase) with peak at 3 h, produced by inducible isoforms of COX (COX-2) and nitric oxide synthase (iNOS), respectively (Seibert *et al.*, 1994; Thomazzi *et al.*, 2010). The second (late) phase is sensitive to most clinically effective anti-inflammatory drugs (Vinegar *et al.*, 1969; Di Rosa *et al.*, 1971). HLE clearly suppressed inflammation induced by carrageenan in both pre-emptive and curative protocols of the anti-inflammatory activity assessment. This is in line with the earlier observation on the ability of the extract to block the inflammatory (late phase) of the formalin test (Section 3.3.3; Figure 3.5). The finding also justifies the use of the extract traditionally in the treatment of inflammatory conditions.

Although the actual mechanism of action of HLE in inflammation is unknown, the fact that it inhibited both early and late phases of oedema suggests that it could be acting through the inhibition of the release and/or action of those inflammatory mediators involved in carrageenan-induced oedema which include cytoplasmic enzymes, histamine, serotonin, bradykinin, prostaglandins and other cyclooxygenase products. The exact mechanism, however, needs to be established. The anti-inflammatory action of HLE can be attributed to one of its chemical constituents. Indeed, HLE has been shown by phytochemical analysis to contain alkaloids, flavonoids, tannins, saponins, phytosterols and terpenoids and one of them may be responsible for the anti-inflammatory effect especially as a lot of these secondary plant metabolites identified have been shown to exhibit anti-inflammatory properties (Whitehouse *et al.*, 1994; Guardia *et al.*, 2001; Rotelli *et al.*, 2003; Barbosa-Filho *et al.*, 2006).

The standard drugs diclofenac and dexamethasone with which the extract was compared both showed a dose-dependent inhibition of carrageenan-induced oedema. The mechanism of anti-inflammatory action of diclofenac and dexamethasone are well known (Seibert *et al.*, 1994; Al-Majed *et al.*, 2003; Wise *et al.*, 2008; Rhen and Cidlowski, 2005).

Adjuvant-induced arthritis (AIA), an experimental chronic inflammatory disorder in rats induced by a single injection of killed mycobacteria in oil, is one of the most widely used animal models to study the effect of anti-rheumatic agents. The adjuvant model represents a systemic inflammatory disease, with bone and cartilage changes similar to those observed in rheumatoid arthritis, but within an accelerated time span. The common pathological features of adjuvant arthritis in rat and rheumatoid arthritis in humans are joint swelling associated with cellular and pannus invasion of the joint space, release of lysosomal constituents into the joint space, and bone resorption (Osterman *et al.*, 1994). The major limitation of this model is its inability to identify disease modifying anti-rheumatic drugs (Rainsford, 1982, Weichman, 1989). In adjuvant arthritis bacterial, peptidoglycan and muramyl dipeptide are thought to be responsible for its induction. It occurs through cell mediated-autoimmunity by structural mimicry between mycobacteria and cartilage proteoglycans in rats (van Eden *et al.*, 1985; Vijayalakshmi *et al.*, 1997).

In this study, oral administration of HLE caused clinical improvement of arthritis and a significant reduction in inflammation shown as decrease in paw thickness. There was, however, not much improvement seen at the joints. The reason for this observation is not immediately clear. But it seems reasonable to infer that HLE probably improved arthritis largely by its anti-

inflammatory activity (reduction in oedema, erythema, pain, etc) than by an immunologic effect on the course of the disease. This argument is supported by the fact that HLE did not significantly prevent the systemic spread of the adjuvant arthritis—a process that is largely immunologic (Donaldson *et al.*, 1995). Nevertheless, further studies are needed to establish these assertions.

Radiographs are necessary to determine true remission of disease and for accurate evaluation of disease status (Kitamura *et al.*, 2007). The extract, as evidenced from X-ray pictures (Plate 4.5-4.6), at doses of 10 mg kg^{-1} and 300 mg kg^{-1} protected against bone loss due to reduced bone formation and increased resorption which are the causes of bone loss in adjuvant-induced arthritis in rats (Aota *et al.*, 1996; Findlay and Haynes, 2005). It is doubtful if this effect is mediated by an immunologic protection of the bones. It is most likely due to protection offered to the bone as a result of the anti-inflammatory effect of HLE, which was most seen at the two doses (i.e. 10 mg kg^{-1} and 300 mg kg^{-1}). Further studies on the effect of HLE on the bone will be necessary to establish exact mechanisms.

Changes in weight reflect arthritic disease progression and general health status, while exaggerated weight loss above that observed in the arthritic control group may be indicative of toxicity (Schopf *et al.*, 2006). Generally, the extract could not protect against arthritic-induced weight loss. While the least dose of the extract (10 mg kg^{-1}) permitted some slight weight gain beyond the arthritic control (CFA) group, higher doses (30-300 mg kg^{-1}) resulted in weight loss. This could be attributed to the fact that aside 10 mg kg^{-1} of HLE, the higher doses could not produce much clinical improvement in arthritis to allow significant weight gain. It is also possible that weight loses are due to HLE toxicity at high doses. Dexamethasone and methotrexate, which are well known to cause weight loss (Orzechowski *et al.*, 2000; Lucas *et al.*, 2003; Kolli *et al.*, 2007), also acted similarly to HLE and were unable to offer protection against arthritis-induced weight loss.

Corticosteroids have been shown to be 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 the spread of arthritis in this study. Dexamethasone is known to inhibit the release of pro-inflammatory cytokines (TNF-α and IL-1β), which are known to play a central role in the propagation of the disease process in

rheumatoid arthritis thus being able to arrest the oedema produced (Issekutz and Issekutz, 1991).

Methotrexate, a DMARD, 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 an immunosuppressant (Cronstein, 2005; Tian and Cronstein, 2007) and 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). It is better tolerated with fewer side effects than other DMARDs, it promotes disease remission and prevents 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). Methotrexate inhibited AIA and dramatically suppressed 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).

HLE exhibited a non-monotonic dose-response pattern in both carrageenan-induced oedema test and the adjuvant-induced arthritis test (fig. 4.3 & fig. 4.6). The exact biochemical mechanism underlying this pharmacological inversion is not exactly clear, and remains to be established, but probably reflects the activation of various pathways at different doses.

In oxidative stress, reactive oxygen species (ROS) such as superoxide (O_2•-, OOH•), hydroxyl (OH•) and peroxyl (ROO•) radicals are generated which play an important role in several human physiopathologies especially neurodegenerative disorders, cancer, cardiovascular diseases and inflammation (Laguerre *et al.*, 2007; Conforti *et al.*, 2008). Several anti-inflammatory agents have been shown to have antioxidant and/or radical scavenging mechanisms as part of their activity (Kirkova *et al.*, 1992; Maffei Facino *et al.*, 1993; Takayama *et al.*, 1994; Burguete *et al.*, 2007; Rojo *et al.*, 2009). The mechanism of inflammation injury is attributed, in part, to the release of ROS from activated neutrophils and macrophages. This overproduction leads to tissue injury by damaging macromolecules and lipid peroxidation of membranes (Cochrane, 1991; Winrow *et al.*, 1993; Gutteridge, 1995). ROS also propagate inflammation through modulation of inflammatory gene expression leading to release of pro-

inflammatory cytokines such as IL-1, TNF-α, and IFN-γ which not only directly enhance inflammatory response but also stimulate recruitment of additional neutrophils and macrophages (Dinarello, 2000; Conforti *et al.*, 2008). Thus free radicals are important mediators that provoke or sustain inflammatory processes and, consequently, their neutralization by antioxidants and radical scavengers can attenuate inflammation (Geronikaki and Gavalas, 2006; Conforti *et al.*, 2008).

One of the possible mechanisms by which HLE exerts anti-inflammatory activity is through the suppression of the effect of free radicals during inflammation. This can be achieved through the direct action of the extract as an antioxidant, or indirectly by boosting the levels of *in vivo* antioxidant enzymes including superoxide dismutase (SOD) and catalase (CAT). The impact of HLE on antioxidant enzymes SOD and CAT was investigated during the adjuvant arthritis study while the direct effect of HLE as an antioxidant was assessed with *in vitro* models.

Evidence suggests oxidative stress is elevated in arthritis (Bae *et al.*, 2003; Mahajan and Tandon, 2004). This initially triggers feedback increases in the activity of antioxidant enzymes but is eventually overwhelmed, resulting ultimately in decreased activity of antioxidant enzymes including SOD and CAT (Vijayalakshmi *et al.*, 1997; Kumar *et al.*, 2002; Jung *et al.*, 2005; Narendhirakannan *et al.*, 2005; He *et al.*, 2006). This was observed in this study—the arthritic control (CFA) group showed significant decrease in the antioxidant enzymes SOD and CAT compared to the non-arthritic control (IFA) group. SOD is a metalloprotein and is the first enzyme involved in the antioxidant defense by lowering the steady state level of O_2•−. SOD which converts superoxide radicals to H_2O_2 is widely distributed to protect such cells against the toxic effects of superoxide anion (Devi *et al.*, 2007). CAT, on the other hand, is a ubiquitous haem protein located in the cytosol and in peroxisomes. This enzyme catalyzes the decomposition of H_2O_2 to H_2O and O_2, thus protecting the cell from oxidative damage by H_2O_2 and OH•. The main function of catalase is to detoxify H_2O_2. HLE at doses of 10 mg kg^{-1} and 300 mg kg^{-1} caused an increase in SOD. This could be attributed to transcriptional activation of the enzyme or amelioration of oxidative stress by HLE at these doses. HLE however did not affect the decreased levels of catalase induced by the arthritis. Dexamethasone and methotrexate (MTX) which are known to increase oxidative stress in rats (Orzechowski *et al.*, 2000; Gao and Horie, 2002; Orzechowski *et al.*, 2002; Miyazono *et al.*, 2004), did not also significantly reverse the arthritis-induced reduction in the levels of SOD and catalase. MTX is reported to increase the production of reactive oxygen species (Gao and Horie, 2002) as well as

MDA levels. It also decreases glutathione levels (Jahovic *et al*., 2004; Sener *et al*., 2006; Kolli *et al*., 2007). Consequently, there is oxidative stress and a reduction in the activities of the protective antioxidant enzymes like SOD and CAT as observed in this study. Glucocorticoids including dexamethasone are capable of inducing oxidative stress (Orzechowski *et al*., 2002; Bjelakovic *et al*., 2007; Bhat *et al*., 2008). Some authors have argued that glucocorticoids can enhance oxidative stress-induced cell death due to inhibition of NF-κB with resultant reduction in gene transactivation by the antioxidant response elements (AREs) (Koide *et al*., 1986; McIntosh *et al*., 1995). The consequences for cell viability are detrimental as antioxidant enzyme activities may drop dramatically (Koide *et al*., 1986; McIntosh *et al*., 1995). This may explain the observed inability to reverse the arthritis-induced fall in SOD and CAT levels in the dexamethasone-treated groups.

To assess the direct antioxidant effects of HLE, *in vitro* antioxidant assays were carried: total phenol, reducing power, total antioxidant capacity, free radical scavenging and lipid peroxidation.

The reducing capacity of a compound is a significant indicator of its potential antioxidant activity. Reducing potential is generally monitored by measuring the absorbance of Perl's Prussian blue complex at 700 nm, which increases as antioxidants reduce the ferric ion/ferricyanide complex to the ferrous form (Yazdanparast and Ardestani, 2007). The results obtained in this study clearly show that HLE has significant, concentration-dependent reducing activity but with less potency compared to standard, *n*-propyl gallate.

DPPH is a stable free radical that accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The reduction in DPPH radical was observed as reduction in its absorbance at 517 nm induced by *n*-propyl gallate and HLE acting as antioxidants. DPPH reaction has been widely used to test the ability of compounds to act as free-radical scavengers or hydrogen donors and to evaluate the antioxidant activity of foods and plant extracts (Scherer and Godoy, 2009). Both HLE and *n*-propyl gallate showed a concentration-dependent free radical scavenging activity.

Lipid peroxidation in biological systems has long been thought to be a toxicological phenomenon that can lead to various pathological consequences (Wu and Ng, 2008). The inhibitory effect of HLE on lipid peroxidation was a significant finding. HLE clearly inhibited peroxide and MDA formation suggesting that it is an antioxidant and can offer protection against biological lipid peroxidation. During the linoleic acid oxidation, peroxides are formed

which further degrade to other carbonyl compounds. Malondialdehyde (MDA) is considered a major oxidation product of peroxidised polyunsaturated fatty acids and increased MDA content is an important indicator of lipid peroxidation (Freeman and Crapo, 1981). The TBA method was used to measure the secondary stage of linoleic acid peroxidation where carbonyl compounds are formed from the degradation of the early stage peroxides (Inatani *et al.*, 1983). TBA forms a purple adduct with MDA measured at 532 nm. Although the measurement of thiobarbituric acid-reactive substances is a test lacking specificity, it is a very good indicator of sarcoplasmic reticulum lipid oxidation (Dinis *et al.*, 1993; Dinis *et al.*, 1994; Goncalves *et al.*, 2005).

From the powerful activity shown by HLE in reference to free radical scavenging, reducing capacity and inhibition of lipid peroxidation, it is clear that HLE is an antioxidant that can be classified as preventive, scavenging or chain-breaking. The mechanism of antioxidant activity of HLE can be stipulated from above findings as the reduction of free radicals as well as scavenging of reactive oxygen species and other free radicals. The observed *in vitro* activities suggest that HLE could exert protective effects also *in vivo* against oxidative and free radical injuries occurring in different pathological conditions including rheumatoid arthritis.

The antioxidant activity of HLE may be due to its phenolic and/or non-phenolic contents. The antioxidant property of many plant sources is due to phenolic compounds (flavonoids, phenolic acids, etc). The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in neutralizing free radicals, quenching singlet and triplet oxygen species, or decomposing peroxides (Rice-Evans *et al.*, 1996). The high potential of phenolic compounds to scavenge radicals may be explained by their phenolic hydroxyl groups (Sawa *et al.*, 1999). It was reasonable, therefore, to determine the total phenolics in HLE and the total phenol determination test has indeed confirmed the presence of appreciable amounts of phenolics in HLE. Though the constituents of HLE, which showed *in vitro* antioxidant activity through the reducing potential, free radical scavenging action and inhibition of lipid peroxidation is still unclear, it is possible that the antioxidant properties of HLE are caused, at least in part, by the presence of phenolic compounds. One cannot, however, rule out the other non-phenolic antioxidant compounds in HLE. These may include alkaloids, carotenoids, lignans, phytosterols and terpenes—which have been reported to possess antioxidant activity in suppressing the initiation or propagation of the chain reactions (Conforti *et al.*, 2008, Wu and Ng, 2008).

4.5 CONCLUSION

It is clear from the study that HLE has anti-inflammatory activity in both acute and chronic inflammation. HLE also has antioxidant activity, which may contribute to its anti-inflammatory activity.

Chapter 5

NEUROBEHAVIOURAL EFFECTS

5.1 INTRODUCTION

Numerous plant medicines are recognized as active on the central nervous system (CNS). In fact, they cover the whole spectrum of central activity such as psychoanaleptic, psycholeptic and psychodysleptic effects (Carlini, 2003). This fascinating incidence of CNS activity among plants can be explained by the many constituents that they contain.

The ethanolic extract of the aerial parts of *Hilleria latifolia* displayed central analgesic activity in the tail immersion test as well as the formalin test. It was therefore worthwhile to assess HLE for other CNS activities including anti-anxiety activity, antidepressant activity and general depressant activity which may have significant bearing on the main activities investigated by this study i.e. analgesic and anti-inflammatory activity. A particular case can be made for the study of the anti-anxiety and antidepressant properties of HLE because anxiety and depression usually comorbid with pain (Lepine and Briley, 2004; Gureje, 2008). Comorbidity of depression and anxiety disorders with pain has implications for the outcome of pain and also possibly for the outcome of depression and anxiety disorders (Mico *et al.*, 2006; Gureje, 2008), making depression and anxiety management during pain treatment very important. Moreover, antidepressants are now being used to treat chronic and neuropathic pain states which are generally unresponsive to conventional analgesics such as non-steroidal anti-inflammatory drugs and opiates. The establishment of HLE as an analgesic with anxiolytic and antidepressant properties means HLE may help treat pain (even chronic and neuropathic) comorbid with anxiety/depression, preventing relapse and enabling the achievement of total symptom remission (Mico *et al.*, 2006).

In this study, therefore, some neurobehavioural effects of the ethanolic extract of the aerial parts of *H. latifolia* were investigated so as to establish the CNS activity of this plant. Experimental paradigms such as elevated plus-maze, light–dark box, tail suspension and forced swimming tests were used to study effects of the plant on anxiety and depression. The hypnosedative potential of *H. latifolia* was also studied using the pentobarbitone-induced sleeping time analysis.

5.2 METHODS

5.2.1 Animals

Male ICR mice (30±5 g) were purchased from Noguchi Memorial Institute for Medical Research, University of Ghana, Accra and housed at the animal facility of the Department of Pharmacology, KNUST, Kumasi, Ghana. The animals were housed in groups of 6 in stainless steel cages (34 cm × 47 cm × 18 cm) with soft wood shavings as bedding, fed with normal commercial pellet diet (GAFCO, Tema, Ghana) and given water *ad libitum*. All behavioural experiments were carried out under dim light. To acclimatize the animals to the test conditions, they were brought to the laboratory a week before the experiments. All procedures and techniques used in these studies were in accordance with accepted principles for laboratory animal use and care (EU directive of 1986: 86/609/EEC). All protocols used were approved by the Departmental Ethics Committee.

5.2.2 Drugs

Diazepam (Sigma-Aldrich Inc., St. Louis, MO, USA) and pentylenetetrazole (Sigma-Aldrich Inc., St. Louis, MO, USA) were used as standard anxiolytic and anxiogenic drugs, respectively. Fluoxetine hydrochloride (Prozac®, Eli Lilly and Company Ltd., Basingstoke, England) was used as a reference antidepressant drug. The doses of drugs were selected based on data from literature and preliminary experiments in our laboratory.

5.2.3 Anxiolytic Effects

5.2.3.1 Elevated Plus Maze Test

This test has been widely validated for measuring anxiolytic and anxiogenic-like activities in rodents (Pellow *et al.*, 1985; Lister, 1987). It has been demonstrated to be bi-directionally sensitive to both anxiolytic drugs, in particular benzodiazepines as well as compounds which induce anxiety in man (Weiss *et al.*, 1998). The test relies on the inherent conflict between exploration of a novel area and avoidance of its aversive features.

The apparatus was made of Plexiglas and consists of two open (30 cm × 5 cm × 0.5 cm) and two closed (30 cm × 5 cm × 15 cm) arms, extending from a central platform (5 cm × 5 cm) and

elevated to a height of 60 cm above the floor. Each arm is positioned at 90° relative to the adjacent arms. A 25-W red fluorescent light 100 cm above the maze served as the source of illumination.

Mice (n=6) were randomly assigned to eleven experimental groups: vehicle-control, HLE (10, 30, 100, 300 mg kg^{-1}), diazepam (0.1, 0.3, 1.0 mg kg^{-1}) and pentylenetetrazole (3, 10, 30 mg kg^{-1}). Diazepam and pentylenetetrazole served as reference anxiolytic and anxiogenic drugs respectively. Drugs were administered 30 min (i.p.) or 1 h (*p.o.*) prior to the test. Animals were placed individually in the central platform of the EPM for 5 minutes and their behaviour recorded with a camcorder (Everio™, model GZ-MG1300, JVC, Tokyo) placed above the maze. The digitized video of each 5 min trial was later scored using public domain software JWatcher™ Version 1.0 (University of California, Los Angeles, USA and Macquarie University, Sidney, Australia available at http://www.jwatcher.ucla.edu/.) for behavioural parameters including: (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.

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:

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

5.2.3.2 *Light–Dark Exploration Test*

Anxiety-related behaviour was tested in the light–dark exploration test as described previously (Miyakawa *et al.*, 2001). The testing apparatus was a large wooden box (45 cm× 30 cm × 30 cm) modified such that two-thirds of the box was open and brightly lit and the remaining one-third

was dark and enclosed. Illumination of the open compartment of the box was by a 60 W light bulb set 50 cm above the floor. Passage between the light and dark compartments was possible via a small opening (7 cm × 7 cm) located at the base, in the middle of the partition wall.

Animals were divided into eleven groups (n=5) and received treatments similar to that described for the elevated plus maze test (Section 5.2.3.1). One hour (*p.o.*) or 30 min (i.p.) after drug administration, each mouse was placed in the dark compartment (head facing a corner) and the compartment was covered. The sessions were videotaped and later scored, in similar manner to that described above (Section 5.2.3.1), to determine the latency to enter the lit compartment, the percentage of time in the lit compartment, and the number of transitions between compartments.

5.2.4 Antidepressant Effects

5.2.4.1 Forced Swimming Test

The forced swimming test (FST) was performed according to the method described by Porsolt *et al.*(1977) with modifications (Detke *et al.*, 1995). Mice were divided into eight groups (n=6) and received HLE (10, 30, 100 or 300 mg kg^{-1}, *p.o.*), vehicle or the standard drug fluoxetine (3, 10 or 30 mg kg^{-1}, *p.o.*). One hour 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 (25-28 °C) up to a level of 20 cm and left therein for 6 minutes. 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 (Everio™, model GZ-MG1300, JVC, Tokyo) suspended approximately 100 cm above the cylinders. After each session, animals were removed from the cylinders, dried with absorbent towels and returned to their home cages.

Behaviours were scored from the videos with the aid of a software (similar to that used in section 5.2.3.1 above) for the following during the last 4 minutes of the 6-minute period: climbing , which is defined as upward-directed movements of the forepaws along the side of the swim chamber; swimming , defined as movement throughout the swim chamber, which include crossing into another quadrant; and immobility time, that was considered when the mice floated in the water without struggling and making only those movements necessary to keep its head above the water.

5.2.4.2 *Tail Suspension Test*

The tail suspension test (TST) was conducted as initially described by Steru *et al.*(1985) but with modifications (Berrocoso *et al.*, 2006). Mice were divided into eight groups (n=6) and received treatments similar to that described for the FST above (Section 5.2.4.1). One hour after administration of the test compound, mice were individually hung by the tail using an adhesive tape placed approximately 1 cm from the tip of the tail attached to a horizontal ring-stand bar 30 cm above the floor. Five animals were suspended and simultaneously videotaped (Everio™, model GZ-MG1300, JVC, Tokyo) in each session. After each session, animals were removed from the horizontal ring-stand bar and returned to their home cages.

Behaviours from recorded videos were scored with the aid of tracking software (same as used in section 5.2.4.1 above) for the last 4 minutes of the 6-minute period. The duration of immobility was measured as previously described (Steru *et al.*, 1985; Nomura *et al.*, 1992). Additional behaviours during the periods of activity (swinging, pedalling and curling) were also determined. Swinging behaviour was defined as when the animal (with the body straight) moved alternately from one side to the other. Pedalling behaviour was defined as when the animal moved its paws continuously without moving its body. Curling behaviour was defined as when the animal raised its head towards it hind paws. Immobility was assigned to when no additional activity was observed (mice hung passively and completely motionless). 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).

5.2.4.3 *Effects on Motor Coordination - Beam Walk Test*

Deficits in motor-coordination and increase in activity could invalidate conclusions drawn from the tail suspension, forced swimming and the analgesic tests. Therefore, the effect of the various treatments was assessed using the beam walk test, which has been shown to be more sensitive than the rotarod test (Stanley *et al*, 2005).

The test was carried out as described previously (Stanley *et al.*, 2005). Mice were trained to walk from a start platform along a square wooden beam (80 cm long, 3 cm wide) elevated 30 cm above the bench by wooden supports to a goal box. Three trials were performed for each mouse each day for a week, and were designed such that the mice tested would be aware that there was a goal box that could be reached.

On the day of the test experiment, mice were divided into twelve groups (n=5) and received either HLE (10, 30, 100 or 300 mg kg^{-1}, *p.o.*), the vehicle or the standard reference drugs diazepam (0.1, 0.3, 1 or 3 mg kg^{-1}, i.*p.*) or fluoxetine (3, 10 or 30 mg kg^{-1}, *p.o.*). Thirty minutes after i.p. and 1 h after oral administration of the test compounds, mice were individually moved onto the test beam (8 mm in diameter, 60 cm long and elevated 30 cm above the bench by wooden supports). The mice were placed on the beam at one end and allowed to walk to the goal box. Mice that fell were returned to the position they fell from, with a maximum time of 60 s allowed on the beam. The walk across the beam was video recorded and later scored for time taken to traverse beam and the number of foot slips (one or both hind limbs slipped from the beam).

5.2.5 CNS depressant Effects

5.2.5.1 Sleeping Time

The effect of HLE on pentobarbitone-induced sleeping time was studied in mice as described previously (de-Paris *et al.*, 2000).

Thirty-six ICR mice were randomly divided into six groups (n=6). The first group served as control and the remaining five groups received either HLE (10, 30,100 and 300 mg kg^{-1}, *p.o.*) or diazepam (1mg kg^{-1}, i.p.). Sodium pentobarbitone (40 mg kg^{-1}) was intraperitoneally administered 30 min after diazepam or 1 hour after HLE. In the case of the control, animals received only the pentobarbitone. Two parameters were registered: time elapsed since the application of pentobarbitone until the loss of the righting reflex (latency/onset of action) and the time elapsed from the loss to regaining of the righting reflex (duration of sleep).

5.2.6 Analysis of Data

All data are presented as mean ± SEM. Data were analysed using one-way analysis of variance (ANOVA) with drug treatment as a between-subjects factor. Whenever ANOVA was significant, further comparisons between vehicle- and drug-treated groups were performed using the Newman–Keuls test. GraphPad Prism for Windows Version 5 (GraphPad Software, San Diego, CA, USA) was used for all statistical analyses. $P < 0.05$ was considered significant.

5.3 RESULTS

5.3.1 Anxiolytic Effects

5.3.1.1 Elevated Plus Maze Test

Figure 5.1 and Figure 5.2 show the effects of HLE, diazepam and PTZ on conventional elevated plus-maze parameters.

ANOVA revealed that the oral treatment of mice with HLE (10-300 mg kg^{-1}) significantly affected open arm activity, that is, the number of entries ($F_{4, 20}$ = 2.679, $P < 0.05$; Figure 5.1a), percentage of open arm entries ($F_{4, 20}$ = 3.269, P = 0.0337; Figure 5.1b), and percentage time spent in the open arm of the elevated plus-maze ($F_{4, 20}$ = 4.146, P = 0.0132; Figure 5.1c). Follow-up Newman-Keuls test showed that HLE increased open arm entries ($P < 0.05$), % open arm entries ($P < 0.05$), and % time in the open arm ($P < 0.01$), with statistical significance at the dose of 300 mg kg^{-1} (Figs. 5.1a, b and c). HLE also significantly reduced risk assessment by decreasing both the percentage protected stretch-attend postures ($F_{4,19}$ = 3.478, P = 0.0271; Figure 5.2a) and percentage protected head-dips ($F_{4,19}$ = 4.952, P = 0.0066; Figure 5.2b) measures from the closed arm, reaching statistical significance at doses 30 mg kg^{-1} ($P < 0.05$) and 300 mg kg^{-1} ($P < 0.01$) (fig. 5.2a, b). HLE did not show any significant effect on any parameter at the dose of 100 mg kg^{-1} ($P > 0.05$).

The anxiolytic, diazepam (0.1 – 1 mg kg^{-1}) also increased the number of open arm entries ($F_{3,16}$ = 3.408, P = 0.0433; Figure 5.1a), percentage open arm entries ($F_{4, 20}$ = 3.720, P = 0.0351; Figure 5.1b), and percentage time spent ($F_{3,14}$ = 4.344, P = 0.0232; Figure 5.1c) in the open arm of the EPM, with statistical significance at 1 mg kg^{-1} (all $P < 0.05$). Percentage protected stretch-attend postures ($F_{3,16}$ = 5.077, P = 0.0117; Figure 5.2a) and percentage protected head-dips ($F_{3,14}$ = 4.962, P = 0.0150; Figure 5.2b) were also significantly reduced, a confirmation of its anxiolytic activity. Statistical significance as regards the effects of diazepam on the risk assessment behaviours was achieved only at 1 mg kg^{-1} ($P < 0.05$)

Pentylenetetrazole (3-30 mg kg^{-1}) significantly increased open arm avoidance by decreasing the number of entries ($F_{3,15}$ = 5.626, P = 0.0087; Figure 5.1a) and percentage time spent ($F_{3,15}$ = 7.376, P = 0.0029; Figure 5.1c) in the open arm of the EPM, with statistical significance at 30 mg kg^{-1} ($P < 0.01$). PTZ also induced an increase in risk assessment behaviour by increasing the percentage protected stretch-attend postures ($F_{3,16}$ = 2.174, P = 0.1309; Figure 5.2a) and

percentage protected head-dips ($F_{3,15}$ = 7.251, P = 0.0031; Figure 5.2b)—statistical significant increases at 30 mg kg^{-1} ($P < 0.05$) compared to the vehicle-treated group.

The extract did not significantly affect locomotor activity as there was no significant difference ($F_{4,18}$=0.833, P=0.5217; fig. 5.2c) between the total distance travelled by the extract group compared to vehicle-treated controls in the EPM. Diazepam and PTZ showed increasing ($F_{3,16}$=0.3659, P=0.7786; fig. 5.2c) and decreasing ($F_{3,12}$=2.637, P=0.0975; fig. 5.2c) trends in the total distance travelled respectively.

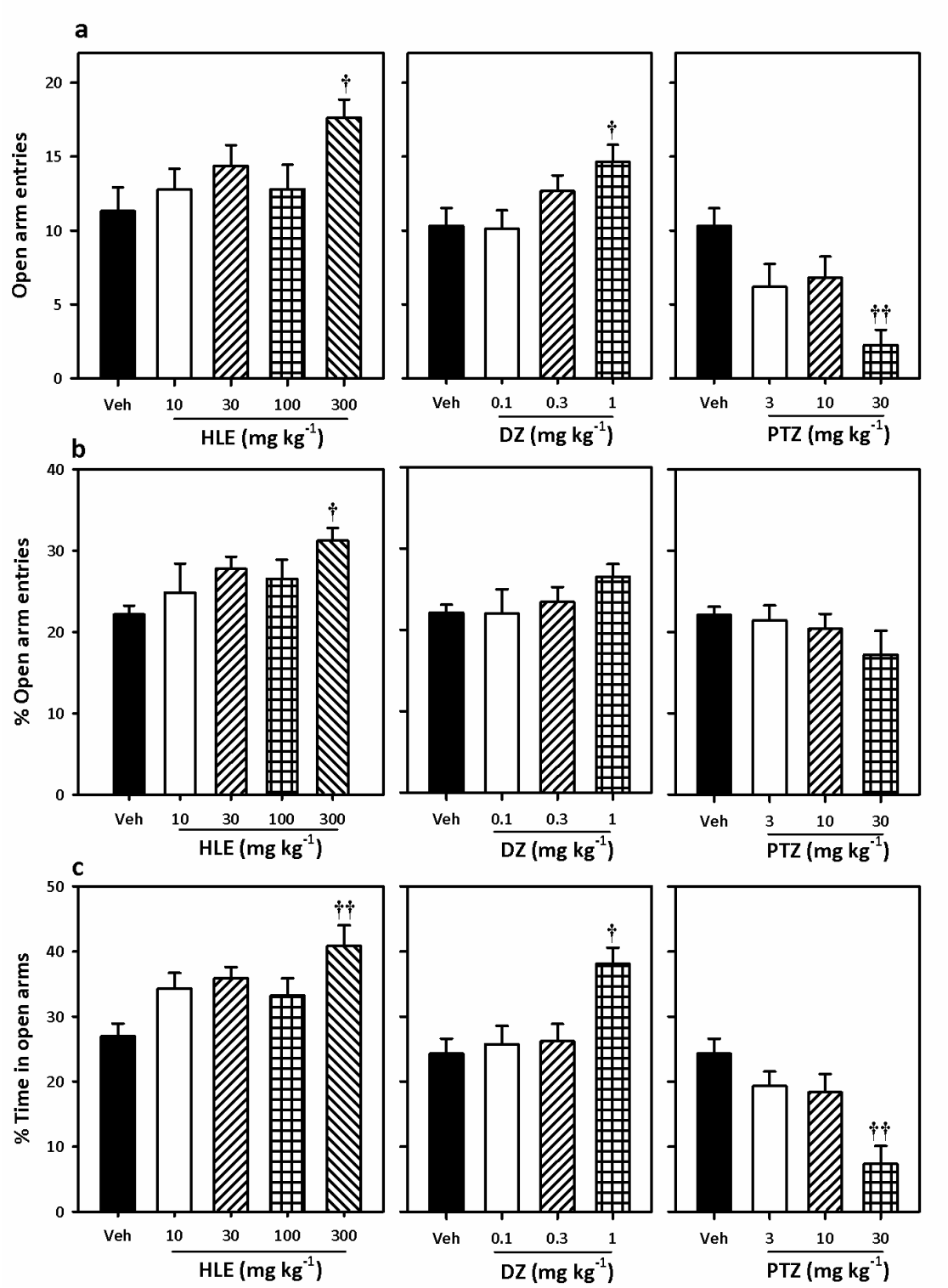

Figure 5.1 Effect of HLE (10-300 mg kg^{-1}, *p.o.*), diazepam (DZ; 0.1-1 mg kg^{-1}, i.p.) and pentylenetetrazole (PTZ; 3-30 mg kg^{-1}, i.p.) on the open arm entries (**a**), percentage open arm entries (**b**) and percentage time spent in open arms (**c**) of the elevated plus-maze over a 5 min test period in the mice. Each bar represents mean± S.E.M. (n=6). *P* values for group comparisons were obtained by one-way ANOVA followed by Student-Newman–Keuls test. †P<0.05; ††P<0.01 compared to vehicle-treated group.

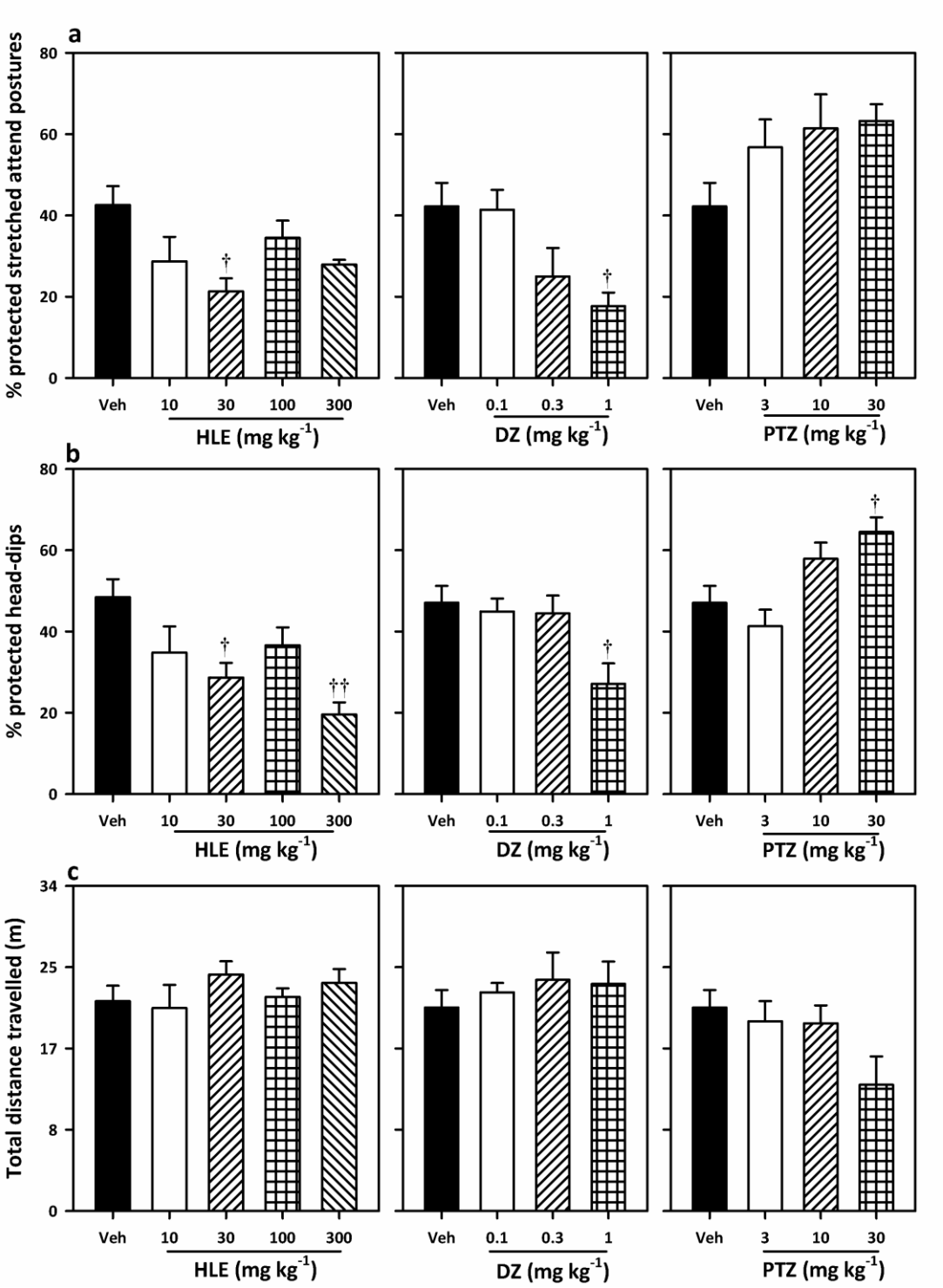

Figure 5.2 Effect of HLE (10-300 mg kg^{-1}, *p.o.*), diazepam (DZ; 0.1-1 mg kg^{-1}, i.p.) and pentylenetetrazole (PTZ; 3-30 mg kg^{-1}, i.p.) on the percentage protected stretch-attend postures (**a**), percentage protected head-dips (**b**) and total distance travelled (**c**) in the elevated plus-maze over a 5 min test period in the mice. Each bar represents mean±S.E.M. (n=6). P values for group comparisons were obtained by one-way ANOVA followed by Student-Newman–Keuls test. †P<0.05; ††P<0.01 compared to vehicle-treated group.

5.3.1.2 Light–Dark Exploration Test

In the light-dark box test, oral administration of HLE (10-300 mg kg^{-1}) induced anti-anxiety effects in mice (fig. 5.3). ANOVA indicated that HLE significantly influenced the latency to enter lit compartment ($F_{4,20}$=6.020, P=0.0024; fig. 5.3a), the number of inter-compartment transitions ($F_{4,20}$ = 5.559; P = 0.0036; fig. 5.3b) and also the time spent in the lit box ($F_{4,20}$ = 14.22; P < 0.0001; fig. 5.3c). Comparisons between the vehicle control and HLE groups (Newman-Keuls test) revealed that the HLE had significantly decreased the latency to enter lit compartment at doses of 30 and 300 mg kg^{-1} (both P < 0.05), increased the number of inter-compartment transitions at the dose of 300 mg kg^{-1} (P < 0.05) and also increased the time spent in the lit box at doses of 30 and 300 mg kg^{-1} (P < 0.05 and P < 0.01, respectively)[Figure 5.3]. HLE at doses 10 and 100 mg kg^{-1}, however, showed no significant effects (P > 0.05) [Figure 5.3].

Diazepam (0.1-1.0 mg kg^{-1}, i.p) also dose-dependently induced anti-anxiety behaviours in mice in the light-dark exploration test (fig. 5.3). Diazepam significantly increased the duration of time spent in the lit box ($F_{3,16}$=5.334; P=0.0097; fig. 5.3c) and decreased the latency to enter lit compartment ($F_{3,16}$= 5.295, P=0.01; fig. 5.3a). Diazepam also increased the number of inter-compartment transitions ($F_{3,16}$=5.416; P=0.0092; fig 5.3b).

In contrast, PTZ significantly decreased the time spent by mice in the lit box ($F_{3,16}$= 4.797; P=0.0144; fig. 5.3c) and increased significantly the latency to enter lit compartment ($F_{3,16}$=3.607; P=0.0367; fig 5.3a). PTZ also decreased the number of inter-compartment transitions ($F_{3,16}$= 4.563; P=0.0171; fig 5.3b).

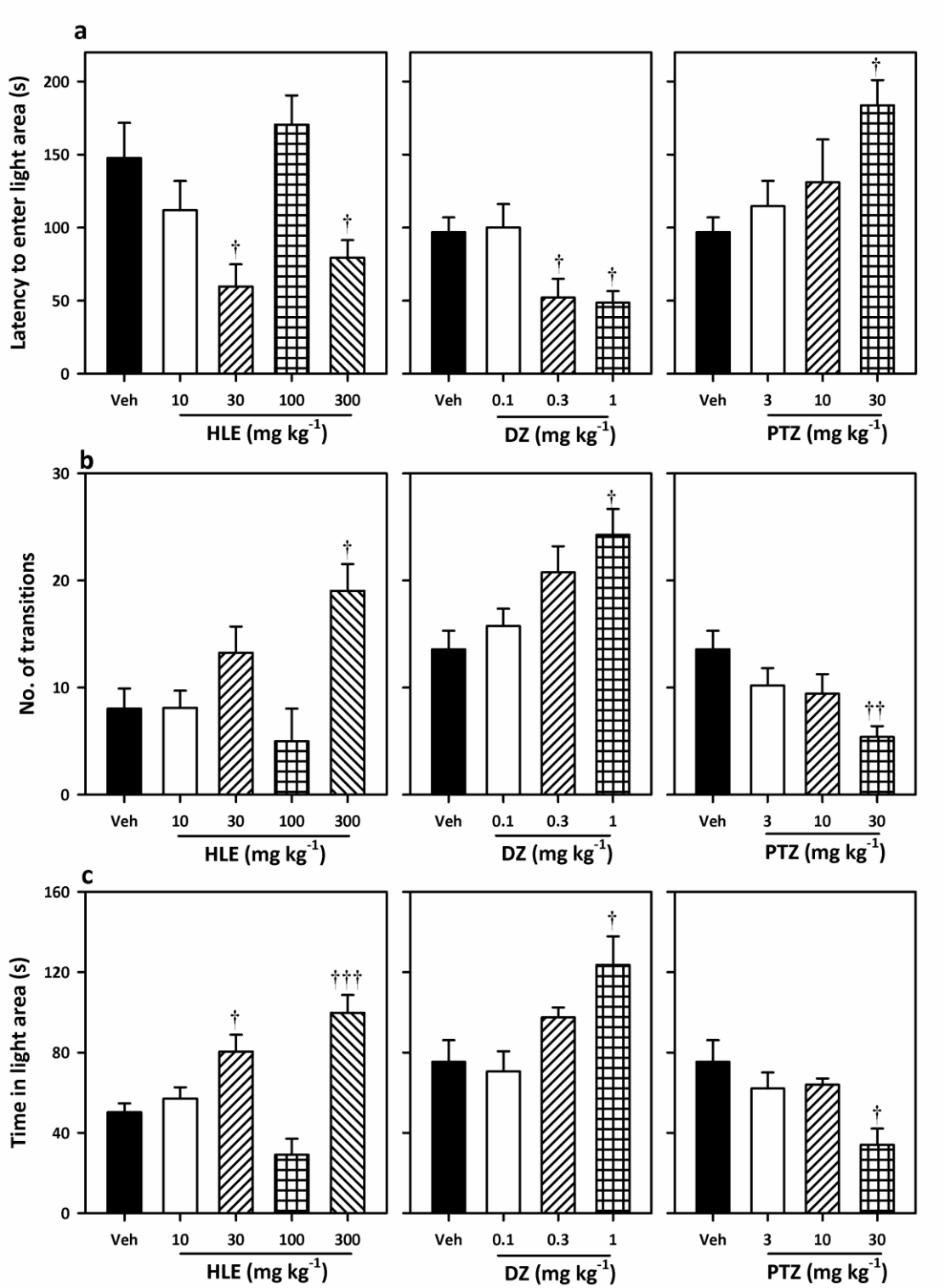

Figure 5.3 Effect of HLE (10-300 mg kg^{-1}, *p.o.*), diazepam (DZ; 0.1-1 mg kg^{-1}, i.p.) and PTZ (3-30 mg kg^{-1}, i.p.) on the latency to enter lit compartment (a), number of inter-compartment transitions (b) and time spent in the lit compartment of the light-dark box over a 5 min test period in the mice. Each bar represents mean± S.E.M. (n=5). *P* values for group comparisons were obtained by one-way ANOVA followed by Student-Newman–Keuls test. †P<0.05; ††P<0.01; †††P<0.001 compared to vehicle-treated group.

5.3.2 *Antidepressant Effects*

5.3.2.1 Forced Swimming Test

HLE (10, 30, 100 and 300 mg kg^{-1}, *p.o.*) and fluoxetine (3, 10 and 30 mg kg^{-1}, *p.o.*), all administered 1 h before the test period, significantly decreased the immobility periods of mice in the forced swimming test when compared to control group, indicating significant antidepressant activity.

HLE (10-300 mg kg^{-1}) and fluoxetine (3 – 30 mg kg^{-1}) significantly influenced the immobility time (HLE: $F_{4, 20} = 4.569$, $P = 0.0088$, fig 5.4a; Fluoxetine: $F_{3,14} = 4.82$, $P = 0.0165$, fig. 5.4b) by a maximum of 58.12 ± 7.46 % and 56.94 ± 11.20 %, respectively. The Newman-Keuls test indicated statistically significant anti-immobility effects of HLE at doses of 30 and 300 mg kg^{-1} (both $P < 0.05$) for HLE and 30 mg kg^{-1} ($P < 0.05$) for fluoxetine (Fig 5.4a, b).

The effects of HLE (10-300 mg kg^{-1}) and fluoxetine (3-30 mg kg^{-1}) on climbing and swimming time in the mouse FST are shown in figure 5.4c-f. ANOVA revealed that HLE did not significantly affect climbing ($F_{4,20}$=1.580, P=0.2183) and swimming ($F_{4,20}$=2.805, P=0.0535) times.. Fluoxetine did not significantly affect climbing ($F_{3,16}$=1.774, P=0.1926) but caused an increase in time spent swimming ($F_{3,16}$=3.641, P=0.0356). Post-hoc analysis revealed statistical significance for the effect of fluoxetine on swimming at doses 3 and 10 mg kg^{-1}(both P<0.05).

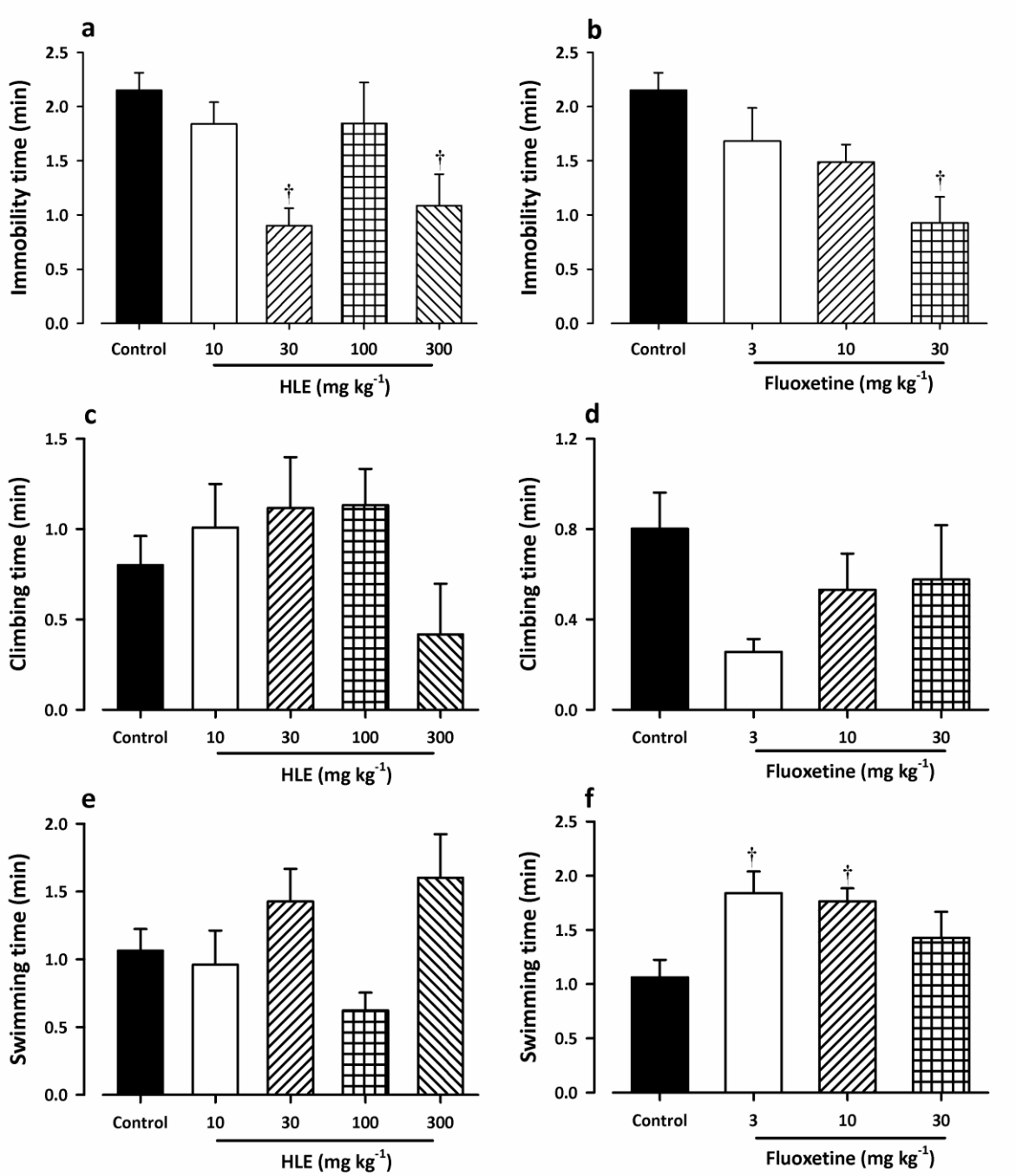

Figure 5.4 Effect of HLE (10-300 mg kg^{-1}, *p.o.*) and fluoxetine (3-30 mg kg^{-1}, *p.o.*) on immobility (**a, b**), climbing (**c, d**) and swimming (**e, f**) behaviours of mice in the forced swimming test. Each bar represents mean±S.E.M. (n=6). P values for group comparisons were obtained by one-way ANOVA followed by Student-Newman–Keuls test. †P<0.05 compared to vehicle-treated group.

5.3.2.2 Tail Suspension Test

Administration of both HLE (10-300 mg kg^{-1}, *p.o.*) and fluoxetine (3-30 mg kg^{-1}, *p.o.*) 1 h before the test period, significantly decreased the immobility periods of mice when compared to control group, indicating significant antidepressant-like activity.

HLE (10-300 mg kg^{-1}) and fluoxetine (3-30 mg kg^{-1}) significantly influenced the immobility time (HLE: $F_{4,19}$=6.034, P=0.0026, fig. 5.5a; Fluoxetine: $F_{3,13}$=9.81, P=0.0012, fig. 5.5b) by a maximum of 61.08±6.89 % and 68.35±16.29 % respectively. Newman-Keuls post-hoc test indicated statistically significant anti-immobility effects of HLE at doses of 10 mg kg^{-1} (P<0.05) and 30-300 mg kg^{-1} (all P<0.01) for HLE as well as 10 and 30 mg kg^{-1} (both P<0.01) for fluoxetine (fig. 5.5a, b).

The effects of HLE (10-300 mg kg^{-1}) and fluoxetine (3-30 mg kg^{-1}) on pedalling, curling and swinging time in the mouse FST are shown in figure 5.5c-h. ANOVA did not indicate any significant effect of fluoxetine on pedalling ($F_{3,13}$=4.747, P>0.05), curling ($F_{3,16}$=2.971, P=0.0631) or swinging ($F_{3,13}$=1.951, P=0.1713) times. HLE did not also significantly affect pedalling ($F_{4,19}$=0.9781, P=0.4428) and swinging ($F_{4,19}$=2.538, P=0.0738) times but caused an increase in time spent curling ($F_{4,20}$=3.733, P=0.0200) which reached statistical significance at doses 100 and 300 mg kg^{-1} (both P<0.05).

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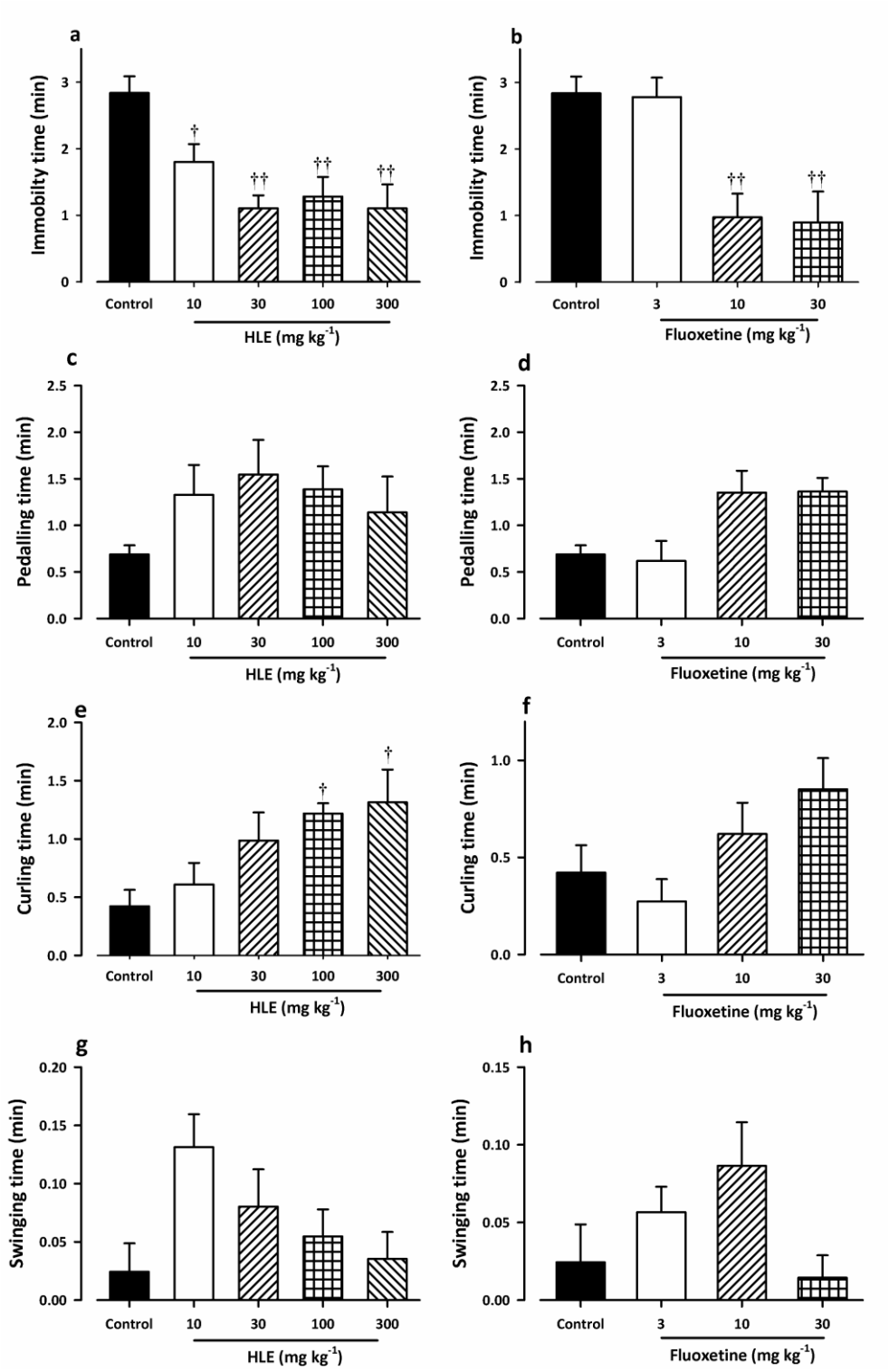

Figure 5.5 Effect of HLE (10-300 mg kg^{-1}, *p.o.*) and fluoxetine (1-10 mg kg^{-1}, *p.o.*) on the immobility (**a**, **b**) pedalling(**c**, **d**), curling (**e**, **f**) and swinging (**g, h**) behaviours of mice in the tail suspension test. Each bar represents mean±S.E.M. (n=6). *P* values for group comparisons were obtained by one-way ANOVA followed by the Newman-Keuls test. †P<0.05; ††P<0.01 compared to vehicle-treated group.

5.3.2.3 *Beam Walk Test*

Figure 5.6 shows the results of the effect of HLE, diazepam and fluoxetine on motor co-ordination in the mouse beam walk test. ANOVA revealed that pre-treatment of mice with HLE (10-300 mg kg^{-1}, *p.o.*) did not significantly affect the time taken by mice to reach the goal box ($F_{4, 19}$ = 1.09, P=0.3913) or the number of foot slips of mice during beam traversal ($F_{4, 19}$=0.18, P=0.9457).

The effect of fluoxetine (3-30 mg kg^{-1}, *p.o.*) was similar to that of HLE: there was no significant effect on the time taken by mice to reach the goal box ($F_{3, 14}$=1.868, P=0.1814) or the number of foot slips of mice ($F_{3, 14}$=2.984, P=0.0673).

In contrast to HLE and fluoxetine, diazepam (0.1-3 mg kg^{-1}, i.p.) significantly and dose-dependently increased ($F_{4, 18}$=4.963, P=0.0071) the time taken by mice to reach the goal box as well as the number of foot slips of mice during beam traversal ($F_{4,16}$=15.15, P<0.0001). Newman-Keuls post-hoc analysis revealed significant effects of diazepam on the time taken by mice to reach the goal box at the dose of 3 mg kg^{-1} (P<0.001). There was also a significant effect on the number of foot slips of mice at doses of 1 and 3 mg kg^{-1} (P<0.05 and P<0.001 respectively).

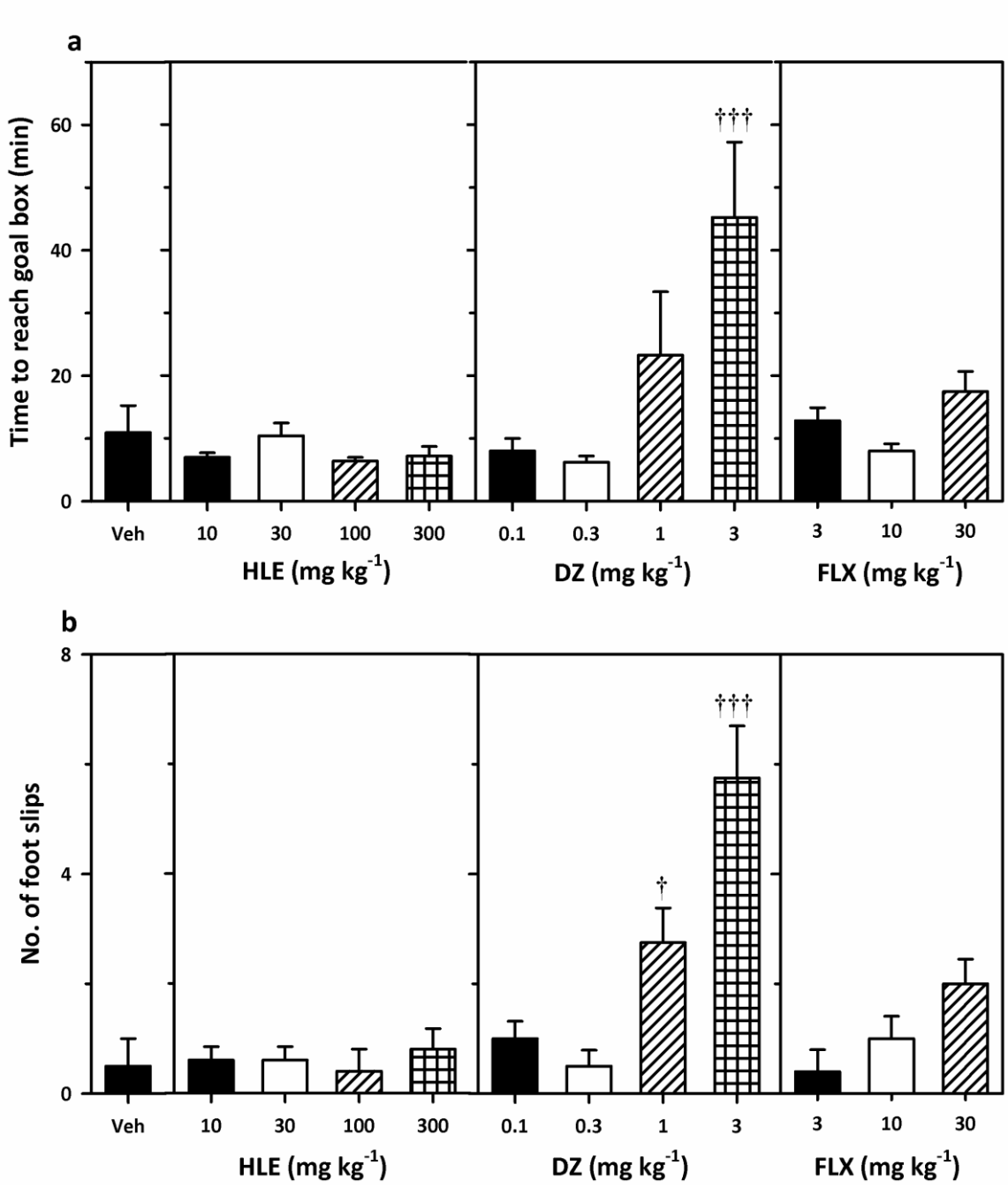

Figure 5.6 Effect of HLE (10-300 mg kg^{-1}), diazepam (DZ; 0.1-3 mg kg^{-1}) and fluoxetine (FLX; 3-30 mg kg^{-1}) on the time taken to reach goal box (**a**) and number of foot slips (**b**) in the mouse beam walk test. Each bar represents mean±S.E.M. (n=5). *P* values for group comparisons were obtained by One-way ANOVA followed by Student-Newman–Keuls *post hoc* test. †P<0.05; †††P<0.001 compared to vehicle-treated group.

5.3.3 General CNS Depressant Effects

5.3.3.1 Sleeping time

Figure 5.7 shows the results of the effect of HLE and reference drug diazepam on pentobarbitone sleeping time. Pre-treatment with HLE (10-300 mg kg^{-1}, *p.o.*) dose-dependently increased ($F_{5, 24}$=6.06, P<0.001) the latency to sleep induced by sodium pentobarbitone (40 mg kg^{-1}, i.p.). There was, however, no significant change in the sleep duration. Diazepam (1 mg kg^{-1}, i.p.) decreased the time of onset of sleep and increased the sleeping time (P<0.05) when administered 30 min before pentobarbitone sodium.

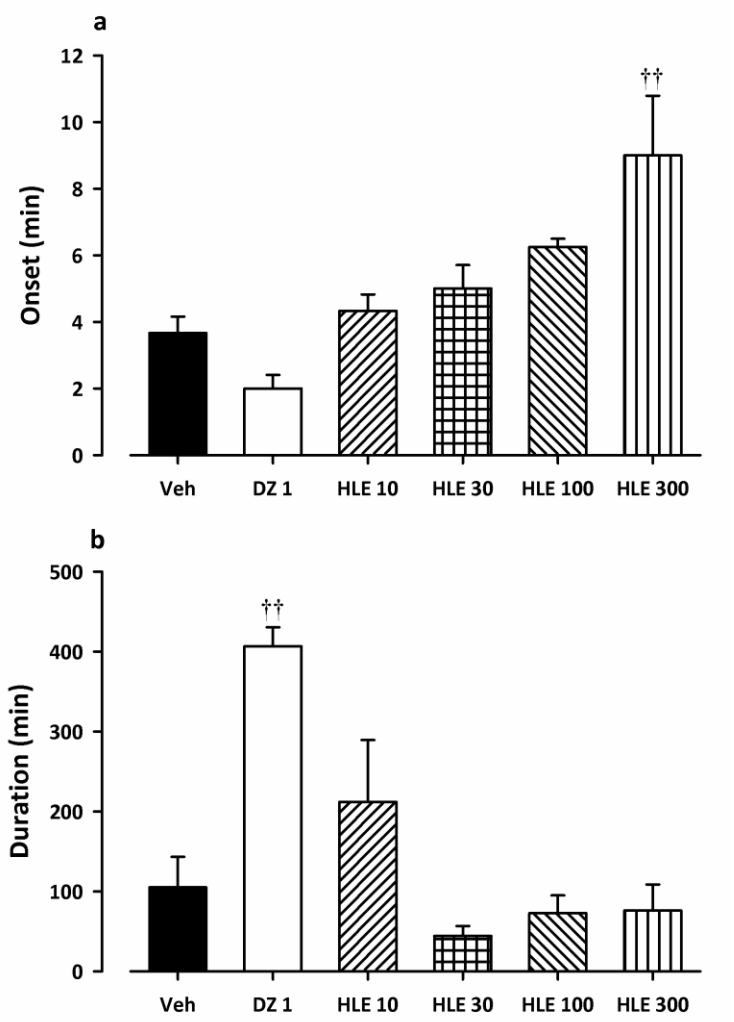

Figure 5.7 Effect of HLE (10-300 mg kg^{-1}) and diazepam (1 mg kg^{-1}) on the onset (**a**) and sleep duration (**b**) induced by sodium pentobarbitone (40 mg kg^{-1}). Each bar represents mean±S.E.M. (n=4-6). †P<0.05; ††P<0.01 compared to vehicle-treated group (One-way ANOVA followed by Newman-Keuls multiple comparison test).

5.4 DISCUSSION

This study assessed some neurobehavioural effects of HLE and has established that it has anxiolytic and antidepressant activities. However, HLE does not have sedative effects at the doses tested.

Anxiety is usually comorbid with pain. Since comorbidity of anxiety disorders with pain has implications for the outcome of pain and also possibly for the outcome of anxiety disorders (Gureje, 2008), anxiety management during pain treatment is very important. In this regard, therefore, drugs with both analgesic and anxiolytic effects will be particularly needful—necessitating the study of the effects of HLE on anxiety. The behavioural models used to assess the anti-anxiety effect of HLE in this study are based on unconditioned responses to stimuli which are thought to be indicative of human generalized anxiety symptoms (Crawley, 1999; Ohl, 2005).

The elevated plus-maze test is probably the most popular of all currently available animal models of anxiety, and affords an excellent example of a model based on the study of unconditioned or spontaneous behaviour (Dawson and Tricklebank, 1995; Rodgers and Dalvi, 1997; Carobrez and Bertoglio, 2005). It derives from some elegant early work on exploratory patterns (Montgomery, 1955), the basic premise of which was that environmental novelty simultaneously evokes fear and curiosity, thereby creating a typical approach-avoid conflict. The EPM test is frequently used as a tool to screen anxioselective effects of drugs (Handley and Mithani, 1984; Pellow *et al.*, 1985; Lister, 1990) and has been demonstrated to be bi-directionally sensitive to both anxiolytic drugs; in particular benzodiazepines (Handley and Mithani, 1984; Pellow *et al.*, 1985; Lister, 1987), as well as compounds which induce anxiety in man (Pellow *et al.*, 1985; Pellow and File, 1986; Lister, 1987). Conventional anxiety indices in the EPM comprise spatiotemporal measures i.e. the number of entries into and the time spent in the open arms of the EPM, with anxiolytics generally increasing and anxiogenics decreasing these measures (Chen *et al.*, 2006). In agreement with previously published reports (Dalvi and Rodgers, 1996; Rodgers *et al.*, 1997), diazepam which is anxiolytic increased the percentage time spent in open arms and open arm entries while PTZ (an anxiogenic) showed opposite effects. In the present study, oral administration of HLE induced an anxiolytic-like effect in mice, since it increased open arm entries and the percentage time spent on open arms of the EPM test.

In addition to using the spatiotemporal indicators of anxiety in the EPM, ethological measures of "risk assessment", such as stretch-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 and Johnson, 1995; Rodgers *et al*., 1997). Both HLE and diazepam were able to markedly decrease the percentage protected forms of both stretch-attend postures and head-dipping indicating reduced anxiety/fear related behaviours. PTZ however increased the protected forms of the risk assessment behaviours which is consistent with its anxiogenic activity.

Changes in locomotor activity can cofound the interpretation of results obtained from the EPM test (Dawson and Tricklebank, 1995; Weiss *et al*., 1998) especially when using only conventional spatiotemporal indicators of anxiety. A non-anxiolytic agent can appear anxiolytic (false positive) if it stimulates motor activity; either 'time on the open arms', or 'number of open-arm entries', or both, are increased and the total number of arm entries is unchanged. Conversely, an agent that causes motor impairment or sedation appears anxiogenic, decreasing time on the open arms, but it does so because it markedly reduces locomotor activity. These scenarios make it imperative to assess locomotor activity of animals in the EPM test. In this study, the extract did not have much effect on the total distance travelled in the EPM implying that HLE actually showed anxioselective behaviours.

The light-dark exploration test is also widely used in rodents as a model for screening anxiolytic or anxiogenic drugs. The test exploits the ethological conflict between the tendencies of mice to explore a novel environment and to avoid a brightly lit, open area (File *et al*., 2004). An increase in inter-compartment transitions without an increase in spontaneous locomotion is considered to reflect anxiolytic activity (Bourin and Hascoet, 2003). Another parameter, the latency time for the first passage from the dark compartment to the light one, has also been used by some other authors (Costall *et al*., 1989; Costall *et al*., 1993). However, the measurement found to be most consistent and useful for assessing anxiolytic-like action is the time mice spend in the lit area, because this parameter provides the most consistent dose–effect results with drugs (Hascoet and Bourin, 1998). In this experiment diazepam and HLE showed anxiolytic effect by decreasing the latency to enter the light compartment and increasing both inter-compartment transitions and time spent in the light. PTZ also showed results consistent with its anxiogenic activity, increasing the latency to enter the light compartment and decreasing both inter-compartment transitions and the time spent in the light.

Pain and depression are often linked, and several studies have indicated that pain and depression share common neurochemical mechanisms (Fishbain, 2000; Blackburn-Munro and Blackburn-Munro, 2001; Suzuki *et al*., 2004). It is therefore not surprising that antidepressants have shown anti-nociceptive effects in various experimental pain models specially those for chronic and neuropathic pain (Sawynok and Reid, 2001; Abdel-Salam *et al*., 2003; Bomholt *et al*., 2005). Antidepressants are also prescribed clinically for their specific analgesic, rather than mood altering effects, against chronic and neuropathic pain and their efficacy in this regard is well established (Onghena and Van Houdenhove, 1992; McQuay *et al*., 1996). This necessitates, from a preclinical viewpoint of pain research, that the search of new analgesics take into account also the antidepressant profile. The antidepressant effect of HLE was, thus, investigated in the FST and TST.

The FST has the ability to induce a state of immobility in animals, which is claimed to reproduce a condition similar to human depression (Willner, 1984). The duration of immobility, which is a measure of despair, is reduced by antidepressants. This model is widely accepted to screen antidepressant drugs, as they are sensitive to all major classes of antidepressants including tricyclics, serotonin-specific reuptake inhibitors, monoamine oxidase inhibitors, and atypicals (Porsolt *et al*., 1977). Modifications have been introduced in the method concerning the water depth and the scoring. The modified FST, besides allowing observing the effects of SSRIs, was found to be very useful in distinguishing that group of compounds from the compounds acting *via* the inhibition of noradrenaline reuptake. The latter group, as for example reboxetine or desipramine, increases climbing, while SSRIs rather swimming behaviour (Kuoemider *et al*., 2007). In the present study, HLE and fluoxetine displayed antidepressant-like activity in the FST by reducing the duration of immobility. Further studies with selective antagonists are necessary to establish the exact mechanism of action of HLE. In agreement with previous reports (Detke *et al*., 1995; Page *et al*., 1999), fluoxetine, a SSRI, increased swimming behaviour without any significant effect on climbing.

The TST is one of the most widely used pharmacological models for assessing antidepressant activity (Cryan *et al*., 2005). Mice develop immobility when they are suspended by the tail from a horizontal ring-stand bar. The immobility behaviour is thought to reflect either a failure to persist in escape-directed behaviour after persistent stress or the development of passive behaviour that disengages the animal from active forms of coping with stressful stimuli. A broad spectrum of antidepressant drugs selectively reduces the development of behavioural

immobility in the TST. However, the paradigm in its traditional form is unreliable at detecting the neurochemical profile of distinct antidepressant-like drugs. Modifications have been made in terms of the measurement of specific behavioural components of active behaviours in the TST to help differentiate between standard/classical antidepressants and other compounds with antidepressant-like effects but with different mechanisms of actions, such as opiates (Berrocoso *et al.*, 2006). Whilst the classical antidepressants have not shown any significant modification to other parameters apart from immobility, opioid compounds produce stereotyped behavioural patterns, decreasing the pedalling behaviour and increasing the curling behaviour in the TST (Berrocoso *et al.*, 2006). In this present study, HLE and fluoxetine showed significant antidepressant-like effects by decreasing immobility. Except for an increase in curling, HLE did not significantly affect pedalling and swinging behaviour. Mechanistic studies with selective antagonists and other pharmacological agents are needed to delineate the exact underlying mechanisms of HLE.

Many drugs are reported to affect several aspects of motor function, resulting in false assumptions about the drugs' effects, which is especially important in the FST and TST which are based on a motor response of the animals. Since one cannot discard the possibility that the results from the FST and TST are merely due to a general stimulation of the animals' motor activity, assessment of the effect of HLE on motor function was carried out. HLE at the doses used in the TST and FST did not impair motor coordination in the beam walk test, which has been shown by Stanley *et al.* (2005) to be more sensitive than the rota rod test in detecting motor deficits. HLE did not also significantly cause changes in locomotor activity when assessed by way of the total distance travelled in the EPM test. These observations strongly indicate that the reduction in the immobility time is due to a selective antidepressant-like effect of HLE and not merely a result of a general stimulation of the animal's motor activity.

It is worthy to point out that HLE showed a non-monotonic dose-response relationship as regards its anti-anxiety and antidepressant activities. Further studies are needed to explain the cause of this observed pharmacological inversion.

To assess, if any, the central nervous system depressant effects, HLE was examined in pentobarbitone-induced sleep test. Decrease in sleep latency and increase in sleeping time are classically related to CNS depressants. However, this test is not specific because compounds that interfere with biotransformation of pentobarbitone by cytochrome P450 complex can

show the same effects as CNS depressants (de-Paris *et al.*, 2000). HLE did not potentiate the induced sleep indicating that it may not exert any depressant effect at the doses tested. A study of the effects of HLE on cytochrome P450 enzymes may be needed to give conclusive comments on the observed dose-dependent increase in the sleep latency.

5.5 CONCLUSION

The ethanolic extract of the aerial parts of *H. latifolia* has anxiolytic and antidepressant properties but not CNS depressant effects at the doses tested.

Chapter 6

TOXICITY STUDIES

6.1 INTRODUCTION

Phytotherapeutic products contain bioactive principles with potential to cause adverse effects and should, therefore, be tested for safety (Bent and Ko, 2004; Gurib-Fakim, 2006). In the determination of the safety of drugs and plant products for human use, toxicological evaluation is carried out in various experimental animals to predict toxicity and to provide guidelines for selecting a 'safe' dose in humans.

The highest overall concordance of toxicity in animals with humans is with haematological, gastrointestinal, and cardiovascular adverse effects (Olson *et al.*, 2000), while certain adverse effects in humans, especially hypersensitivity and idiosyncratic reactions, are poorly correlated with toxicity observed in animals. Furthermore, it is quite difficult to ascertain certain adverse effects in animals such as headache, abdominal pain, dizziness and visual disturbances. In addition, interspecies differences in the pharmacokinetic parameters make it difficult to translate some adverse effects from animals to humans (Rhiouani *et al.*, 2008). Nevertheless, the evaluation of adverse effects of acute and chronic dosing in experimental animals is crucial in determining the overall toxicity of drug or plant preparations. Acute toxicity (single dose) 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). Repeated-dose (sub-acute, sub-chronic and chronic) toxicity study provides data for predicting the maximum tolerated levels for the species during potential lifetime exposure. The results of repeated-dose toxicity studies together with 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).

Notwithstanding the widespread use of *Hilleria latifolia* in Ghanaian traditional medicine, no scientific study has been reported on its toxicity. Information regarding toxicity of *H. latifolia* from traditional use has also been controversial. While the plant is reported to be fatal to sheep, goats, snails and humans in some parts of west Africa, it is also known to be used as vegetable, a relish to tortoise meat and in soup (Dokosi, 1998; Schmelzer and Gurib-Fakim, 2008). The present study, therefore, determined the toxicity of the ethanolic extract of the aerial parts of *Hilleria latifolia* after acute and sub-acute oral administration in rats, with the aim to obtain

information on the safety *Hilleria latifolia* and provide guidance for selecting a safe dose of *Hilleria latifolia* in its use in traditional medicine.

6.2 METHODS

6.2.1 ACUTE TOXICITY

Male Sprague-Dawley rats (200-230 g) were randomly divided into seven groups (n=5) and kept in the experimental environment for an acclimation period of 1 week. The animals were fasted overnight, but with access to water *ad libitum*, and then treated orally with *H. latifolia* extract in doses of 300, 450, 600, 900, 1200, and 3000 mg kg^{-1} of body weight. The control group received 10 ml kg^{-1} *p.o.* of saline. The rats were observed up to 24 hours for general changes in behaviour and physiological function as well as mortality. The assessment of behaviour and physiological function was carried out similar to the primary observation procedure (Irwin test) originally described by Irwin (1968). In accordance with the Irwin test, the rats were observed at 0, 15, 30, 60, 120 and 180 min, and 24 h after treatment for behaviours specifically related to neurotoxicity, such as convulsions and tremor, for behaviours related to CNS stimulation, such as excitation, Straub tail, jumping, hypersensitivity to external stimuli, stereotypies, and aggressive behaviour, and for behaviours related to CNS depression, such as sedation, rolling gait, loss of balance, loss of traction, motor incoordination, hyposensitivity to external stimuli, decreased muscle tone, akinesia, catalepsy, and hypothermia. Effects on autonomic functions, such as respiration, body temperature, salivation, urination and defaecation, were also noted.

6.2.2 SUB-ACUTE TOXICITY

Male Sprague-Dawley rats (200-230 g), 5 per group, were treated orally with *H. latifolia* (300, 1200 and 3000 mg kg^{-1}) 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 daily doses of extract i.e. 300, 1200 and 3000 mg kg^{-1} *p.o.* respectively. The extract was prepared such that not more than 2 ml was given orally. The animals were monitored closely for signs of toxicity. Appearance and behaviour pattern were assessed daily and any abnormalities in food and water intake were registered.

6.2.2.1 Preparation of Serum and Isolation of Organs

The rats were sacrificed on the fifteenth day by cervical dislocation, the jugular vein was cut and blood flowed freely. About 1.5 ml of blood was collected into vacuum tubes 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 sample tubes without anticoagulant. The blood without the anticoagulant was allowed to clot before centrifugation (4000 rpm at 4 °C for 10 min) to obtain serum, which was collected and stored at -20ºC until assayed for biochemical parameters the next day. After collecting blood, the rats were quickly dissected and the organs (spleen, liver, kidney and stomach) removed, freed of fat and connective tissue, blotted with clean tissue paper and then weighed on a balance.

6.2.2.2 Effect of Extract on Haematological Parameters

Haematological parameters including haemoglobin (HGB), 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 (BC-3000 Plus Auto Hematology Analyzer, Shenzhen Mindray Bio-Medical Electronics Co. Ltd, China).

6.2.2.3 Effect of Extract on Serum Biochemical Parameters

Biochemical analyses were performed on serum collected for the determination of the following parameters: fasting blood glucose, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total bilirubin (T-BIL), direct bilirubin (D-BIL), indirect bilirubin (I-BIL), total protein, albumin, calcium, urea and creatinine. All analyses were carried out using an automated clinical chemistry analyser (Flexor Junior®, Vital Scientific, AC Dieren, The Netherlands).

6.2.2.4 Effect of Extract on Body and Organ Weights in Rats

Body weights of the rats were taken on days 0 and 15. The relative organ weight (ROW) of each organ was calculated as follows:

$$\text{ROW} = \frac{\text{Absolute Organ Weight (g)}}{\text{Rat body weight on sacrifice day (g)}} \times 100$$

6.2.2.5 Histopathological Examination

Portions of the tissue from liver, kidney, spleen and stomach were used for histopathological examination. Tissues were fixed in 10 % neutral 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 (BX-51) and photographed by INFINITY 4 USB Scientific Camera (Lumenera Corporation, Otawa, Canada).

1.1.4 Analysis of Data

Data were presented as mean±SEM. The presence of significant differences among means of groups was determined by one-way ANOVA using GraphPad Prism for Windows version 5 (GraphPad Software, San Diego, CA, USA). Significant difference between pairs of groups was calculated using the Newman-Keuls Multiple Comparison Test.

6.3 RESULTS

1.1.5 Acute Toxicity

All the rats survived throughout the study period (24 h). During observation, the animals at the dose of 300 mg kg^{-1}(NOAEL) did not exhibit any toxic signs. However at 450, 600, 900, 1200 and 3000 mg kg^{-1}, the rats displayed asthenia and increased defaecation, salivation, urination compared to untreated controls (Table 6.1; Appendix 8.2).

Table 6.1 Observations in the acute toxicity test after oral administration of HLE in rats.

Dose(mg kg^{-1})	Mortality		Toxicity signs
	D/T	Latency(h)	
0	0/5	-	None
300	0/5	-	None
450	0/5	-	Asthenia, defaecation, salivation, urination
600	0/5	-	Asthenia, defaecation, salivation, urination
900	0/5	-	Asthenia, defaecation, salivation, urination
1200	0/5	-	Asthenia, defaecation, salivation, urination
3000	0/5	-	Asthenia, defaecation, salivation, urination

The ethanolic extract of *H. latifolia* in distilled water, was administered orally; each dose was administered to groups of 5 rats. Observation for signs of toxicity were performed at 15, 30, 60, 120, 180 min and 24 hr after administration. The symptoms that did not necessitate handling were also observed up to 15 min immediately following administration. D/T: dead/treated rats; None: no toxic symptoms were seen during the observation period; latency: time to death (in hours) after the dose.

1.1.6 Sub-Acute Toxicity

All the rats survived throughout the 14 days. There were no extract-related changes noted in behaviour, activity, posture, or external appearance in rats that received 300 mg kg^{-1} of HLE. However, there were signs of sedation, defaecation and urination on the first two days after administration of HLE in doses of 1200 and 3000 mg kg^{-1}. These signs slightly receded from the third day.

1.1.6.1 Effect of Extract on Haematological Parameters

There were generally no significant differences noted between control and treated groups for the parameters measured (Table 6.2).

Table 6.2 Haematological values of control and rats treated with *H. latifolia* for 14 days.

Parameters	*Hilleria latifolia* extract (mg kg^{-1})				F	*P* Value
	0	300	1200	3000		
WBC ($\times 10^9$/L)	4.00±0.47	2.90±0.23	4.48±0.75	3.44±0.53	$F_{3,15}$=1.695	0.2107
LYM (%)	64.45±3.33	68.76±2.02	62.10±6.21	60.38±2.92	$F_{3,13}$=1.001	0.4233
MID (%)	8.58±0.31	7.28±0.42	9.73±0.93	8.13±0.34	$F_{3,13}$=3.611	0.0529
GRAN (%)	26.98±3.06	23.96±1.85	28.18±5.30	31.50±3.06	$F_{3,13}$=0.890	0.4724
RBC ($\times 10^{12}$/L)	8.50±0.23	7.97±0.20	7.54±0.34	7.74±0.12	$F_{3,13}$=2.863	0.0776
HGB(g/dL)	15.58±0.35	14.76±0.40	13.73±0.59	14.42±0.18	$F_{3,13}$=2.782	0.0829
HCT (%)	45.95±0.94	43.82±1.23	40.35±1.85	42.80±0.80	$F_{3,13}$=2.233	0.1329
MCV(fL)	54.18±0.86	55.00±0.70	53.63±0.67	55.38±0.47	$F_{3,15}$=1.601	0.2310
MCH (pg)	18.30±0.25	18.44±0.20	18.18±0.23	18.60±0.15	$F_{3,15}$=0.990	0.4240
MCHC (g/dL)	33.83±0.15	33.64±0.13	33.98±0.15	33.66±0.24	$F_{3,15}$=0.702	0.5657
RDW-CV (%)	14.70±0.17	15.00±0.17	15.00±0.14	15.08±0.15	$F_{3,15}$=1.101	0.3795
RDW-SD(fL)	29.78±0.60	29.60±0.54	29.43±0.34	30.14±0.45	$F_{3,15}$=0.559	0.6499
Platelets ($\times 10^9$/L)	704.25±42.56	975.80±178.93	1020.25±283.65	853.60±229.51	$F_{3,15}$=0.434	0.7321

Values are mean±S.E.M. (n=4-5). Treated groups were compared to control group using one-way ANOVA followed by Newman-Keuls *post hoc* test.

Table 6.3 Clinical biochemistry values of control and rats treated with *H. latifolia* for 14 days.

Parameters	*Hilleria latifolia* extract (mg kg^{-1})				F	*P* value
	0	300	1200	3000		
ALBUMIN(g/L)	35.10±0.35	28.54±2.33	20.18±3.28**	25.50±2.26*	$F_{3,14}$=5.146	0.0132
GLOBULINS(g/L)	29.81±0.91	24.90±2.53	13.34±3.13**	14.78±0.61**	$F_{3,7}$=16.28	0.0015
TOTAL PROT(g/L)	65.53±1.06	49.52±4.22	34.86±6.245**	40.13±3.52*	$F_{3,16}$=6.340	0.0070
A/G RATIO	1.18±0.03	1.40±0.12	1.43±0.09	1.50±0.03	$F_{3,16}$=0.590	0.6355
D-BIL(µmol/L)	1.65±0.12	0.90±0.20*	0.80±0.15**	0.93±0.07**	$F_{3,8}$=10.43	0.0039
I-BIL(µmol/L)	0.36±0.06	0.12±0.05	0.25±0.09	0.19±0.02	$F_{3,8}$=2.679	0.1179
T-BIL(µmol/L)	1.98±0.09	1.05±0.25*	1.07±0.20**	1.10±0.12**	$F_{3,8}$=10.70	0.0036
ALT(U/L)	107.55±6.19	75.80±11.90	67.91±13.17	80.90±5.42	$F_{3,8}$=3.430	0.0658
AST(U/L)	165.10±19.41	118.15±34.65	96.37±25.68	115.60±11.42	$F_{3,8}$=2.283	0.1478
ALP(U/L)	467.68±81.24	471.55±50.45	416.93±128.29	499.27±44.47	$F_{3,8}$=0.144	0.9305
UREA(mmol/L)	3.80±0.84	2.89±0.98	3.57±1.18	5.39±1.25	$F_{3,8}$=0.853	0.5033
CREATININE(mmol/L)	45.00±2.27	42.45±4.45	43.77±4.67	52.17±3.11	$F_{3,8}$=2.619	0.1228
GLUCOSE(mmol/L)	5.99±0.32	6.50±0.73	5.90±1.05	6.75±0.25	$F_{3,8}$=0.321	0.8101
CALCIUM(mmol/L)	2.42±0.13	1.94±0.16	1.99±0.50	2.30±0.17	$F_{3,8}$=0.687	0.5848

Values are mean±S.E.M. (n=4-5). *P<0.05; **P<0.01 compared to vehicle-treated group (one-way ANOVA followed by Newman-Keuls *post hoc* test).

1.1.6.2 Effect of Extract on Serum Biochemical Parameters

There were no statistically significant differences between control and treated groups for the biochemical parameters measured except for decrease in the levels of albumin ($F_{3,14}$=5.146, P<0.05), globulins ($F_{3,7}$=16.28; P<0.01), total protein ($F_{3,16}$=6.34; P<0.01), direct bilirubin ($F_{3,8}$=10.43; P<0.01) and total bilirubin ($F_{3,8}$=10.70; P<0.01)(Table 6.3).

1.1.6.3 Effect of Extract on Body and Organ Weights in Rats

Generally, rats in all experimental groups gained weight over the course of this study. The percentage changes in body weights were greater for groups treated with HLE (300 and 1200 mg kg^{-1}) when compared to controls but were not statistically significant (P>0.05 in all comparisons) (Fig. 6.1). There was also no statistically significant differences in relative organ weights (ROW) between treated and control groups (Fig. 6.2).

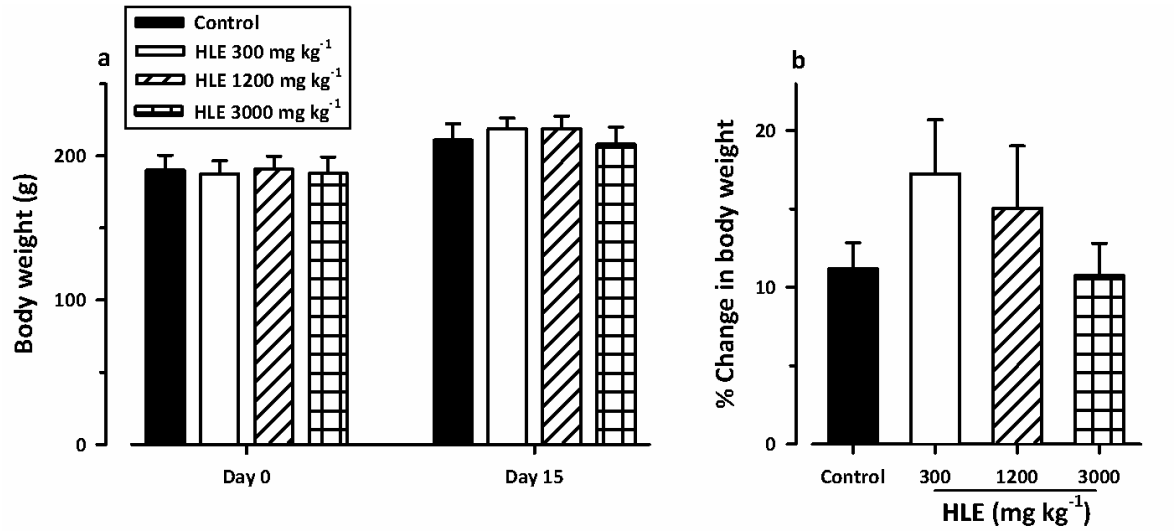

Figure 6.1 Effect of oral administration of *Hilleria latifolia* extract on the absolute body weight (**a**) and % change in body weights (**b**) of rats in the sub-acute toxicity test. Data are expressed as mean±S.E.M. (n=5). Treated groups were compared to controls using a one-way ANOVA followed by Newman-Keuls *post hoc* test.

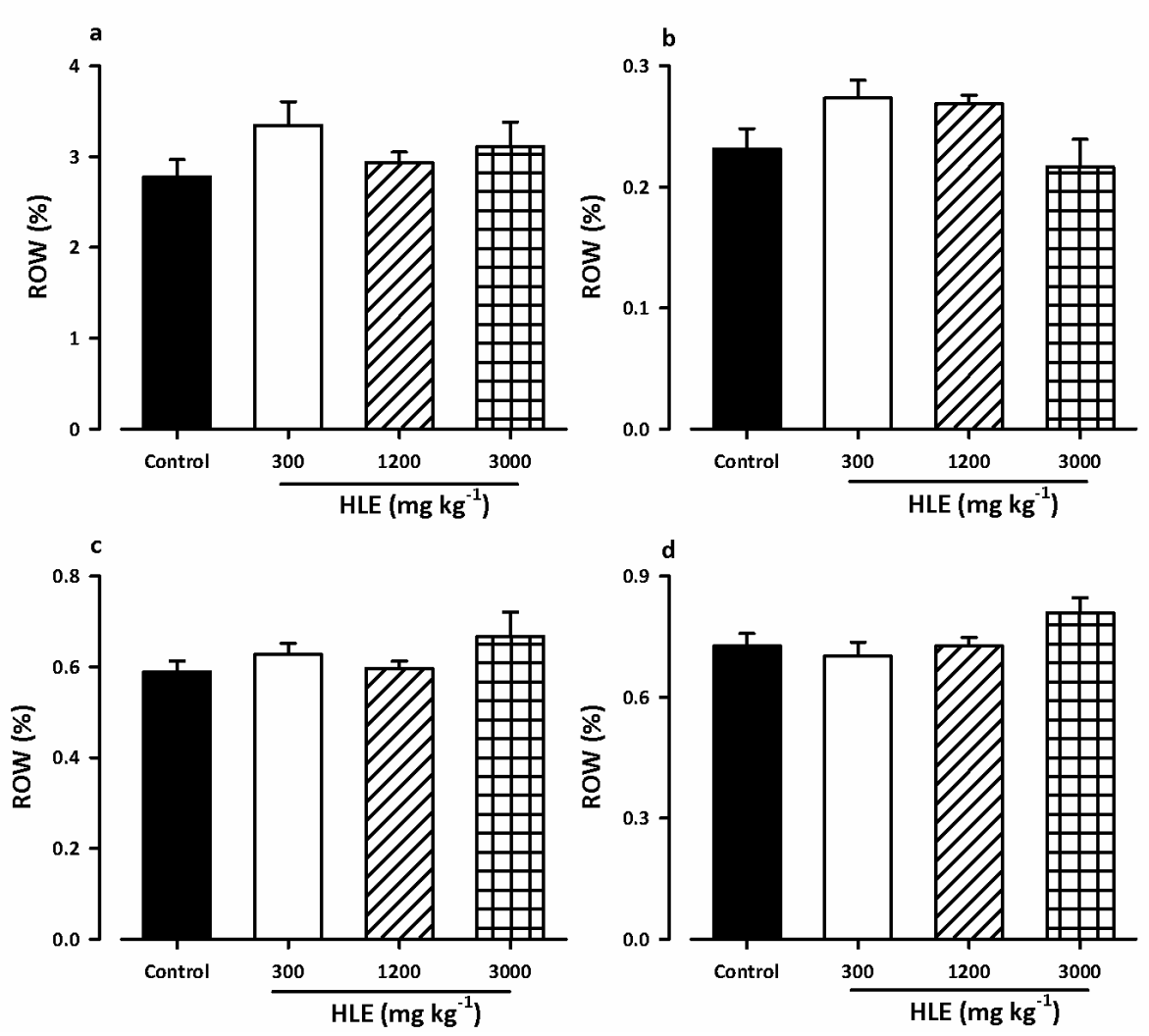

Figure 6.2 Effect of oral administration of *Hilleria latifolia* extract on the relative organ weights (ROW) with organs: liver (**a**), spleen (**b**), kidney (**c**) and stomach (**d**). Data are expressed as mean±S.E.M. (n=5). Treated groups were compared to controls using a one-way ANOVA followed by Newman-Keuls *post hoc* test.

6.3.1.1 Histopathological Changes

Plates 6.1-6.4 show the photomicrographs of sections of the isolated organs of control rats, and rats treated orally with HLE (300-3000 mg kg^{-1}) for 14 days in the sub-acute toxicity study.

Histopathological evaluation of the organs isolated from rats sacrificed at the end of the sub-acute toxicity experiment revealed significant HLE-related changes for the kidneys and stomach. All the other organs showed largely, normal histological features when compared with untreated controls. No alterations were observed in the organs of the control animals.

Comparison of liver morphological structure in extract-treated rats to controls (Plate 6.1) showed no remarkable abnormalities. The morphological structure of the capsule and hepatic lobules were normal and no necrosis or cellular degeneration was found. There was neither observed infiltration of inflammatory cells in the portal area nor hyperplasia in connective tissues. Additionally, there were no micro- and macro- cellular fatty changes, central and periportal fibrosis or vascular congestion.

The spleen samples from both control and extract-treated animals (Plate 6.2) showed normal architecture. Splenic congestion and haemorrhage, which are most common findings when there is toxicity, were not observed. These indeed evidenced the lack of detrimental changes and morphological disturbances due to administration of HLE for 14 days.

All stomach samples showed normal zymogenic cells, parietal cells and normal grooves in the mucosa with neither atrophy nor inflammatory cell infiltration. There were, however, mild mucosal erosions in samples from rats treated with 3000 mg kg^{-1} of HLE (Plate 6.3).No extract-induced alterations were observed in the kidneys of treated animals except those that received 3000 mg $kg^{-1}$of HLE in which case there were evidences of mild focal tubular casts and moderate glomerular hyperaemia (Plate 6.4).

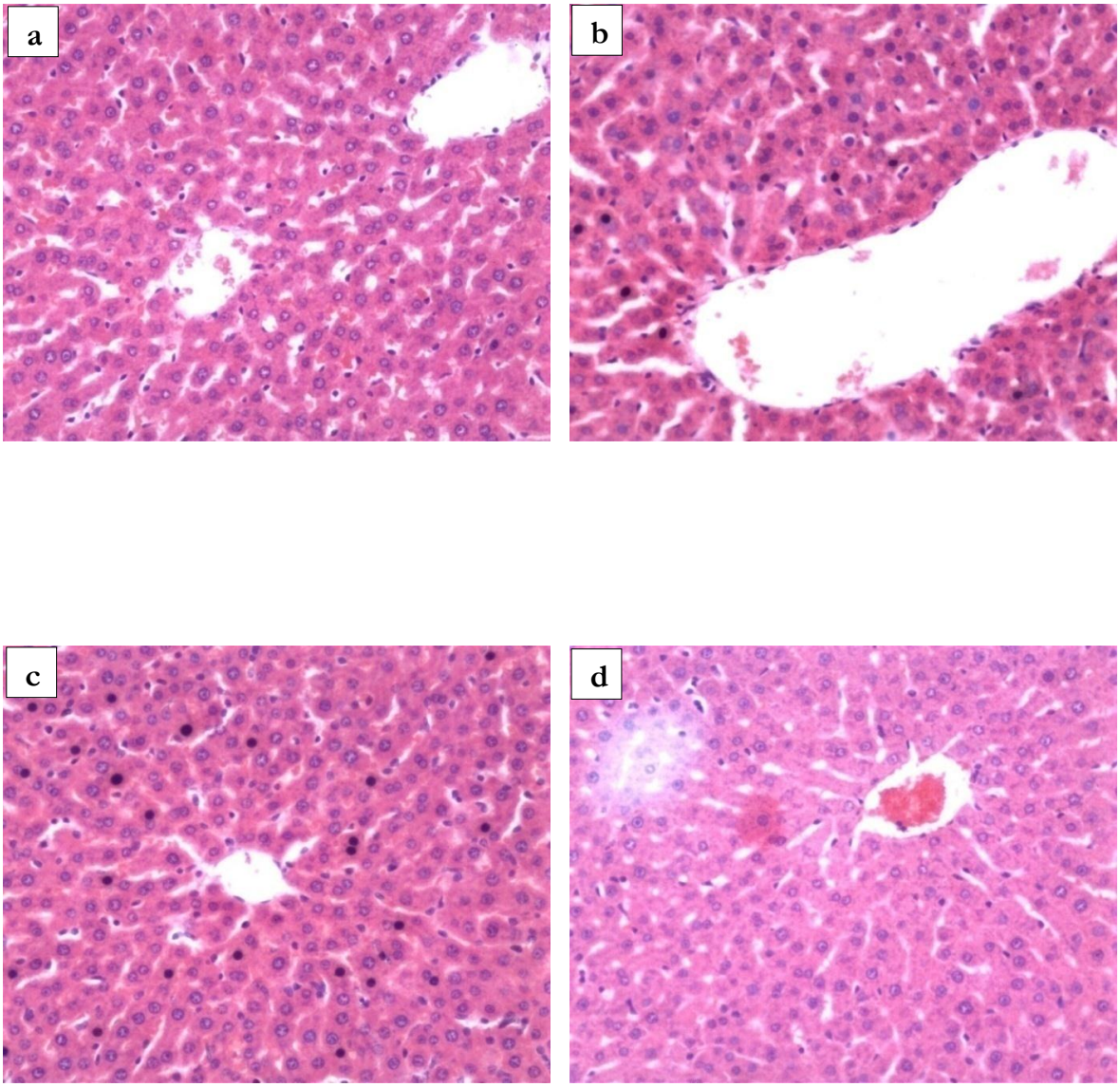

Plate 6.1 Photomicrograph of the sections of the liver in control rats (**a**), and rats treated orally with 300 mg kg^{-1} (**b**), 1200 mg kg^{-1} (**c**) and 3000 mg kg^{-1}(**d**) of HLE for 14 days in the sub-acute toxicity study (H & E, ×400)

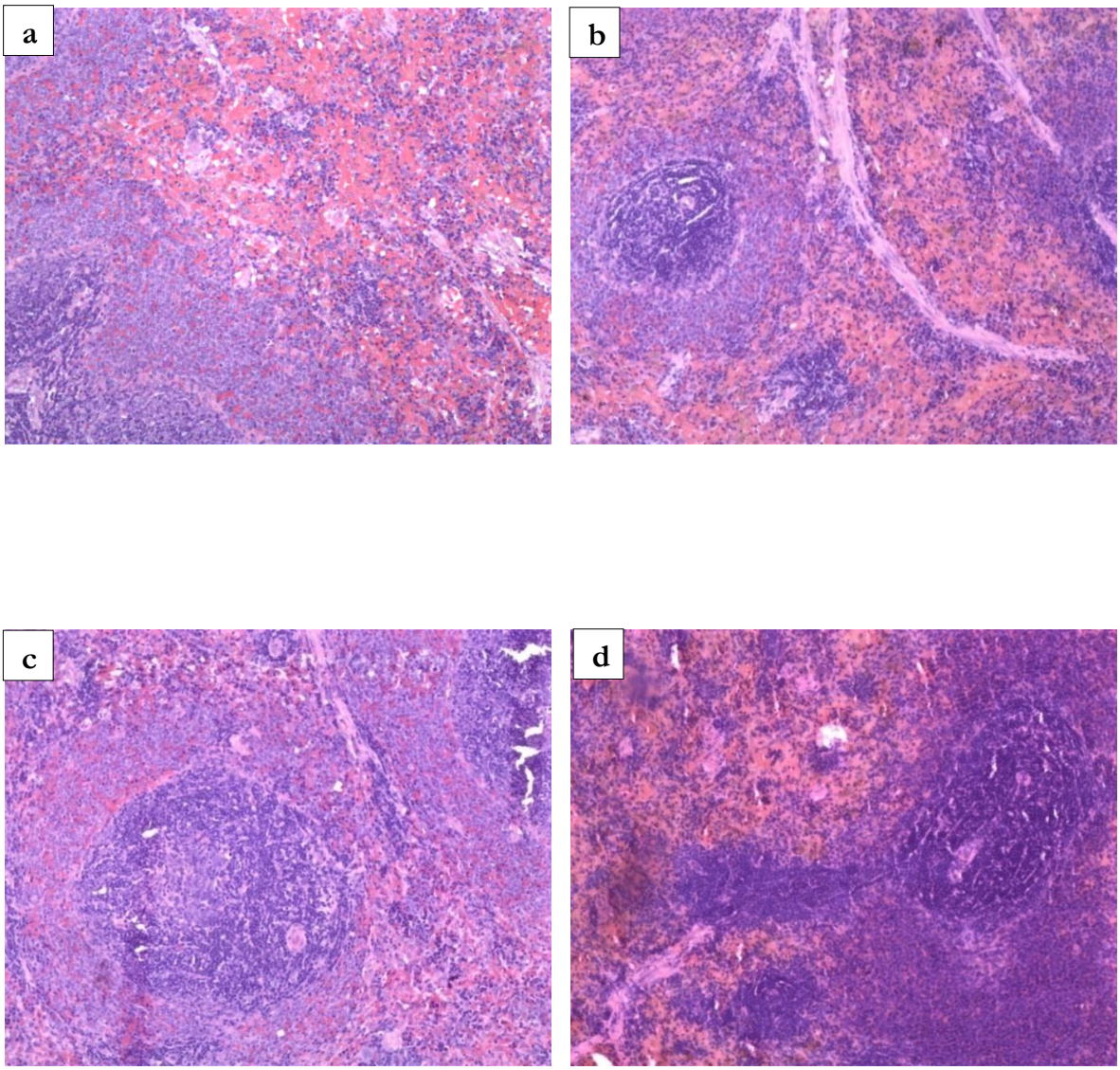

Plate 6.2 Photomicrograph of the sections of the spleen in in control rats (**a**), and rats treated orally with 300 mg kg^{-1} (**b**), 1200 mg kg^{-1} (**c**) and 3000 mg kg^{-1}(**d**) of HLE for 14 days in the sub-acute toxicity study (H & E, ×400)

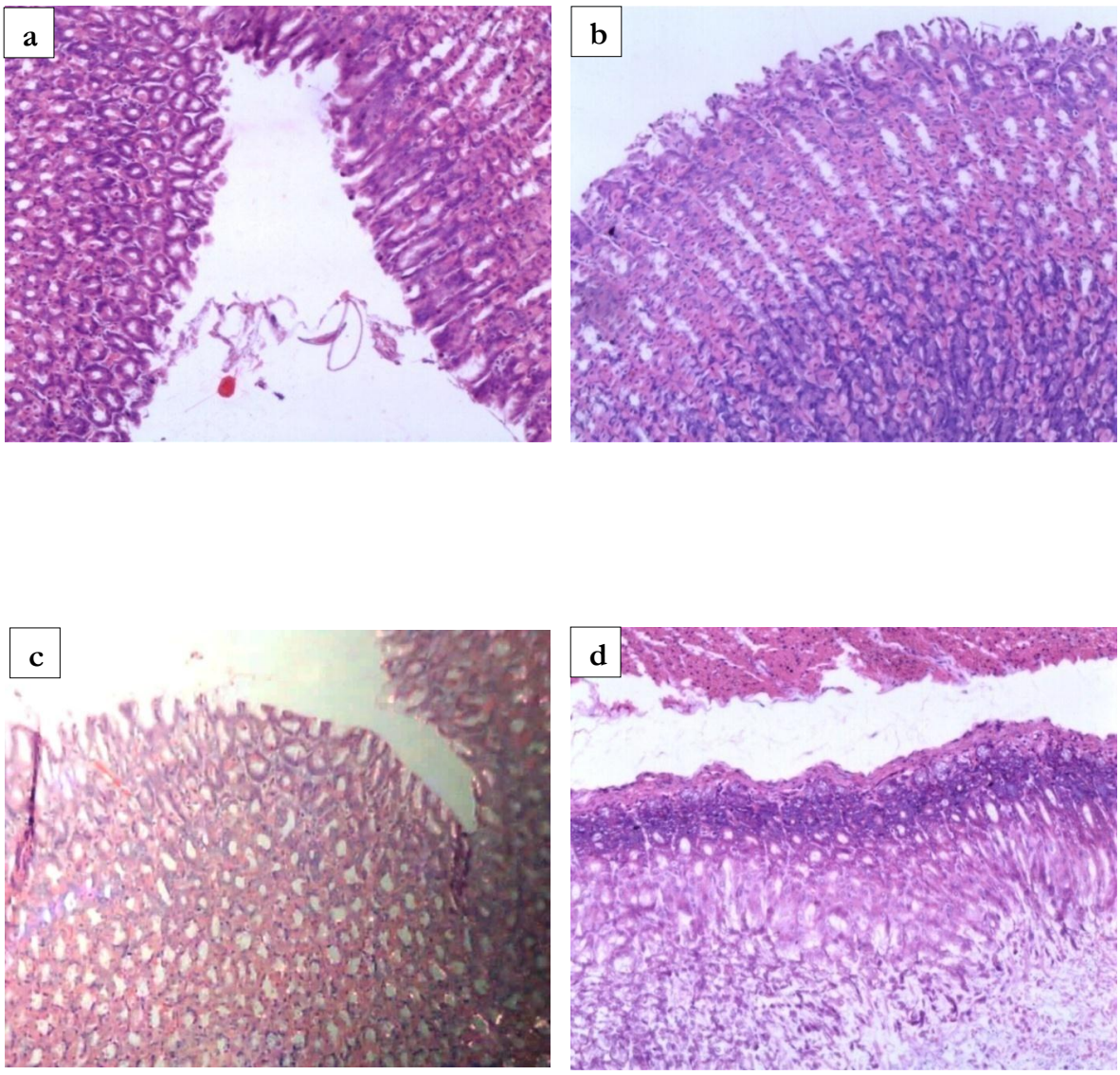

Plate 6.3 Photomicrograph of the sections of the stomach in control rats (**a**), and rats treated orally with 300 mg kg^{-1} (**b**), 1200 mg kg^{-1} (**c**) and 3000 mg kg^{-1} (**d**) of HLE for 14 days in the sub-acute toxicity study (H & E, ×400)

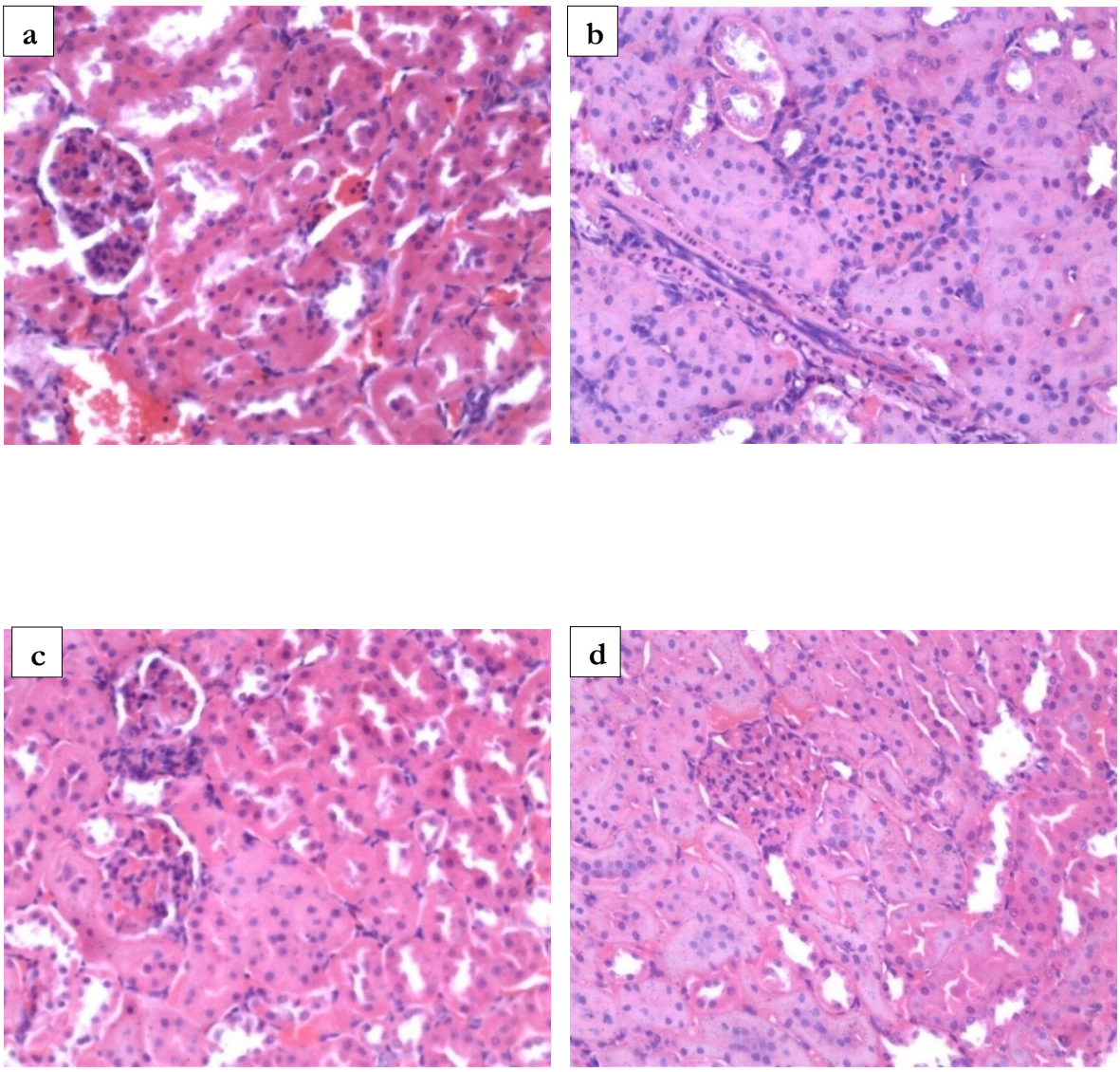

Plate 6.4 Photomicrograph of the sections of the kidney in control rats (**a**), and rats treated orally with 300 mg kg^{-1} (**b**), 1200 mg kg^{-1} (**c**) and 3000 mg kg^{-1} (**d**) of HLE for 14 days in the sub-acute toxicity study (H & E, ×400)

6.4 DISCUSSION

Phytotherapeutic products are often mistakenly regarded as safe because they are "natural". Nevertheless, these products contain bioactive principles with the potential to cause adverse effects (Bent and Ko, 2004). In addition, the poor pharmacovigilance services in this area make it difficult to determine the frequency of adverse effects caused by the use of phytotherapeutic products (Feres *et al.*, 2006). Thus, all the "natural" products used in therapeutics must be

submitted to efficacy and safety tests by the same methods used for new synthetic drugs (Talalay and Talalay, 2001). The aim of this study was to assess, if any, the potential toxicity of *H. latifolia* using rodent models.

In the acute toxicity study (Table 6.1), none of the animals died in the doses administered. Rats treated with an oral dose of 300 mg kg^{-1} of HLE did not exhibit any signs of adverse effects (NOAEL). However, adverse signs, mainly cholinergic in nature, were observed at doses above 300 mg kg^{-1} but were all reversible in a maximum period of 24 h after the administration of the extract. From the data obtained in this study, the LD_{50} of HLE can be estimated to be above 3000 mg kg^{-1}. This strongly suggests that HLE is relatively non-toxic since substances with an LD_{50} value of 1000 mg kg^{-1} by the oral route are regarded as being safe or of low toxicity (Obici *et al.*, 2008). It is worth noting, however, that the observation of toxic effects especially death in acute toxicity experiments with natural products is generally infrequent since natural products do not present purity similar to the synthetic ones that are able to promote the appearance of toxicity in acute assays. It is in this context that the popular perception that natural products do not present toxicity arises, since the arousal of toxic effects of a product only is connected to its use when the effects occur immediately after the administration (Feres *et al.*, 2006).

Acute toxicity data are of limited clinical application since cumulative toxic effects do occur even at very low doses. Hence multiple dose studies are almost always invaluable in evaluating the safety profile of phytomedicines. Acute toxicity studies along with pharmacological activity studies in animals are important in order to choose the doses to be used in a repeated-dose study. Selected doses must be larger than that suggested for use in humans. A sub-acute toxicity study was therefore carried out with doses 300, 1200 and 3000 mg kg^{-1}.

Daily clinical observations are of major importance as well as the final observations (end point) in repeated dose studies (Feres *et al.*, 2006). In daily clinical evaluation, changes, such as sedation, defaecation and salivation were observed in animals in the groups treated with 1200 and 3000 mg kg^{-1} of HLE but all the changes receded with time. As for water and food consumption, no significant changes were observed. The determination of such parameters is important in the study of safety of a product with therapeutic purpose, as proper intake of nutrients and water are essential to the physiological status of the animals and to the accomplishment of the proper response to the drug tested instead of a "false" response due to improper nutritional conditions (Feres *et al.*, 2006).

In addition to these parameters, body weight changes (fig 6.1) are an indicator of adverse side effects, as the animals that survive cannot lose more than 10 % of the initial body weight (Raza *et al*., 2002; Teo *et al*., 2002; Feres *et al*., 2006; Obici *et al*., 2008). 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 and stomach 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 (fig 6.2) provides support for the safety of HLE in rats.

The criteria for assessing histopathological changes include necrosis, cloudy swelling, fatty infiltration of cells, inflammatory infiltrations, and fibrosis among other parameters (Greaves, 2007). Except for the kidneys and stomach, there were no significant findings at the end of the histopathological examinations of the selected organs that indicated toxicity due to treatment with the extract. In animals treated with 3000 mg kg^{-1} of HLE, the kidneys showed some minor changes i.e. mild focal tubular casts and moderate glomerular hyperaemia (Plate 6.4). These may not be considered clinically significant since serum urea and creatinine levels, which are considered markers of renal function, were not significantly elevated (Table 6.3). Caution should however be taken in using this extract beyond 3000 mg kg^{-1}. Further studies may be needed in other animal species and with more chronic toxicity tests to ascertain the safety of HLE, especially with regards renal toxicity. Since mild mucosal erosions were observed at 3000 g kg^{-1} dose of HLE (Plate 6.3), further tests would also help substantiate gastrointestinal safety of HLE on chronic usage especially in doses at and above 3000 mg kg^{-1}.

Analysis of blood parameters is relevant to risk evaluation as the haematological system has a higher predictive value for toxicity in humans (91%) when assays involve rodents and non-rodents (Olson *et al*., 2000). Moreover, certain medicinal herbal preparations have been reported to adversely affect various blood components causing conditions like haemolytic anaemia and thrombocytopaenia (Synder *et al*., 1977; Yunis *et al*., 1980; King and Kelton, 1984; Gandolfo *et al*., 1992). Blood forms the main medium of transport for many drugs and xenobiotics in the body and for that matter 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 are the red blood

cells. HLE did not have any significant effect on the haematological parameters measured, confirming its haematological safety in rats (Table 6.2).

In the biochemical analysis, few significant non-dose-dependent changes were observed. The biochemical evaluation is important since there are several reports of liver and kidney toxicity related to the use of phytotherapeutic products (Corns, 2003; Obici *et al.*, 2008; Rhiouani *et al.*, 2008). In preclinical toxicity studies renal changes are particularly liable to occur because of the high doses given and the fact that the kidneys eliminate many drugs and their metabolites. The kidney has a high blood flow that exposes renal parenchyma to high peak concentrations of chemicals even if they are only present transiently in the circulation. Its high oxygen consumption makes it sensitive to ischaemia and volume depletion. Its ability to concentrate toxic solutes in parenchymal cells and in tubular luminal fluid is a further risk factor (Schreiner and Maher, 1965; Greaves, 2007). In the present study, creatinine and urea determinations (Table 6.3) were critical as markers of kidney function (Arneson and Brickell, 2007b; Obici *et al.*, 2008). Urea and creatinine are compounds derived from proteins, which are eliminated by the kidney and when kidneys are damaged, their levels rise. There were no significant differences in serum levels of creatinine and urea in the HLE treated groups compared to controls. However, histopathological assessment data (Plate 6.4) revealed some mild changes in kidney ultrastructure at highest dose of HLE (3000 mg kg^{-1}). The lack of marked changes in serum levels of creatinine and urea implies that the histopathological changes seen were not significant enough to affect kidney function and can be considered as clinically unimportant.

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. Among the biochemical parameters evaluated, AST, ALT, and ALP are considered markers of liver function (Feres *et al.*, 2006; Arneson and Brickell, 2007a; Obici *et al.*, 2008). Hepatocellular damage is characterized by a mutual rise in serum levels of AST and ALT. Usually, about 80% of AST is found in the mitochondria whereas ALT is purely cytosolic. Therefore, AST appears in higher concentrations in a number of tissues (liver, kidneys, heart, pancreas, etc) and is released slowly in comparison to ALT. But since ALT is localized primarily in the cytosol of hepatocytes, this enzyme is considered a more sensitive marker of hepatocellular damage than AST and within limits can provide a quantitative assessment of the degree of damage sustained by the liver (Aniagu *et al.*, 2004). ALP is most often measured to indicate bile duct obstruction. High levels

of ALP exist in cells that are rapidly dividing or are otherwise metabolically active. These cells include the epithelium of the biliary tract and liver, osteoblasts laying down new bone, granulocytes of circulating blood, intestinal epithelium, proximal tubules of the kidney, placenta and lactating mammary glands. ALP levels reach spectacular heights in primary biliary cirrhosis, in conditions of disorganized hepatic architecture (cirrhosis), and in diseases characterized by inflammation, regeneration, and obstruction of intrahepatic bile ductules (Witthawaskul *et al.*, 2003). There were no significant changes in the serum levels of AST, ALT and ALP demonstrating that liver function was preserved in animals exposed to HLE over the 14 days period (Table 6.3). This conclusion correlates well with findings on histopathological examination of the liver, since it did not indicate any significant cellular lesions (Plate 6.1).

Bilirubin is the main pigment that is formed from the breakdown of haem in red blood cells. It is conjugated in the liver and then secreted into the bile (Marshall and Bangert, 2008). 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 (Limdi and Hyde, 2003). In cases of increased production, or decreased conjugation, the unconjugated (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 (Marshall and Bangert, 2008). When the bile ducts are obstructed, there is a build-up of direct bilirubin. This escapes from the liver and ends up in the blood increasing plasma levels. 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 (Limdi and Hyde, 2003). Since there were no elevations in direct, indirect and total bilirubin fractions after treatment with the extract, it can be inferred that the extract did not have any deleterious effects on hepatic metabolism or biliary excretion.

A striking trend in the levels of direct bilirubin, indirect bilirubin, total bilirubin, AST and ALT (Table 6.3) deserves some comment. Though not statistically significant, the serum levels of AST and ALT were lower in extract treated groups than controls. The same can be said of serum bilirubin in extract-treated groups (in this case with statistical significance). These observations seem to suggest that HLE may have some hepatoprotective effects. This is especially plausible when one considers the reported (Iwu, 1993) use of the decoction of the leaves and twigs of *Hilleria latifolia* traditionally in the treatment of jaundice. Nevertheless,

further studies need to be carried out to scientifically establish this speculated hepatoprotective effects.

The liver produces most of the plasma proteins in the body including albumin and globulin. After 14 days of *H. latifolia* extract administration, the amount of total proteins, albumin and globulin in blood serum were significantly lower when compared to vehicle-treated animals. Since liver histology, relative liver weight, albumin-globulin ratio and serum markers of liver function were all normal in extract-treated animals, the fall in plasma proteins cannot be attributed to liver damage. Also, because there was no significant impairment observed in renal function, renal loss is untenable. It may be reasonable to attribute the low serum proteins to either HLE-induced reduction in synthesis or HLE-induced intestinal protein malabsorption due to rapid gastrointestinal transit. These may need to be studied further since the observed fall in serum proteins could have been purely artefactual (Aniagu *et al.*, 2004). In any case, however, the implications of the observed fall are pharmacologically significant. Plasma proteins bind many molecules including drugs and shuttle them through circulation. With the above observation, chronic concurrent administration of high doses of HLE with another drug that is normally highly bound to plasma proteins may result in an exaggerated response (or even toxicity) of that drug due to its increased free plasma concentration.

The present investigations could be regarded as preliminary probes, necessitating further studies to firmly establish the toxicity of HLE. Prospective studies should include amongst other things a battery of reproductive toxicity, genetic toxicity, mutagenicity and carcinogenicity tests in addition to effects on drug metabolising enzymes (especially cytochrome P450s) and toxicokinetic profiling. When such data are available, a conclusive remark can then be made on the safety profile of HLE.

6.5 CONCLUSION

Oral toxicity of the ethanolic extract from the aerial parts of *Hilleria latifolia* in rats is low. However, since this finding cannot be directly extrapolated to humans, caution should be exercised in its use especially at high doses.

Chapter 7

GENERAL DISCUSSION

Pain and inflammation management remains a real and current problem in clinical medicine. This does not merely reflect inadequate management strategies at a local level but also poor efficacy and poor tolerability of the analgesic and anti-inflammatory agents. To enable more effective therapy multi-modal strategies are now employed; however, new agents with improved efficacy are required to help combat problematic pain and inflammation management. Medicinal plants have become important sources of new chemical substances with potential therapeutic effects (Ebadi, 2007). With the vast resource of medical plants available, the research into those with alleged traditional use as pain and inflammation relievers is therefore a fruitful and logical research strategy in the search for new analgesic and anti-inflammatory drugs. The current study investigated the analgesic and anti-inflammatory properties of the aerial parts of *Hilleria latifolia* (Lam.) H. Walt., a perennial herb commonly used in Ghanaian traditional medicine for the treatment of various diseases including pain and inflammation. The safety as well as some neurobehavioural effects that could be advantageous or limiting to the prospected analgesic and anti-inflammatory effects of the plant was also studied. It is clear from the study that the ethanolic extract of aerial parts of *Hilleria latifolia* has significant anti-nociceptive, anti-inflammatory and antioxidant effects. The study has also established the antidepressant and anxiolytic activity of the plant extract. Additionally, the ethanolic extract is relatively non-toxic in rats.

Due to the analgesic effect shown in various animal models of nociception in this study, HLE can be said to be effective against both nociceptive and inflammatory pain. Various studies have found pharmacological correlation between the second phase of the formalin test and neuropathic pain behaviours (Vissers *et al*., 2003, Vissers *et al*., 2006, Ellis *et al*., 2008). Though HLE was not assessed for activity in any specific neuropathic pain model, it is likely to be also effective against neuropathic pain since HLE was very effective in the second phase of the formalin test.

One major drawback that greatly limits the effectiveness and usage of centrally acting analgesics especially opioids in current clinical practice is the development of tolerance. Although HLE is centrally acting and works partly through the opioid pathway, it does not develop tolerance on chronic treatment. This makes HLE a useful option for chronic pain treatment. An added

advantage to the use of HLE is also that it interferes with tolerance development when given concurrently with morphine. HLE can, therefore, be used in opioid tolerant patients; a combination therapy of HLE and opioids may also prevent or delay opioid tolerance development.

Pain is a subjective experience inextricably linked to the sufferer's emotion (Fishbain, 2002; Keefe *et al.*, 2004; Gureje, 2007; Gureje, 2008). On a neural level, cognitive and emotional factors have been shown to interact with the ascending regulation of pain transmission in the spinal cord (Woolf, 2004). Again, several midbrain and hindbrain areas (amygdala, hypothalamus, periaqueductal gray and raphe magnus) that are involved in fear, anxiety, mood regulation and autonomic responses are associated with pathways that are activated by painful stimuli (Millan, 2002). Therefore, it is logical to expect chronic pain conditions to likely lead to disturbances of mood of sufficient severity to achieve diagnostic status. The clinical effectiveness of antidepressants as analgesics has been confirmed in preclinical studies using various tests of nociceptive activity, which use thermal, mechanical, electrical or chemical stimuli (Schreiber *et al.*, 1999; Otsuka *et al.*, 2001; Rojas-Corrales *et al.*, 2003; Duman *et al.*, 2004), as well as in animal models of chronic pain (Zarrindast *et al.*, 2000; Marchand *et al.*, 2003; Anjaneyulu and Chopra, 2004). HLE by virtue of its potent antidepressant activity has an added advantage of helping manage, in addition to pain, depression that usually worsens chronic pain and makes total remission difficult. The antidepressant effects of HLE also goes further to support the assertion that HLE may be effective in neuropathic pain since there is substantial evidence for the effectiveness of antidepressants in neuropathic pain (Sawynok, 2001; Jasmin *et al.*, 2003; Mico *et al.*, 2006).

Oxidative stress is primarily or secondarily involved in the pathogenesis of major depression (Zafir *et al.*, 2009). A number of studies 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 (Maes *et al.*, 2000; Bilici *et al.*, 2001; Khanzode *et al.*, 2003; Ozcan *et al.*, 2004; Tsuboi *et al.*, 2006; Sarandol *et al.*, 2007). Zafir *et al.* (2009) have shown that antidepressants can improve oxidative stress in depression. In examining this, their results showed a significant recovery in the activities of superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST), glutathione reductase (GR) and glutathione (GSH) levels by antidepressant treatments following a restraint stress-induced decline of these parameters. The severely accumulated lipid peroxidation product

malondialdehyde (MDA) and protein carbonyl contents in stressed animals were also significantly normalized by antidepressant treatments. It was thus suggested that augmentation of *in vivo* antioxidant defenses could serve as a convergence point for multiple classes of antidepressants and also as an important mechanism underlying the neuroprotective pharmacological effects of these drugs observed clinically in the treatment of various stress disorders. Consequently—even though the *in vivo* antioxidant effect of HLE was not clearly established in this study—it is speculated that the antidepressant activity of HLE may be dependent in part on its antioxidant activity shown *in vitro*.

Just like depression, anxiety disorders are often comorbid with pain. Hitherto a neglected area of research, there is now evidence from large epidemiologic studies suggesting that the association of anxiety disorders with chronic pain is comparable with that of mood disorders (Gureje, 2008). Since comorbidity of anxiety disorders with pain has implications for the outcome of pain and also possibly for the outcome of anxiety disorders, anxiety management during pain treatment is very important. HLE with its combined analgesic and anxiolytic effects makes it particularly advantageous in this regard.

HLE exhibited a unique feature, evidenced in almost all the results obtained in this study: a biphasic dose- response relationship. This non-monotonic dose-response pattern was shared by the anti-nociceptive, anti-inflammatory, anti-anxiety and antidepressant actions of HLE. This suggests that HLE may be acting through a mechanism common to the nociceptive, inflammatory, anxiety and depression pathways. Further studies are required establish the exact nature of this mechanism. However, it will not be surprising if this has to do with the neuronal nicotinic effects of HLE in the nervous system since nicotine and many neuronal nicotinic agonists are known to have anti-nociceptive (Damaj *et al.*, 1998; Decker and Meyer, 1999), anti-inflammatory (Pavlov *et al.*, 2003; Liu *et al.*, 2009), anti-anxiety (File *et al.*, 2000; Picciotto *et al.*, 2000) and antidepressant actions (Picciotto *et al.*, 2000; Vazquez-Palacios *et al.*, 2004).

One of the aims of research into new analgesic and anti-inflammatory agents is to develop such agents with reduced side effects. Most anti-inflammatory agents especially NSAIDs 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). In this study conducted, histology of the stomach after sub-acute toxicity studies did not show any difference between control and extract-treated animals up to 1200 mg kg^{-1}.

Similarly, the extract did not show any overt effect on the kidneys up to 1200 mg kg^{-1}. At 3000 mg kg^{-1}, however, there were mild mucosal erosions in the stomach as well as mild focal tubular casts and moderate hyperaemia in the kidneys. Since these effects cannot be directly extrapolated to humans, the possibility of them occurring at lower doses in humans cannot be ruled out. Until further studies conclusively reveal the renal and GI safety, it is difficult to speculate any real advantages of HLE over NSAIDs in terms of their two worrisome side effects of gastric ulceration and nephrotoxicity.

Chapter 8

CONCLUSIONS

From all the results from this study, it can be concluded that the ethanolic extract of the aerial parts of *H. latifolia*:

- has central and peripheral anti-nociceptive effect in chemical and thermal animal models of nociception—without tolerance induction—through mechanisms that involve an interaction with adenosinergic, nicotinic cholinergic, muscarinic cholinergic and opioid pathways;
- has anti-inflammatory effects in acute and chronic animal models of inflammation;
- has antioxidant property which may contribute to its anti-inflammatory activity;
- has anxiolytic properties;
- has antidepressant properties which may also contribute to its analgesic property;
- has neuronal nicotinic receptor agonist effects;
- increases gastrointestinal transit and
- is relatively non-toxic in rats.

This study has provided evidence to support the use of *H. latifolia* aerial parts in Ghanaian traditional medicine for the treatment of pain and inflammatory conditions.

RECOMMENDATIONS

The following are recommended:

- The active component(s) in HLE that are responsible for the analgesic and anti-inflammatory effects should be isolated and characterised.
- The exact mechanism(s) underlying the analgesic and anti-inflammatory effects should be established.
- Chronic toxicity test should be carried out in rodents and other animal species to establish the safety of HLE.
- The hepatoprotective effects of HLE should be investigated.

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APPENDIX

8.1 PHARMACOLOGICAL METHODS

8.1.1 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 additions. It was then left for 2-3 hours before use.

8.1.2 PREPARATION OF COMPLETE FREUND'S ADJUVANT (CFA)

80 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 4 mg ml^{-1} suspension.

8.1.3 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 0.2 M 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 0.1 M sodium phosphate buffer, pH 7.4.

8.1.4 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 $V_t - V_0$. 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

$$\%\,\text{Change in foot volume} = \frac{V_t - V_0}{V_0} \times 100$$

Where

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

V_0 is the foot volume before injection (0 h).

8.1.5 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 concentrations were made such that the required dose was always given in equivalent volumes not exceeding a total volume of 1 ml for oral administration and 0.5 ml for intraperitoneal route except in the toxicity studies.

8.2 DETAILED OBSERVATIONS IN THE IRWIN'S TEST

Dose (mg/kg)	0							300							450							600						
Time(min)	0-15	15	30	60	120	180	24h	0-15	15	30	60	120	180	24h	0-15	15	30	60	120	180	24h	0-15	15	30	60	120	180	24h
Lethality	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Convulsions	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Tremor	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Straub	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sedation	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	2+	2+	2+	0	0	0	#	2+	2+	1+	0	0	0
Excitation	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0
Abnormal gait(rolling)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Abnormal gait(tiptoe)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Jumps	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Motor incoordination	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Loss of balance	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Fore-paw treading	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Writhes	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Piloerection	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Stereotypies(sniffing)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Stereotypies(chewing)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Stereotypies(head movements)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Head twitches	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Scratching	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Respiration	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Aggressiveness	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Fear	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	2-	2-	2-	0	0	0	#	2-	2-	1-	0	0	0
Reactivity to touch	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	2-	2-	0	0	0	0	#	2-	2-	2-	0	0	0
Muscle tone	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0
Loss of writhing reflex	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0
Ptosis	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0
Exophthalmos	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0

Loss of grasping	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0
Akinesia	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0
Catalepsy	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0
Loss of traction	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0
Loss of corneal reflex	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0
Analgesia	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	2+	2+	0	0	0	#	0	0	2+	2+	0	0
Defaecation	#	0	0	0	2	0	0	#	0	0	0	0	0	0	#	3+	3+	3+	2+	0	0	#	3+	3+	3+	2+	0	0
Salivation	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	1+	1+	0	0	0	#	1+	2+	2+	1+	0	0
Lacrimation	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0
Urination	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	3+	3+	3+	0	0	#	0	3+	3+	3+	0	0
Change in Rectal temperature	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0	#	0	0	0	0	0	0

Dose (mg/kg)	900								1200								3000						
Time(min)	**0-15**	**15**	**30**	**60**	**120**	**180**	**24h**		**0-15**	**15**	**30**	**60**	**120**	**180**	**24h**		**0-15**	**15**	**30**	**60**	**120**	**180**	**24h**
Lethality	0	0	0	0	0	0	0		0	0	0	0	0	0	0		0	0	0	0	0	0	0
Convulsions	0	0	0	0	0	0	0		0	0	0	0	0	0	0		0	0	0	0	0	0	0
Tremor	0	0	0	0	0	0	0		0	0	0	0	0	0	0		0	0	0	0	0	0	0
Straub	0	0	0	0	0	0	0		0	0	0	0	0	0	0		0	0	0	0	0	0	0
Sedation	#	3+	3+	3+	1+	0	0		#	4 (+)	4 (+)	2 (+)	0	0	0		#	5 (++)	5 (++)	1 (+)	0	0	0
Excitation	#	0	0	0	0	0	0		#	0	0	0	0	0	0		#	0	0	0	0	0	0
Abnormal gait(rolling)	0	0	0	0	0	0	0		0	0	0	0	0	0	0		0	0	0	0	0	0	0
Abnormal gait(tiptoe)	0	0	0	0	0	0	0		0	0	0	0	0	0	0		0	0	0	0	0	0	0
Jumps	0	0	0	0	0	0	0		0	0	0	0	0	0	0		0	0	0	0	0	0	0
Motor incoordination	0	0	0	0	0	0	0		0	0	0	0	0	0	0		0	0	0	0	0	0	0
Loss of balance	0	0	0	0	0	0	0		0	0	0	0	0	0	0		0	0	0	0	0	0	0
Fore-paw treading	0	0	0	0	0	0	0		0	0	0	0	0	0	0		0	0	0	0	0	0	0
Writhes	0	0	0	0	0	0	0		0	0	0	0	0	0	0		0	0	0	0	0	0	0
Piloerection	0	0	0	0	0	0	0		0	0	0	0	0	0	0		0	0	0	0	0	0	0

Stereotypies (sniffing)	0	0	0	0	0	0	0		0	0	0	0	0	0	0		0	0	0	0	0	0	0
Stereotypies (chewing)	0	0	0	0	0	0	0		0	0	0	0	0	0	0		0	0	0	0	0	0	0
Stereotypies(head movements)	0	0	0	0	0	0	0		0	0	0	0	0	0	0		0	0	0	0	0	0	0
Head twitches	0	0	0	0	0	0	0		0	0	0	0	0	0	0		0	0	0	0	0	0	0
Scratching	0	0	0	0	0	0	0		0	0	0	0	0	0	0		0	0	0	0	0	0	0
Respiration	0	0	0	0	0	0	0		0	0	0	0	0	0	0		0	0	0	0	0	0	0
Aggressiveness	0	0	0	0	0	0	0		0	0	0	0	0	0	0		0	0	0	0	0	0	0
Fear	#	2-	3-	0	0	0	0		#	2-	2-	0	0	0	0		#	3-	3-	0	0	0	0
Reactivity to touch	#	2-	3-	0	0	0	0		#	3-	2-	0	0	0	0		#	3-	3-	0	0	0	0
Muscle tone	#	0	0	0	0	0	0		#	0	0	0	0	0	0		#	0	0	0	0	0	0
Loss of writhing reflex	#	0	0	0	0	0	0		#	0	0	0	0	0	0		#	0	0	0	0	0	0
Ptosis	#	0	0	0	0	0	0		#	0	0	0	0	0	0		#	0	0	0	0	0	0
Exophthalmos	#	0	0	0	0	0	0		#	0	0	0	0	0	0		#	0	0	0	0	0	0
Loss of grasping	#	0	0	0	0	0	0		#	0	0	0	0	0	0		#	0	0	0	0	0	0
Akinesia	#	0	0	0	0	0	0		#	0	0	0	0	0	0		#	0	0	0	0	0	0

Catalepsy	#	0	0	0	0	0	0		#	0	0	0	0	0	0		#	0	0	0	0	0	0
Loss of traction	#	0	0	0	0	0	0		#	0	0	0	0	0	0		#	0	0	0	0	0	0
Loss of corneal reflex	#	0	0	0	0	0	0		#	0	0	0	0	0	0		#	0	0	0	0	0	0
Analgesia	#	0	3+	1+	0	0	0		#	0	2+	2+	0	0	0		#	0	0	3+	2+	0	0
Defaecation	#	0	4+	4+	3+	0	0		#	4+	4+	3+	3+	0	0		#	5+	4+	4+	2+	0	0
Salivation	#	0	4+	4+	4+	0	0		#	4+	5+	4+	4+	0	0		#	4+	4+	4+	4+	0	0
Lacrimation	#	0	0	0	0	0	0		#	0	0	0	0	0	0		#	0	0	0	0	0	0
Urination	#	0	4+	4+	3+	0	0		#	0	3+	3+	3+	0	0		#	0	0	3+	3+	0	0
Change in Rectal temperature	#	0	0	0	0	0	0		#	0	0	0	0	0	0		#	0	0	0	0	0	0

HLE was administered at 300, 450, 600, 900, 1200 and 3000 mg kg^{-1}, *p.o.*; five rats were used per group. Data is presented as the number of animals showing symptoms during the test, with an indication of intensity for sedation, analgesia, fear, reactivity to touch, defaecation, urination, salivation and lacrimation (+ = slight increase, ++ = moderate increase,+++=marked increase, - = slight decrease). Observations were performed at 15, 30, 60, 120, 180 min and 24 hr after administration. The symptoms that did not necessitate handling were also observed up to 15 min immediately following administration. # Parameters not measured.