ANTICONVULSANT AND NEUROBEHAVIOURAL EFFECTS OF THE AQUEOUS LEAF EXTRACT OF *LEEA GUINEENSIS* G. DON (FAMILY: LEEACEAE)

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DECLARATION

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

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ABSTRACT

Leea guineensis is an evergreen shrub found in tropical forest zones in Africa and other parts of the world and the leaves are used traditionally to treat convulsions and epilepsy. This study investigated the anticonvulsant, anxiolytic, antinociceptive and neuromuscular effects of the aqueous leaf extract of the plant.

Three widely used animal models of epilepsy namely the pentylenetetrazole, picrotoxin and maximal electroshock-induced seizure tests were used to evaluate the anticonvulsant effects of the extract, the elevated plus-maze and the light/dark box were used to evaluate its anxiolytic effects and the formalin test used to investigate its antinociceptive effects. The neuromuscular effects of the extract were determined in the beam traversal test, the rotarod test and the isolated toad rectus abdominis muscle.

In the pentylenetetrazole-induced seizures, *Leea guineensis* (30-300 mg/kg *p.o*) caused marked and dose-dependent delay of the onset of myoclonic jerks ($F_{3,16}$=6.29, P=0.0051) and decreased the duration of tonic-clonic convulsions ($F_{3,16}$=9.20, P=0.0009). The extract delayed the onset of tonic convulsions and decreased the frequency of convulsions induced by pentylenetetrazole ($F_{3,16}$=0.95, P=0.4385 and $F_{3,16}$=2.39, P=0.1065, respectively). Onset of tonic convulsions induced by picrotoxin was also significantly delayed by *Leea guineensis* and the frequency and duration of convulsions significantly reduced. The onset of picrotoxin-induced myoclonic jerks was delayed by the extract ($F_{3,16}$=0.53, P=0.6682). Again, the extract showed marked anticonvulsant activity by significantly reducing the duration ($F_{3,36}$=7.35, P=0.0006) of maximal electroshock-induced tonic hind limb extension. Administration of *Leea*

guineensis (30-300 mg/kg *p.o*) in mice caused significant anxiolytic effects similar to that of diazepam (0.1-1.0 mg/kg *i.p*) and opposite to that of pentylenetetrazole (3-30 mg/kg *i.p*) in the elevated plus-maze and the light/dark box tests. It caused significant increase in the number of open arm entries and time ($F_{3,16}$=5.21, P=0.0106 and $F_{3,16}$=4.18, P=0.0230, respectively), increased the frequency and duration of unprotected stretch-attend postures ($F_{3,16}$=5.39, P=0.0093 and $F_{3,16}$=3.47, P=0.0411, respectively) as well as unprotected head dips ($F_{3,16}$=3.95, P=0.0277 and $F_{3,16}$=5.15, P=0.0111, respectively). Similar to diazepam and opposite to pentylenetetrazole, *Leea guineensis* significantly delayed the emergence latency of mice into the light box from the dark box and increased the time spent in the light area of the light/dark box.

Leea guineensis caused potent antinociceptive activity in both the early phase and late phase of formalin-induced pain ($F_{3,16}$=17.18, P<0.0001 and $F_{3,16}$=40.17, P<0.0001, respectively). Morphine (3-10 mg/kg *i.p*) also produced antinociceptive activity in both phases similar to the extract. The extract was approximately equipotent in both phases (ED_{50} =16.37±4.57 and ED_{50}=14.06±4.39, respectively). The effect of morphine was also almost equipotent in both phases. However, *Leea guineensis* was less potent than morphine in both phases (about 181.88 and 127.82 times respectively). Naloxone and theophylline significantly reversed the antinociceptive activity of the extract in the first phase but had no effect on the second phase.

The extract caused concentration-dependent contractions on the isolated toad rectus abdominis muscle similar to ACh and did not impair motor coordination and balance. In conclusion, the results indicate that the leaves of *Leea guineensis* produces anticonvulsant, anxiolytic and analgesic effects through central mechanisms which support the traditional use of the plant to treat epileptic fits.

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To all loved ones who prayed and wished me well "I pray that God will help you overflow with hope in him through the Holy Spirit's power within you".

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ABBREVIATIONS

AEDs	Antiepileptic Drugs
GABA	Gama-amino butyric acid
i.p	Intraperitoneal
IPSCs	Inhibitory Post-Synaptic Currents
TLE	Temporal Lope Epilepsy
IGluR	Ionotropic Glutamate Receptors
MGluR	Metabotropic Glutamate Receptors
NMDA	N-Methyl-D-Aspartate
AMPA	Alpha-3-Hydroxy-5-Methoxy-4-Isoxazole Propionic Acid
CNS	Central Nervous System
MEST	Maximal Electroshock Test
ScPTZ	Subcutaneous Pentylenetetrazole
THLE	Tonic Hind Limb Extension
BIC	Bicuculline
PTX	Picrotoxin
STN	Strychnine
GAERS	Genetic Absence Epilepsy Rats from Strasbourg
PD	Panic Disorder
GAD	Generalised Anxiety Disorder

PTSD	Post-Traumatic Stress Disorder
EPM	Elevated Plus-Maze
LDB	Light/Dark Box
NSAIDs	Nonsteroidal Anti-inflammatory Drugs
MES	Maximal Electroshock
SAPs	Stretch-Attend Postures
U/PSAPs	Unprotected/Protected Stretch-Attend Postures
Ach	Acetylcholine
p.o	Per Os
U/PHDs	Unprotected/Protected Head Dips
LGE	Leea guineensis extract
DZP	Diazepam
TH	Theophylline
NLX	Naloxone

Chapter 1

INTRODUCTION

1.1 GENERAL INTRODUCTION

People tend to rely on traditional and other forms of complementary and alternative medicine for chronic conditions which do not respond well to conventional or modern drug treatments. Among these are neurological disorders such as anxiety, pain and epilepsy (Spinella, 2001). Centuries before the advent of modern medicine, synthetic chemistry and the pharmaceutical industry, virtually all medicines came from plants (Agosta, 1997). These medicinal plants have been an important source for the discovery of novel bioactive compounds which served and continue to serve as lead molecules for the development of new drugs (Cragg *et al.*, 1997). Aspirin, atropine, scopolamine, taxol, theophylline, tubocurarine, vincristine and vinblastine are a few examples of such invaluable therapeutic tools for today's physicians (Cox *et al.*, 1994; Jones, 1996).

Epilepsy is one of the major neurological disorders affecting approximately 0.8% of the population (Pitkanen *et al.*, 2009). There has been considerable progress in the pharmacotherapy of epilepsy over the last few decades, including the introduction of new antiepileptic drugs such as felbamate, lamotrigine, etc. (Bazil *et al.*, 1998; McCabe, 2000). However, current drug therapy of epilepsy is complicated by side-effects, teratogenic effects; long term toxicity and about a third of patients are refractory to pharmacotherapies (Loscher, 2002b; Raza *et al.*, 2001). Furthermore, there is currently no drug available which prevents the development of epilepsy e.g. after head trauma (Temkin, 2001; Temkin *et al.*, 2001) and all currently available AEDs drugs are synthetic molecules.

Medicinal plants used for the therapy of epilepsy in traditional medicine practice possess promising anticonvulsant activities in animal models of anticonvulsant screening and these can be an invaluable source for search for new antiepileptic compounds (Stafford *et al.*, 2008). Majority of epilepsy patients rely on medicinal plants for therapy. For example, a sample in Nigeria found 52% of epilepsy patients using some form of traditional medicine (Danesi *et al.*, 1994). Also, the use of traditional medicine and medicinal herbs is currently enjoying a renaissance in popularity in the West as well, and in fact, it is the primary form of medicine in many parts of the world. Epilepsy in particular is a condition where traditional healers are very critical in providing treatment in the rural settings (Stafford *et al.*, 2008).

Considering the great reliance on traditional medicinal plants for treatment of diseases and the potential for drug discovery, it becomes relevant to search for potent, effective and relatively safe plant medicines as well as to scientifically validate success claims about plants already in use by traditional medicine practitioners.

Leea guineensis G. Don (Leeaceae) is an evergreen shrub or small tree native to the tropics and used in the management of various conditions. Some of the traditional uses include the management of pain (Op de Beck *et al.*, 2000) epilepsy and convulsions (Burkill, 1985). This thesis investigated the anticonvulsant activities of the aqueous leaf extract of the plant in classical animal models of epilepsy namely maximal electroshock (MES), pentylenetetrazole (PTZ) and picrotoxin (PTX) -induced seizure tests in mice. Many anticonvulsant agents act via GABA and glutamate neurotransmission and so offer promise for novel anxiolytic therapies (Sinclair *et al.*, 2007). Also, some antiepileptic agents are now being used in managing certain types of pain including neuropathic and acute pain (Lopes *et al.*, 2009; Thienel *et al.*, 2004). Apart from that, *L. guineensis* has been reported to have analgesic effects (Op de Beck *et al.*, 2000). This

thesis therefore, also investigated the anxiolytic effect of *L. guineensis* in the elevated plus-maze and the light/dark box tests and its antinociceptive activity in the formalin test. Most anticonvulsant agents also impair neuromuscular function and hence the neuromuscular effect of the extract was determined in the beam traversal and rotarod tests and on isolated toad rectus abdominis muscle.

1.2 THE PLANT LEEA GUINEENSIS

1.2.1 Name

Botanical name: *Leea guineensis* (G. Don)

Family: Leeaceae

Local name(s): Okatakyi (Twi)

1.2.2 Description

Leea guineensis is an evergreen, erect or sub-erect shrub or small tree about 6 m or 17 feet tall by 9 feet wide, very variable with bright-red, orange or yellow flowers, brilliant-red turning black fruit, found mostly in moist shaded places in the tropics. It has light green new growth and dark green foliage. Flowers are in much-branched cymes and are up to 8 inches across, with individual flowers 1/8 inches across. On the next page is a picture of the plant.

Fig. 1.1: Leaves of *Leea guineensis*

1.2.3 *Ecological and geographical distribution*

The plant is native to moist, intermediate temperate zones in tropical Africa including Cote d'Ivoire, Liberia, Sierra Leone, Ghana, Cameroon, and Nigeria.

1.2.4 Traditional uses

The medicinal uses include treatment for diarrhoea, dysentery, as a diuretic; oral treatments, as a pain-killer, paralysis, epileptic fits (juice of fresh leaves used as an enema), convulsions, spasm and stomach troubles (Burkill, 1985).

1.2.5 Previous work done on Leea guineensis

1.2.5.1 Chemical constituents

Op de Beck *et al.*, (2000) investigated the volatile constituents from the leaves and wood of the plant. The volatile constituents were obtained by hydrodistillation and

analysed by gas chromatography-mass spectrometry (GC-MS). The analysis indicated the presence of 69 compounds and prominent among them were terpenoids.

In another study, Op de Beck *et al.*, (2003) isolated and identified three hydrophilic flavonoids named quercetin-3′-sulphate-3-*O*-*α*-L-rhamnopyranoside, quercetin-3,3′-disulphate and quercetin-3,3′4′-trisulphate, together with kaemferol, quercitrin, mearnsitrin, gallic acid and ethyl gallate. These flavonoids were found to have free radical scavenging effect which was evaluated in the 2, 2-Diphenyl-1-Picrylhydrazyl (DPPH) assay.

1.2.5.2 Anti-oedematogenic activity

The aqueous leaf extract of the leaves of *Leea guineensis* showed significant anti-inflammatory activity in the carrageenan-induced rat paw oedema (Falodun *et al.*, 2007).

1.3 EPILEPSY, ANTIEPILEPTIC DRUGS AND ANTICONVULSANT SCREENING

1.3.1 Introduction

Epilepsy is a major neurological disorder characterized by recurrent, spontaneous brain seizures or convulsions and its prevalence in developing countries is generally higher than in developed countries (Sander *et al.*, 1996; Stafford *et al.*, 2008). Epilepsy is the second most common neurological disorder after stroke and it is estimated that approximately 0.8% of the population is affected by some form of epilepsy (Pitkanen *et al.*, 2009). Recent studies suggest an increased risk of dying and a greater proportion of deaths that are epilepsy-related in Africa as high as a six-fold increase in mortality in people with epilepsy. This is higher than the two-to-three fold increase reported in

developed countries (Christianson *et al.*, 2000; Diop *et al.*, 2005). Though not clear, the reasons for this gap might be due to social deprivation (Sander, 2003). Recent data suggest that people from socio-economically deprived backgrounds in developed countries are more likely to develop epilepsy (Heaney *et al.*, 2002). This neurological disorder is viewed as a shameful disorder and has severe social implications in African communities as it carries a stigma. Sufferers are often shunned and discriminated against with respect to education, employment and marriage (Baskind *et al.*, 2005; Stafford *et al.*, 2008).

Drug therapy of epilepsy with currently available Antiepileptic Drugs (AEDs) is associated with side effects, dose-related and chronic toxicity that involve virtually every organ system. Moreover, all the currently available AEDs have potential for adverse effects on cognition and behaviour (Duncan, 2002; Samren *et al.*, 1997). The practice of polypharmacy in the therapy of epilepsy that has doubtful background increases the risk of side effects and drug interactions. It can be said that all problems with the current AED therapy of epilepsy are more prevalent in underdeveloped countries due to lack of facilities for proper diagnosis, treatment and monitoring of serum levels of AEDs. Another critical issue associated with currently available AEDs is recent clinical and experimental data that strongly suggest that AED therapy does not alter the course or natural history of epilepsy and though AEDs suppress the seizures, they may not affect the underlying disorder (Chadwick, 1995; Loscher, 2002b; Shinnar *et al.*, 1996). Only a very few AEDs have been shown to be antiepileptogenic including valproate and phenobarbitone (Duncan, 2002; Silver *et al.*, 1991) and levetiracetam (Duncan, 2002; Loscher *et al.*, 1998) but these are not well substantiated. There is pressing need for further research especially in the field of pharmacotherapy of epilepsy to find drugs which are not only anticonvulsant but also antiepileptogenics that either

prevent epilepsy or alter its natural course. Natural products and plants for that matter, used in traditional medicine can be an invaluable source for search for novel antiepileptic compounds (Meldrum, 1997; Stafford *et al.*, 2008).

1.3.2 Pathophysiology of epilepsy

A variety of different electrical or chemical stimuli can easily give rise to a seizure in any normal brain. The epileptic seizure always reflects abnormal hypersynchronous electrical activity of neurones caused by an imbalance between excitation and inhibition in the brain. Neurones are interconnected in a complex network in which each individual neurone is linked through synapses with hundreds of others. A small electrical current is discharged by neurones to release neurotransmitters of synaptic levels to permit communication with each other. More than hundred neurotransmitters or neuromodulators have been shown to play a role in neuronal excitation. However, the major excitatory neurotransmitter in the brain is L-glutamate and the major inhibitory neurotransmitter in the brain is gamma-amino butyric acid (GABA). An abnormal function of either of these could result in a seizure. An excited neurone will activate the next neurone whereas an inhibitory neurone will not. A normal neurone discharges repetitively at a low baseline frequency, and it is the integrated electrical activity generated by the neurones of the superficial layers of the cortex that is recorded in a normal electroencephalogram. If neurones are damaged, injured or suffer electrical or metabolic insult, a change in the discharge pattern may develop. In the case of epilepsy, regular low-frequency discharges are replaced by bursts of high-frequency discharges usually followed by periods of inactivity. An epileptic seizure is triggered when a whole population of neurons discharges synchronously in an abnormal way. This abnormal discharge may remain localized or it may spread to adjacent areas, recruiting more neurons as it spreads.

1.3.3 Role of GABA and glutamate in the pathogenesis of epilepsy

It is important to emphasize the role of neurotransmitters especially, γ-amino butyric acid (GABA) and glutamate in epileptogenesis, since they are the major inhibitory and excitatory transmitters in the central nervous system, respectively, and the fact that generation of seizures has been attributed to imbalance between excitatory and inhibitory neurotransmission in epileptic brains.

GABA plays an important role in regulation of neuronal excitability and impairment of GABA function produces seizures (Olsen *et al.*, 1997). Compounds that enhance GABA-mediated inhibition are convulsants (Scholze *et al.*, 1996; Sieghart, 1992). GABA exerts its major inhibitory effect via $GABA_A$ receptor (which is a ligand-gated ion channel) by increasing neuronal membrane conductance for chloride ions causing membrane hyperpolarization resulting in reduced neuronal excitability and most rapid inhibition in brain (Sieghart, 1992). $GABA_A$ receptor is target for many important neuroactive drugs including antiepileptic drugs benzodiazepines and barbiturates (Scholze *et al.*, 1996; Sieghart, 1992). $GABA_A$ receptor consists of five subunits that form a chloride ion channel (Macdonald *et al.*, 1994). The subunits consist of various subtypes and pharmacological studies have shown that individual subunits and subtypes confer different sensitivities to agents acting on $GABA_A$ receptors (Neelands *et al.*, 1998). It is postulated that exposure of GABA to postsynaptic receptors for a brief period of time results in generation of Inhibitory Post-Synaptic Currents (IPSCs) (Hill *et al.*, 1998). $GABA_A$ receptor-mediated miniature IPSCs play important physiological role in preventing the development of neuronal hyperexcitability (Salin *et al.*, 1996). Decrease in $GABA_A$ from receptor-mediated IPSCs is observed in cells from hippocampi of animals with chronic experimental epileptic seizures and humans with chronic intractable temporal lope epilepsy (Isokawa, 1996).

Glutamate is the most important excitatory neurotransmitter in all rapidly conducting relay pathways of the motor and sensory systems of the outer tube of the central nervous system. It produces fast or prolonged synaptic excitation and triggers various calcium dependent processes in the target cells, including production of nitric oxide (Bienvenu *et al.*, 2002). Glutamate is a transmitter in the corticospinal, corticostriatal pathways, intrahemispheric and interhemispheric association pathways, hippocampal circuits, primary afferents, and somatosensory and special sensory pathways, cerebellar afferents and excitatory inter-neurones. Glutamate acts via two types of receptors, ionotropic glutamate receptors (iGluR) which are ligand-gated cation specific channels and metabotropic glutamate receptors (mGluR) which are G-protein-coupled receptors. Ionotropic glutamate receptors are classified according to their prototype agonists: NMDA (N-methyl-D-aspartate), kainite and AMPA (alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid). Practically all agonists are able to induce epileptic seizures and brain damage whereas antagonists have been shown to be anticonvulsant (Mares *et al.*, 2004a; Mares *et al.*, 2004b). The role played by metabotropic glutamate receptors depends on the type of receptors: activation of type I is proconvulsant and convulsants, whereas activation of type II and III is anticonvulsant (Moldrich *et al.*, 2003).

Epilepsy may arise as a consequence of a dramatic release of glutamate from central nerve terminals. Sustained seizures of the limbic system in experimental animals result in brain damage that resembles that due to glutamate toxicity. Similar changes are seen at autopsy in patients with intractable epilepsy. In animals such seizure-related brain damage may be reduced by the administration of non-competitive NMDA receptor antagonists, but it would appear that not all seizure activity is suppressed by drugs (Leonard, 2003). The precise mechanism whereby persistent seizure activity results in neuronal degeneration is not completely understood. It seems possible that repetitive

depolarization and repolarization of the nerve membrane eventually leads to an energy-deprived state within the cell, thereby preventing the restoration of the cell membrane potential. Each depolarization will also lead to an influx of calcium ions and efflux of potassium ions, which if prolonged, can result in cell death. The reduced efficiency of glial cells to remove potassium ions, and the ability of high extracellular concentration of potassium ions to depolarize neurons and cause neurodegenerative changes also play a critical role in causing the degenerative changes that are a feature of status epilepticus and intractable epilepsy (Leonard, 2003). Recent advances have indicated that $GABA_A$ receptors work synergistically with NMDA receptors to increase the influx of calcium ions into neuroblasts and immature neurons. This is essential for the modulation of early CNS development (DeLorey *et al.*, 1999). It is evident that GABA is a critical inhibitory transmitter and seizures can rapidly be elicited by pharmacological disruption of GABAergic mechanism (Feldman *et al.*, 1991). Drugs have also been developed to modulate glutamic acid function. Reduction of excitatory glutaminergic neurotransmission is potentially important; AMPA receptor blockade probably contributes to the antiepileptic effect of drugs such as lamotrigine (Lee *et al.*, 2008).

1.3.4 Types of epilepsy

The clinical classification of epilepsy recognizes two categories, namely; partial seizures and generalized seizures, although there are some overlaps and many varieties of each. A seizure is said to be partial if it is restricted to a regional disturbance. Partial seizures are those in which the discharge begins locally and often remains localized. These may produce relatively simple symptoms without loss of consciousnesses, such as involuntary muscle contractions, abnormal sensory experiences or autonomic discharge or they may cause more complex effects on consciousness, mood and behaviour, often termed psychomotor epilepsy (Rang *et al.*, 2003).

Generalized seizures involve the whole brain, including the reticular system, thus producing abnormal electrical activity throughout both hemispheres. Immediate loss of consciousnesses is characteristic of generalized seizures (Bienvenu *et al.*, 2002). The main categories are generalized tonic-clonic seizures (*grand mal*) and absences seizures (*petit mal*). A generalized tonic-clonic seizure consists of an initial strong contraction of the whole musculature, causing a rigid extensor spasm. Respiration stops and defaecation, micturition and salivation often occur. This tonic phase lasts for about 1 minute and is followed by a series of violent, synchronous jerks, which gradually dies out in 2-4 minutes.

Most types of epilepsy are characterized by more than one type of seizure. Patients with focal (or partial) epilepsy may have simple partial, complex partial and secondarily generalized tonic-clonic seizures (e.g. partial seizures with secondary generalization). Patients with generalized epilepsy may have one or more of the following seizure types: absence, myoclonic, and tonic, clonic, tonic-clonic and atonic. Thus, no seizure type is specific for a single type of epilepsy. Seizures are symptoms, and patients should be treated for a type of epilepsy, not for a type of seizure (Benbadis *et al.*, 2001).

1.3.5 Causes of epilepsy

Approximately 1% of the world's population has epilepsy, the second most common neurological disorder after stroke (Porter and Meldrum, 2001). The cause of convulsions must be clearly understood through some precise observations. The type of seizure depends on the site of the focus in the brain. Epileptic attack can be caused by biochemical insults to the brain, such as hypoglycaemia, anoxia, hypocalcaemia, hyperventilation, water intoxication and sudden withdrawal of certain drugs such as barbiturates or alcohol (Bienvenu *et al.*, 2002). Epilepsy can also be caused by previous

active pathology, such as birth trauma to the brain, during or following meningitis, trauma to the skull and brain later in life, cerebral abscesses, cerebral infarction, cerebral haemorrhage or subarachnoid haemorrhage (Bienvenu *et al.*, 2002). Further analysis shows that the blockade of post-synaptic gamma-amino butyric acid receptors or an inhibition of GABA synthesis is the principal origin of brain discharge. According to Bienvenu and co-workers (Bienvenu *et al.*, 2002), an epileptic attack can be triggered by a sensory stimulus, which is specific for individuals. To date, there is no single unifying explanation as to how these diverse factors cause seizures. Hence, it is difficult to determine the exact cause of epilepsy, even though it has been possible to investigate the physiological events which participate in the genesis of epilepsy.

1.3.6 Mechanism of action of antiepileptic drugs

With the exception of valproate, the established AEDs tend to have clearly defined, single mechanisms of which facilitates the prediction of effectiveness of treatment on the basis of pharmacology.

At the cellular level, three major mechanisms of action of antiepileptic drugs are recognised; modulation of ion channels, enhancement of GABA inhibitory neurotransmission, and attenuation of glutamate mediated excitatory transmission (Kwan *et al.*, 2001).

1.3.6.1 Modulation of ion channels

The intrinsic excitability of the nervous system is ultimately controlled by voltage-gated ion channels which regulate the flow of cations across surface and internal cell membranes.

The sodium channel is arguably the most important and responsible for depolarization of the cell membranes and the characteristic upstroke of the neuronal action potential.

Blockade of voltage-gated sodium channels is the most common mechanism of action amongst currently available AEDs (Deckers *et al.*, 2003). Well established AEDs, phenytoin and carbamazepine are prototype sodium channel blockers and this mechanism is shared by the newer drugs lamotrigine, felbamate, topiramate and oxcarbazepine (Deckers *et al.*, 2003). These drugs mainly bind to the inactivated state of the sodium channel and produce a voltage- and frequency-dependent reduction in channel conductance, resulting in a limitation of repetitive neuronal firing with little or no effect on the generation of single action potentials (Kwan *et al.*, 2001).

Voltage-gated calcium channels, likewise sodium channels, are involved in depolarization, often recruited in response to initial sodium-dependent action potential generation. Calcium channels are distributed throughout the nervous system on dendrites, cell bodies and nerve terminals. The N-, P- and Q-type calcium channels have been implicated in the control of neurotransmitter release at the synapse, whereas the T-type channel, expressed predominantly in the thalamocortical relay neurones, is believed to play a role in the distinctive rhythmic discharges of generalised absence seizures (Kwan *et al.*, 2001). These channels represent a major target for AEDs. Ethosuximide efficacy against generalised absence seizures is believed to be mediated by blockade of the T-type calcium channel (Deckers *et al.*, 2003). Evidence suggests that valproate may have similar effects (Deckers *et al.*, 2003). Lamotrigine has also been reported to limit neurotransmitter release by blockade of the N- and P- subtypes of voltage-sensitive calcium channel while gabapentin binds to the $\alpha_{2\delta}$-subunit of the L-type channel (Kwan *et al.*, 2001).

1.3.6.2 Enhancement of inhibitory neurotransmission

GABA is the predominant inhibitory neurotransmitter in the mammalian central nervous system. Following synaptic release, GABA acts at three specific receptors, $GABA_A$, $GABA_{B,}$ and $GABA_C$ (Deckers *et al.*, 2003). The GABA belongs to the ligand-gated ion channel superfamily and responds to GABA binding by increasing chloride conductance, resulting in neuronal hyperpolarization. GABA is removed from the synaptic cleft into localised nerve terminals and glial cells by specific transport molecules. Thereafter, GABA is either recycled to the readily releasable neurotransmitter pool or metabolized by the action of the mitochondrial enzyme GABA-transaminase, thereby completing the cycle (Kwan *et al.*, 2001). Phenobarbital and the benzodiazepines bind to distinct sites on the $GABA_A$ receptor complex and exert an allosteric influence on the opening of the chloride ion channel in response to GABA. Phenobarbital increases the duration of channel opening, while the benzodiazepines increase the frequency of opening (Deckers *et al.*, 2003). Vigabatrin and tiagabin exert their antiepileptic actions by selective effects at the GABA synapse. Vigabatrin is an irreversible inhibitor of the enzyme GABA-transaminase, while tiagabin prevents the uptake of GABA from the synaptic cleft by blockade of the GAT-1 transporter.

1.3.6.3 Attenuation of excitatory neurotransmission

Glutamate is the principal excitatory neurotransmitter in the mammalian brain. Following synaptic release, it exerts its effects on both ionotropic and metabotropic receptor types. The ionotropic glutamate receptors are arguably the best characterized and are classified into three subtypes, AMPA, kainite and NMDA, which form ligand-gated ion channels permeable to sodium and depending on subtype and subunit

composition, calcium ions. The AMPA and kainite subtypes are implicated in fast excitatory neurotransmission, whereas the NMDA receptor, quiescent at resting membrane potential, is recruited during periods of prolonged depolarization (Kwan *et al.*, 2001). None of the current AEDs available exerts its pharmacological effects solely by an action on the glutamatergic system (Deckers *et al.*, 2003). However, blockade of the NMDA subtype of glutamate receptor has been reported to contribute to the antiepileptic effects of felbamate (Deckers *et al.*, 2003). Topiramate is similarly distinguished by an inhibitory action on AMPA receptors. Furthermore, several AEDs have been reported to reduce glutamate release, although this effect may be more indicative of their actions on calcium channels than a direct effect on the glutamate system (Kwan *et al.*, 2001).

1.3.7 Anticonvulsant screening

The first important neuropharmacological step in detecting the potential value of candidate anticonvulsant compounds is the classical maximal electroshock test (MEST) in mice, introduced by Putnam and Merritt (Putnam *et al.*, 1937). The MEST is the most widely used animal model in AED discovery, because seizure induction is simple and the predictive value for detecting clinically effective AEDs is high (Loscher, 2002a). A powerful detection system is ensured when the MEST is combined with the pentylenetetrazole (PTZ) seizure test. These are the two primary bioassays employed in the in vivo screening of new anticonvulsant compounds (Krall *et al.*, 1978; Loscher *et al.*, 1988; Raza *et al.*, 2001). AEDs such as phenytoin, carbamazepine, valproic acid, that inhibit the hind limb tonic extension phase (HLTE) of the electroshock seizure in MEST are effective in the therapy of generalised tonic-clonic and partial seizures, while AEDs that inhibit seizures induced by pentylenetetrazole (PTZ) in PTZ test e.g. ethosuximide and phenobarbitone are effective in the treatment of generalised

myoclonic and absence seizures (Raza *et al.*, 2001; White, 1997). The subcutaneous administration of bicuculline (BIC), picrotoxin (PTX) and strychnine (STN) are also valuable tests to induce seizures and evaluate the effectiveness and mechanisms of anticonvulsant compounds (Porter *et al.*, 1984; Raza *et al.*, 2001). The MEST and the PTZ test are often classified as acute animal models (Loscher, 2002a). Clinically efficacious drugs have been discovered by these acute models including ethosuximide, trimethadone and valproate. These show similar anticonvulsant effects in different genetic models of absence epilepsy such as Genetic Absence Epilepsy Rats from Strasbourg (GAERS) or lethargic mice (Loscher, 2002a). This suggests that there is no need to use chronic models in the search for new AEDs (Loscher, 2002a). However, these models used alone might not detect all compounds with antiepileptic activity. The MEST preselects drugs with certain mechanisms, but misses others (Meldrum, 1997). Although the MEST is often considered a mechanism-independent model (Kupferberg, 2001), electroconvulsive seizures are particularly sensitive to drugs blocking sodium channels (Meldrum, 1997). This means that several clinically efficacious AEDs which act by other mechanisms (such as levetiracetam, vigabatrin and tiagabin) and were initially not screened or detected by using MEST would have been missed using MEST as the only drug discovery model (Loscher *et al.*, 1994). Also, the PTZ test might not be able to detect all antiepileptic drugs against non-convulsive seizures (Loscher, 2002a). This is due to the fact that lamotrigine, which is very efficacious against non-convulsive seizures in patients, is ineffective in the PTZ test, while vigabatrin and tiagabin, which are quite effective in the PTZ test, are ineffective in patients and even aggravate non-convulsive seizures (Loscher, 2002a). In these instances, chronic seizure models including kindling, genetic models such as GAERS or lethargic mice have been used (Loscher, 2002a). Also, even after the primary screening of anticonvulsants, advanced

experiments on primate models and 'Kindling in rodents' which may follow include monkey models of absence (*petit mal*) seizures, aluminium hydroxide induced partial or secondary generalised (grand mal) seizures in monkeys, experimental temporal lobe epilepsy in monkeys and amygdala kindled seizures in rats.

1.4 EFFECT OF ANTICONVULSANTS IN ANXIETY

1.4.1 Background

Anxiety is both a normal emotion and a psychiatric disorder. Anxiety is a feeling of apprehension or fear, combined with symptoms of sympathetic activity. It is a normal response to stress and only becomes a clinical problem only if the anxiety becomes severe or persistent, and interferes with everyday performance. It has a lifetime prevalence of over 5% of the population (Sinclair *et al.*, 2007). A number of pharmacological theories exist which suggest that anxiety is caused by either amine or excitatory amino acid function and anxiolytics have therefore, been developed to target specific brain neurotransmitter systems. GABA is the main inhibitory neurotransmitter in the brain. The $GABA_A$ receptor is the brain's main inhibitory receptor and so regulates the activity of many types of neurons, including dopaminergic, noradrenergic and serotonergic. There is considerable evidence that a down-regulation of $GABA_A$ function may underlie some forms of anxiety, some of which comes from imaging studies (Malizia *et al.*, 1998).

Most anticonvulsant drugs act via GABA and glutamate neurotransmission and so offer promise for novel anxiolytic therapies. Benzodiapines act on $GABA_A$-BZ receptor and are effective predominantly in panic disorder (PD), general anxiety disorder (GAD) and social phobia (Malizia *et al.*, 1998). Gabapentin and pregabalin are effective in certain anxiety disorders. Pregabalin, which works via voltage-gated Ca^{2+} channels, causing

decreased release of several neurotransmitters, has shown short-term efficacy GAD (Sinclair *et al.*, 2007). The anticonvulsant properties of lamotrigine are mediated via NMDA glutamate receptor antagonism. Efficacy has been shown in post-traumatic stress disorder (PTSD) (Sinclair *et al.*, 2007). Tiagabine, a $GABA_A$ reuptake inhibitor, has had mixed results in clinical trials but has shown efficacy in GAD as well as in PD.

1.4.2 Animal models used for screening anxiolytics

1.4.2.1 Introduction

Animal tests of anxiety are used to screen for novel compounds for anxiolytic or anxiogenic activity, to investigate the neurobiology of anxiety, and to assess the impact of other occurrences such as exposure to predator odours or early rearing experiences. There is a diversity of animal models of anxiety currently available. These behavioural models involve exposure of animals to stimuli (exteroceptive or interoceptive) that appear capable of causing anxiety in humans. Despite their apparent diversity, animal anxiety models may be grouped into two general categories involving either conditioned (e.g. Geller-Seifter conflict, potentiated startle) or conditioned (social interaction and light/dark exploration tests) responses (Rodgers *et al.*, 1997). An ideal model of anxiety should have predictive, face and construct validities. A model that has predictive validity should display reduced anxiety when treated with anxiolytics, while in a model with face validity, the response of an animal to a threatening stimulus should be comparable to the response known for humans, and the mechanisms underlying anxiety should be exhibited by a model with construct validity (McKinney *et al.*, 1969). Naturally, one or more models usually combine to achieve these parameters. Conditioning models require considerable training of subjects, food or water deprivation and/or the use of electric shock as an aversive stimulus. However, some of

these procedures, such as conditioned defensive burying, take advantage of the natural tendency of rodents to make faster stimulus-response associations when faced with ecologically relevant (versus arbitrary) environmental challenges (Treit, 1990). The study of unconditioned responses to various forms of external threat represents a logical extension of this refinement of laboratory methods, providing a high degree of ecological validity for the research and allowing for a very much more complete behavioural characterization of the effects of experimental manipulations. Hence models involving unconditioned behavior were adopted in this study as discussed below:

1.4.2.2 The elevated plus-maze test

The elevated plus-maze (EPM) is 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 (Carobrez *et al.*, 2005; File, 1992; Handley *et al.*, 1993; Rodgers *et al.*, 1997). It has been initially described by Pellow and co-workers (Pellow *et al.*, 1985) as a simple method for assessing anxiety responses by rodents. Handley and company (Handley *et al.*, 1984) described it as made of four arms (two open and two closed) that are arranged to form a plus shape. These authors described the assessment of anxiety behaviour of rodents by using the ratio of time spent on the open arms to the time spent on the closed arms. In the test, mice or rats are placed at the junction of the four arms of the maze, facing an open arm, and entries/duration in each arm are recorded by a video-tracking system and observer simultaneously for 5 minutes. Other ethological parameters (i.e. rears, head dips, and stretch-attend postures) reflect anti-anxiety behaviour (Walf *et al.*, 2007). Unlike other behavioural assays used to assess anxiety responses that rely upon the presentation of noxious stimuli (i.e. electric shock, food/water deprivation, loud noises, exposure to

predator odour, etc) that typically produce a conditioned response, the EPM relies upon rodents' proclivity toward dark, enclosed spaces (approach) and an unconditioned fear of heights/open spaces (avoidance) (Walf *et al.*, 2007). However, the focus of this study using the EPM was to assess the anxiolytic effect of the plant extract. This model was chosen because it has face validity, which is the ability of a task to appear to measure what it is supposed to measure. For instance, in the EPM, the anxiety or fear of open spaces/heights of rodents seems to be measured. In this task, the open arms are avoided and rodents spend the majority of the time in this task in the closed arms of the maze. Other anxiety-related behaviours of rodents, such as freezing/immobility and defaecation, are increased on the open arms of the maze compared to the closed arms (Pellow *et al.*, 1985). The EPM also has construct validity, which refers to whether an observable dependent variable, such as time spent in the open arms of the EPM, used measures an unobservable construct, such as anxiety. This is demonstrated by anxiogenic drugs reducing time spent on the open arms and anxiolytic drugs increasing the time spent on the open arms of the EPM (Pellow *et al.*, 1985). Predictive validity, defined as the extent to which the dependent measure predicts behaviour on a related measure is characteristic of the EPM. It has been shown that increased open arm activity occurs in rodents that also demonstrate increased central square entries in a brightly lit open field (Frye *et al.*, 2000). Furthermore, plasma Corticosterone is increased with open arm exposure and is positively correlated with risk assessment behaviour (i.e. stretch-attend postures) in the EPM (File *et al.*, 1994; Rodgers *et al.*, 1999).

1.4.2.3 The light/dark box test

Apart from the elevated plus-maze, the light/dark box and the open field are other test models that provide unconditioned anxiety-like behaviour. All of these tests do not

require conditioning, do not cause physical discomfort and are considered to have ecological validity (Rodgers *et al.,* 1997). Some anxiety-related behavioural tests appear to be highly variable (Carobrez *et al.*, 2005; Hogg, 1996; Milner *et al.*, 2008). Researchers have also found that anxiety-like behavioural measurements are not always highly correlated across tasks, suggesting that different anxiety-like behavioural tests do not necessarily address the same underlying construct (Milner *et al.*, 2008). Griebel and friends (Griebel *et al.*, 2000) tested the behaviour of various mouse strains in the LDB and EPM tests and found that basal anxiety-related behaviour in LDB test was not strongly associated with basal anxiety-like behaviour in the EPM and that diazepam's anxiolytic effects in one assay were not necessarily observed in the other. These discrepancies can be explained by the idea that different tests reflected different types of anxiety (Belzung *et al.,* 2001). However, other differences such as sensitivity to light (DeFries *et al.,* 1966) or locomotor behaviour (Henderson, 1986) could be responsible.

The light/dark test is based on the innate aversion of rodents to brightly illuminated areas and on the spontaneous exploratory behaviour of rodents in response to mild stressors, that is, novel environment and light (Crawley *et al.,* 1980). A natural conflict situation occurs when an animal is exposed to an unfamiliar environment or novel objects (Bourin *et al.*, 2003). The light/dark box apparatus is based on the initial model described by Crawley *et al.* (Crawley et al., 1980). However, many structural modifications have been made to it. The typical dimensions of the compartment are generally one third for the dark compartment and two thirds for the light compartment. The model is based on the observation that although nocturnal rodents such as mice will naturally tend to explore a novel environment, open fields appears to have aversive properties which inhibit exploratory behaviour (Bourin *et al.*, 2003). Here, the safe area is the small dark compartment (one third) and the aversive area is the large illuminated

compartment. When a rodent is placed in the box, the conflict is between the tendency to explore and the initial tendency to avoid the unfamiliar (neophobia). Thus in the light/dark test, drug-induced increase in behaviours in the white part of a two-compartment box, in which a large white compartment is illuminated and a small compartment is darkened, is suggested as an index of anxiolytic activity. This model differs from other purported models of anxiety which are not equivalent in terms of elicited/induced emotional state (De Vry *et al.*, 1993; File, 1992; Njung'e *et al.*, 1991; Treit, 1990).

1.5 EFFECT OF ANTICONVULSANTS IN PAIN

1.5.1 Background

Pain is considered to be an unpleasant sensory and emotional experience associated with potential or actual tissue damage, or described in terms of such damage. However, there is evidence that this might not occur in certain painful conditions, but is the result of interaction between neuromediaters, neurotransmitters and signal transducers (Lopes *et al.*, 2009). It often has a protective function and is essential for survival. Pain in many cases represents the only symptom for the diagnosis of several diseases. It is associated with various diseases, inflammatory conditions, tissue trauma and surgical interventions. Pain is different from nociception which is the encoding and processing of noxious stimuli by the nervous system. Pain is a subjective experience, and includes a strong emotional component.

Pain is often called ‘chronic’ when it lasts longer than 6 months. In chronic pain, there is often no clear causal relationship between nociception and pain: pain does not reflect tissue damage, but may be due to psychological and social factors. Chronic pain might

also result from persistent nociceptive process, and be accompanied by neuroendocrine dysregulation, impaired physical and even mental performance (Schaible *et al.*, 2004).

Pathophysiological nociceptive pain occurs when tissue is inflamed or injured; it may appear as spontaneous pain, as hyperalgesia or allodynia, and be effectively treated with nonsteroidal anti-inflammatory drugs (NSAIDs) and opiates (Schaible *et al.*, 2004). Pain caused by neurone injury or neuropathic pain in the peripheral or central nervous system is less effectively managed by these drugs and often, anticonvulsants or tricyclic antidepressants are used (Schaible *et al.*, 2004).

Throughout history man has used many different forms of therapy for the relief of pain, among them; medicinal herbs have been popularly used. An example is *Papaver somniferum* from which morphine was isolated (Almeida *et al.*, 2001). Current conventional drugs for treatment of various types of pain have important issues of pharmacological concern in terms of their efficacy, side effects and safety (Almeida *et al.*, 2001). Morphine is regarded as the prototype of opiate analgesic drugs. This group of drugs generally acts on the central nervous system exercising their effects through three opioid receptors (μ, κ, and δ) and are important for the treatment of chronic pain and moderate to severe pain (Martin *et al.*, 2001). However, there are concerns of morphine's addictive properties and side effects which include respiratory depression, drowsiness, nausea and several alterations of the endocrine and autonomic systems (Almeida *et al.*, 2001; Lopez-Munoz *et al.*, 2008). The non-opioid analgesics or NSAIDs have peripheral actions and produce their analgesic effects by inhibiting prostaglandin synthesis. These drugs are commonly used to treat musculoskeletal complaints and for minor aches and pain. Their use is responsible for numerous adverse reactions worldwide and they feature in reports of drug-related deaths (Rang HP, 2003). Their toxic effects occur mainly in the gastrointestinal tract and bone marrow.

Furthermore, pathological pain or pain of neuropathic origin is typically resistant to these conventional treatments. Anticonvulsants have been used in these situations due to the belief that they are effective in alleviating certain forms of neuropathic pain (McQuay *et al.*, 1995; Todorovic *et al.*, 2003) and even in acute pain (Lopes *et al.*, 2009; Thienel *et al.*, 2004). Medicinal plants with anticonvulsant and/or antinociceptive activity may lead to the development substances devoid of side effects related to morphine or NSAIDs and may be useful in managing pain non-responsive to currently available pharmacological treatments.

1.5.2 Effect of anticonvulsants in animal models of pain

Tissue injury and inflammation generate bradykinin, which not only activates nociceptors, but increases their sensitivity by triggering prostaglandin production (Baker, 2005). The process of pain transduction, gating and modulation involves neurotransmitters (L-glutamate, N-methyl-D-aspartic acid, γ-amino butyric acid) ionic channels (calcium and sodium), and neuropeptides (calcitonin gene-related peptide, substance P) (Okuse, 2007). These mechanisms make anticonvulsants useful in the treatment of many neuropathic and even acute pain conditions (Lopes *et al.*, 2009; Thienel *et al.*, 2004). Lopes and friends (Lopes *et al.*, 2009) used acute pain models to investigate the antinociceptive effect of topiramate and found that it shows antinociceptive activity in these models. It has also been shown in animal models that gabapentin prevents nociceptive responses from hyperalgia in animal models (Field *et al.*, 1997; Taylor *et al.*, 1998) and also has analgesic actions in clinical reports (McGraw *et al.*, 1997; Mellick *et al.*, 1997; Rosner *et al.*, 1996).

There are many models of pain available for investigating the antinociceptive activity of drugs including anticonvulsants with potential antinociceptive activity. These include chemical and thermal methods in rodents. The formalin test is the most commonly used, and involves intradermal injection of formalin solution (Le Bars *et al.*, 2001). It is the most predictive of acute pain (Le Bars *et al.*, 2001) and a valid model for clinical pain (Costa-Lotufo *et al.*, 2004; Vasconcelos *et al.*, 2003; Vissers *et al.*, 2003).

The subcutaneous administration of formalin into the plantar surface of the rodent paw produces a biphasic nocifensive behavioural response. The early phase consists of intense licking and biting of the injected paw and lasts up to 10 minutes but a second late phase of licking and biting occurs from 10 to 60 minutes after injection (Dubuisson *et al.*, 1977). The late phase is a state of facilitated pain response (hyperalgesia) associated with inflammation. This behavioural response has been shown to correlate with a biphasic increase in the activity of C-fibre primary afferent neurons after formalin injection (McCall *et al.*, 1996). The formalin behavioural test involves sensitization of sensory neurons of the spinal dorsal horn in response to injury or intense artificial activation of C-fibre afferents (Woolf *et al.*, 1986).

1.6 PURPOSE AND OBJECTIVES OF PRESENT STUDY

The primary aim of this study was to investigate the anticonvulsant activity of *Leea guineensis* and other neurobehavioural effects that may be associated with antiepileptic drugs. The specific objectives of the thesis therefore included to:

- *Evaluate the anticonvulsant activity of the aqueous leaf extract against pentylenetetrazole, picrotoxin and maximal electroshock -induced seizures.*
- *Examine the anxiolytic effect of the extract using classical models of anxiety including the elevated plus-maze and the light/dark box tests.*

- *Evaluate the analgesic activity of the plant's extract in the formalin-induced pain test*
- *Investigate the effect of the extract on motor coordination and balance using the beam traversal task and the rotarod test and the in vitro neuromuscular effects using the isolated toad's rectus abdominis muscle.*
- *Phytochemical tests on the extract for the presence of secondary metabolites.*

Chapter 2

MATERIALS AND METHODS

2.1 PLANT COLLECTION AND EXTRACTION

The fresh leaves of *Leea guineensis* G. Don (Family: Leeaceae) were collected at the KNUST Botanical Gardens in September, 2007 and authenticated by Dr. Kofi Annan of the Department of Pharmacognosy, KNUST. A voucher specimen of the plant (LEEACEAE/FP/08/26) has been deposited in the University's Pharmacognosy Departmental Herbarium.

The plant extract was prepared by blending and macerating the fresh leaves of *L. guineensis* with distilled water at room temperature for 24 hours. The mixture was filtered and the extract obtained by concentrating the filtrate to dryness using a freeze-dryer. The dried aqueous extract, herein referred to as the extract, LGE, was stored in a refrigerator at 0 ^{0}C. During experimentation, the dried aqueous extract was always suspended in distilled water for administration.

2.2 DRUGS AND CHEMICALS

The drugs and chemicals used in this study include: diazepam (INTAS, Gujarat, India), pentylenetetrazole, picrotoxin, acetylcholine bromide, formalin (39% with 10% methanol as a stabilizer), and tubocurarine, (Sigma, St. Louis, MO., USA), carbamazepine (Tegretol®, Novartis, Basel, Switzerland).

2.3 EXPERIMENTAL ANIMALS

Male and female ICR or Balb C mice (20-35g) were used in this study. The animals were purchased from the Noguchi Memorial Institute for Medical Research (NMIMR),

University of Ghana, Legon, and housed in the animal house of the Department of Pharmacology, Kwame Nkrumah University of Science and Technology, Kumasi. The animals were housed in groups of 10 in stainless steel cages (34 cm x 47 cm x 18 cm) with soft wood shavings as bedding, fed with normal commercial pellet diet (GAFCO, Tema), given water *ad libitum* and maintained under laboratory conditions (temperature 24 - 28°C, relative humidity 60 - 70%, and 12 h light-dark cycle). All procedures and techniques used in this study were in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals (NIH, Department of Health and Human Services publication no. 85 - 23, revised 1985) and were approved by the Departmental Ethics Committee.

2.4 PHYTOCHEMICAL TESTS

The freshly prepared aqueous extract was analysed for phytochemical constituents as described by Trease and Evans (1989) for the detection of alkaloids, saponins, cardiac glycosides, reducing sugars, flavonoids and tannins. These are described as follows:

2.4.1 Test for alkaloids

A sample of the freeze-dried extract (0.5 g) was boiled with 10 ml of dilute hydrochloric acid in a test tube for 5 minutes. The supernatant liquid was filtered into another test tube and 1 ml of the filtrate was taken, into which 3 drops of Dragendorff's reagent (potassium bismuth iodide solution) was added. The mixture was shaken and observed for the appearance of an orange-red spot and a precipitate formation.

2.4.2 Test for Tannins

An amount of the extract (0.5 g) 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. A small

quantity of water (10 ml) and 2 drops of 1 % ferric chloride were added to 1 ml of the filtrate and observed for the appearance of a blue-black precipitate.

2.4.3 Test for saponins

A small amount (0.2 g) of the extract was shaken with a few mls of water in a test tube and the mixture observed for the presence of a froth which does not break readily upon standing.

2.4.4 Test for reducing sugars

A portion of the extract (0.2 g) was boiled in 5 ml of water. The mixture was cooled and filtered. An equal quantity (5 ml) of Fehling's A and B solutions were added to the filtrate, heated and observed for a red-brown precipitate.

2.4.5 Test for cardiac glycosides

A sample of the extract (500 mg) was boiled in 5 ml of 70% ethyl alcohol for 2 minutes. The mixture was filtered and 10 ml of water and 5 ml of chloroform added to the filtrate and shaken. The lower chloroform layer was separated off and evaporated to dryness in a water bath. The cooled chloroform residue was dissolved in 3 ml of glacial acetic acid containing 0.1 ml of ferric chloride. The solution was carefully transferred to the surface of 2 ml of sulphiric acid and observed for a reddish-brown layer formed at the interface and whether the upper layer gradually acquired a bluish-green colour.

2.4.6 Test for flavonoids

About 10 g of the extract was boiled for 3 minutes in 100 ml of water in a water bath. To 3 ml of the filtrate, 3 ml of acid alcohol (ethanol: water: concentrated hydrochloric acid in a ration of 1:1:1), a solid magnesium (1 cm) and 1 ml of t-amyl-alcohol were added. The mixture was observed for a rose-orange or violet colour change.

2.5 ASSESSMENT OF ANTICONVULSANT ACTIVITY

2.5.1 Pentylenetetrazole-induced seizure test

The method used was adapted from that described by Swinyard *et al.*, (1985). Female ICR mice were divided into seven groups (n=5). The extract (30, 100 and 300 mg/kg *p.o.*) was administered to three groups while diazepam (0.1, 0.3 and 1.0 mg/kg *i.p*) was given to three other groups and the last group administered 10 ml/kg *p.o* of the vehicle to serve as control. After 1 hour and 30 minutes of treatment with drugs orally and intraperitoneally respectively, each mouse was administered pentylenetetrazole, 85 mg/kg subcutaneously. The animals were placed individually in clear plastic observation chambers (15 cm x 15 cm x 15 cm) placed on a large plain glass elevated above the floor (80 cm) and a mirror placed behind the glass at an angle of 45^0 to the glass on the floor to enable clear and complete view of the animals. A digital video camera was positioned in front of the mirror to videotape test sessions. The Behaviour Tracker Software Version 1.5 (http://www.behaviortracker.com/) was used to analyse the videos for the latency to the first myoclonic jerks, the latency to tonic convulsions and the frequency and duration of tonic convulsions for each mouse.

2.5.2 Picrotoxin-induced seizure test

The procedure used was the same as in the case of pentylenetetrazole-induced seizure test except that mice were administered picrotoxin, 10 mg/kg intraperitoneally (Avallone *et al.*, 2000; Mackenzie *et al.*, 2002; Ngo Bum *et al.*, 2004; Swinyard, 1969) 30 minutes and 1 hour after treatment with diazepam and LGE, respectively instead of pentylenetetrazole. The latency to myoclonic jerks, latency to tonic convulsions and the

frequency and duration of tonic convulsions were recorded from the videos for each mouse as in PTX-induced convulsions.

2.5.3 Maximal electroshock-induced seizure test

The method used has been previously described by Schmutz *et al.*, (1990). Male ICR mice were grouped into seven (n=10). Three groups were treated with the extract (30, 100 and 300 mg/kg *p.*o), three other groups treated with carbamazepine (3, 10 and 30 mg/kg *p.*o) and the last grouped administered distilled water (10 ml/kg *p.o*), to serve as control. After 1 hour and 30 minutes of oral and intraperitoneal drug treatments, respectively tonic convulsions of the hind limb extremities of mice were induced by passing alternating electrical current (50 Hz, 60 mA and 0.2 s) through ear electrodes. This was the maximal current (60 mA) that induced tonic hind limb extension in all the trial mice and it was determined previously before commencement of the experiment. The number of animals protected from tonic hind limb extension seizure and the time spent in this position were determined in each dose group.

2.6 ASSESSMENT OF ANXIOLYTIC ACTIVITY

Anxiolytic activity was assessed using the light/dark box and the elevated plus-maze tests.

2.6.1 The elevated plus-maze test

This test was adapted from the methods described by Pellow *et al.*, (1985) and Lister, (1987). The apparatus was made of Plexiglas and consisted of two open arms (15 cm x 5 cm x 1 cm) and two enclosed arms (15 cm x 15 cm x 5 cm). The arms extended from a central platform (5 x 5 cm^2) forming a plus-sign with like arms opposite each other. The maze was elevated 60 cm from the floor. Male ICR mice weighing 20-35 g were

divided into ten groups (n=5). Three groups were administered the extract (30, 100 and 300 mg/kg *p.o*), three groups received diazepam (0.1, 0.3 and 1.0 mg/kg, *i.p.*), three other groups given pentylenetetrazole (3, 10 and 30 mg/kg, *i.p.*) and the last group given distilled water (10 ml/kg *i.p*). Thirty minutes after *i.p* injection with diazepam and pentylenetetrazole and 1 h after oral treatment with the extract and vehicle, mice were placed individually in succession in the central platform of the maze for 5 minutes and their behaviour videotaped with a digital video camera placed above the maze. Behavioural parameters were scored from the videotapes using the Behaviour Tracker software Version 1.5 (http://www.behaviortracker.com/) as follows:

(1) Number of entries into and time spent in each arm i.e. closed and open arms,

(2) Number and duration of protected and unprotected stretch-attend postures,

(3) Number and duration of protected and unprotected head dipping,

(4) Number and duration of grooming and

(5) Number and duration of rearing.

Entry into an arm was defined as the animal placing all four paws into the arm. Protected head dipping was defined as the mouse stretching to dip its head into the open space and observing the environment with the body remaining in a closed arm or the central platform while in nonprotected head dipping, the mouse dips its head into the open space and observing the environment with the body being in an open arm. Protected stretch attend postures were defined as the mouse stretching forward and retracting without moving forward its feet whilst in the closed arm or central platform of the maze whereas unprotected stretch-attend postures were defined as the mouse

exhibiting this behaviour whilst in the open arms. To compute total distance travelled by mice, the software Behavior Collect (http://cas.bellamrmine.edu/tietjen/DownLoads/computer_programs_for_data_colle.htm) was used to obtain raw data from the videos. These data were then exported into Microsoft® Office Excel 2007 and further analysed. Distance between X-Y coordinate pairs was calculated from the formula: $\sqrt{[(X_1-X_2)^2+ (Y_1-Y_2)^2]}$.

2.6.2 The light/dark box test

The method used in this test has been described by Ardayfio *et al.*, (2006) with little modification. The apparatus was a wooden box (36 cm long x 33 cm wide x 30 cm deep) divided into two compartments by a wooden board with a small opening (8 cm x 8 cm) connecting the compartments. The larger compartment comprised two-thirds of the apparatus, painted white, open and illuminated by a 60-W lamp placed above the compartment. The smaller compartment was painted black and had a cover that was closed during testing. Male ICR mice were divided into seven groups (n=5) and treated with LGE, diazepam and the vehicle as described above for the elevated plus-maze test. At the beginning of the experiments, mice were placed individually at a far corner of the dark compartment facing the light compartment and videotaped with a digital video camera for a period of 5 minutes. Behaviours of the animals from the videotapes were scored manually with the aid of the computer software, Behaviour Tracker Version 1.5 for the following parameters:

(1)The latency to emerge from the dark compartment with all four paws into the light compartment,

(2) Total time spent in each compartment, and

(3) Total number of transitions between the compartments. Animals were placed in a far corner of the dark compartment facing the light compartment at the beginning of experimentation because previous studies have found that when started with the light side of the box, mice given anxiogenic treatments did not explore early enough to discover and enter the dark compartment and generally thought to reflect anxiolytic-like effects. Instead, it is been found that the emergence latency to leave the dark compartment and enter the light compartment is a more reliable indicator of anxiety-like behaviour sensitive to both anxiogenic and anxiolytic treatments (Ardayfio *et al.*, 2006).

2.7 ASSESSMENT OF ANALGESIC EFFECT

2.7.1 The formalin test

The antinociceptive effect of the extract was evaluated using the formalin test. The method used was an adaptation of the method described by Malmberg *et al.*, (1995). Mice were randomly divided into eleven groups (n=5) and each mouse acclimatized to the testing environment (clear plexiglas chambers, 15x15x15 cm^3) for 30 minutes before formalin injection.

Two sets of experiments were carried out in this test. In the first set, mice were pre-treated with LGE (30, 100 and 300 mg/kg, *p.o.*), morphine (1, 3 and 10 mg/kg, *i.p.*) and 10 ml/kg of vehicle *p.o* to serve as control. The second set involves determination of possible involvement of opioid and adenosinergic systems in the mechanisms by which LGE may inhibit formalin-induced nociception. Hence two groups of mice were pre-treated intraperitoneally with naloxone (2 mg/kg, a nonselective opioid receptor antagonist) and two other groups pre-treated with theophylline (10 mg/kg *i.p* a nonselective adenosine receptor antagonist) before treatment with LGE and morphine.

Fifteen minutes after treatment with the antagonists, the animals then received LGE (100 mg/kg *p.o.*: one group from naloxone pre-treatment and one from theophylline pre-treatment), and morphine (3 mg/kg *i.p.*: one group from naloxone pre-treated groups and one from that of theophylline).

After the above treatments, each mouse was subjected to 0.01ml of 5% formalin right hind paw intraplanter injection (1 hour for oral route and 30 minutes for *i.p* route of test drug pre-treatment). Each animal was immediately returned after formalin injection into one of the testing chambers which were placed on a large plain glass elevated 80 cm above the floor. A mirror placed at an angle of 45^0 to the glass on the floor of the chambers allowed a complete view of the paws of the mice. The mice were videotaped with a digital video camera positioned in front of the mirror for 60 minutes. From the videos, pain response was scored for 60 minutes, starting immediately after formalin injection. The first phase of the formalin test was defined as 0-10 min and the second phase 10-60 min post formalin injection (Wilson *et al.*, 2002). Nociceptive behaviour was quantified by counting the incidents of spontaneous licking/biting of the injected paw (Hayashida *et al.*, 2003) using the public domain software JWatcher version 1.0 (University of California, Los Angeles, USA and Macquarie University, Sydney, Australia, http://www.jwatcher.ucla.edu/). Nociceptive score was determined for each 5 minute time block in each phase by measuring the amount of time spent licking/biting the injected paw. The product of the frequency and duration of licking/biting was used as nociceptive score.

2.8 EFFECT ON MOTOR FUNCTION AND NEUROMUSCULAR EFFECT *IN VITRO*

The effect of *Leea guineensis* on motor coordination and balance was determined using the beam traversal task and the rotarod test while *in vitro* neuromuscular effect was determined on isolated toad rectus abdominis muscle.

2.8.1 The beam traversal test

The beam walking task (also known as the raised-bridge test) was adapted from the methods described by Carter *et al.*, (2001) and Meredith *et al.*, (2006). The apparatus consisted of a wooden beam 12 mm wide and 1m long elevated 50 cm above the floor on two narrow support stands (3 cm cross-section, 50 cm high) one end on each stand. A goal box (20 cm on each side, with a 4 cm x 5 cm entrance hole) was secured on one of the narrow support stands. A 60-W desk lamp was positioned above the start end (the one without the goal box) of the beam to create an aversive stimulus (bright light). The mice were trained to traverse the beam (three consecutive trials each day for three days) to the goal box in less than 20 seconds (Carter *et al.*, 2001). Mice that could not traverse into the goal box within 20 seconds were not used for the test. In the middle of the beam, 80 cm was defined by drawing lines 10 cm from the beginning and 10 cm from the end of the beam using a permanent marker. Mice were divided into seven groups (n=5) and treated with the extract (30, 100 and 300 mg/kg *p.o*; a dose for a group), diazepam (0.1, 0.3 and 1.0 mg/kg *i.p*; a dose for a group) and on group received 10 ml/kg *p.o.* distilled water to serve as control. During testing, mice were placed individually at the start end of the beam and allowed up to 60 seconds to traverse the beam. The test sessions were recorded with a video camera and tapes were then analysed for stepping errors, total number of steps, and time to traverse (Carter *et al.*,

2001; Meredith *et al.*, 2006). Mice which did not cross the beam or fell down from the beam were given a score of 60 seconds.

2.8.2 The rotarod test

The method used was adapted from the one described by Dunham and Miya (1957). The rotarod apparatus (Ugo Basile, model 7600, Milan, Italy) consisted of a rotating bar suitably machined to provide grip. Latency to fall from the bar is automatically recorded in seconds. Mice were initially selected for their ability to remain on the rotarod for at least two consecutive 60 seconds trials before the test day. On the test day (24 h after selection), the latency to fall from the rotarod (one trial of 60 seconds) was determined. Mice were randomly divided into seven groups (n=5) and treated as described for the beam traversal task before being placed on the rotarod. The rotarod was rotating at a constant speed of 25 rev/min. Mice that stayed on the bar for more than 60 seconds were given the maximum score, 60 seconds.

2.8.3 Effect on isolated toad rectus abdominis muscle preparation

The *in vitro* neuromuscular effect of the extract was assessed on an isolated toad rectus abdominis muscle preparation in Ringer solution. The procedure used has previously been described by Basuray *et al.*, (1968). A strip of the rectus abdominis muscle was isolated from a toad (20 g) and mounted in aerated Ringer solution. Experiments were done at room temperature. Contractions were recorded with a frontal writing-lever attached to a rotating smoked kymograph paper. The load on the tissue was between 0.5-1.0 g. A 1 g weight was attached to the lever at the same distance from fulcrum as is the preparation, but on the other side. This was raised and lowered as necessary to relax the muscle to the baseline after drugs were added and washed out. The preparation was

stretched and allowed to equilibrate under this tension (1 g) after initial setup for about 1 hour before experiments began in order to obtain a stable basal condition. In all experiments, ACh was allowed in contact with the tissue for 35 seconds whereas the extract was allowed 45 seconds. The tissue was then washed out, the preparation gently stretched by lowering the 1 g weight, then washed again. Tissue was then left for 3 minutes and the next application of ACh or extract was made exactly 3 minutes after the previous one. In this time cycle of 3 minutes, a total of 3 washings were given. The recovery of the tissue after addition of drugs was complete. Contractions were recorded with graded doses of ACh or LGE till a maximum response was reached in absence and presence of varying concentrations of tubocurarine (tubocurarine was always added to the bath and allowed for 45 seconds before ACh or LGE added). Also contractions were recorded with graded doses of ACh in the presence of different concentrations of the extract till a maximum response was attained. Five separate experiments were done each for ACh, LGE, ACh in the presence of tubocurarine, LGE in the presence of tubocurarine and ACh in the presence of LGE. The mean ± S.E.M of these five experiments were used to plot log concentration-response curves. Submaximal responses to only ACh, only LGE, ACh in the presence of tubocurarine, LGE in the presence of tubocurarine and ACh in the presence of LGE were expressed as a percentage of the maximal response to the full agonist (ACh) in the absence of tubucurarine.

2.9 STATISTICAL ANALYSIS

In all experiments, a sample size of five or ten (n=5 or 10) was utilized. All data are presented as mean ± SEM. To compare differences between groups, one-way ANOVA was performed with Newman-Keuls' test as *post hoc.* Also in some instances (the

light/dark box test, elevated plus-maze test and the formalin test), behavioural data were analysed using two-way ANOVA followed by Bonferroni's test as *post hoc*.

GraphPad Prism for Windows 5 (GraphPad Software, San Diego, CA, USA) was used for all statistical analysis and ED_{50} determinations. $p<0.05$ (Newman-Keuls' test or Bonferroni's test) was considered statistically significant.

Chapter 3

RESULTS

3.1 PHYTOCHEMICAL TESTS

Phytochemical analysis of the extract revealed the presence of alkaloids, saponins, reducing sugars, cardiac glycosides and flavonoids. However, the tests show that the leaves of *L. guineensis* do not contain tannins.

Table 3.1: Phytochemical analysis of *L. guineensis*

Test	**Results**
Alkaloids	Present
Saponins	Present
Reducing sugars	Present
Flavonoids	Present
Tannins	Absent
Cardiac glycosides	Present

3.2 ANTICONVULSANT EFFECTS

3.2.1 Effect of extract on pentylenetetrazole-induced seizures

The extract showed significant anticonvulsant activity against PTZ-induced seizures. It significantly and dose-dependently delayed the onset of myoclonic jerks ($F_{3,16}$ =6.29, P=0.0051; fig. 3.1) and decreased the duration of tonic convulsions ($F_{3,16}$ =9.20, P=.0009, fig. 3.2b). LGE significantly delayed the onset of myoclonic jerks at doses of 100 mg/kg and 300 mg/kg ($p<0.01$ and 0.05, respectively) but 30 mg/kg did not cause a significant delay in the onset of myoclonic jerks ($p>0.05$). Reduction in the duration of tonic convulsions by the extract was profound at all the doses used ($p<0.01$ at 30-300 mg/kg). Again, it delayed the onset of PTZ-induced tonic convulsions and reduced the frequency of convulsions though not statistically significant ($F_{3,16}$=0.95, P=0.4385 and $F_{3,16}$=2.39, P=0.1065, respectively fig. 3.2a).

Diazepam, an anticonvulsant, produced effects similar to that of the extract against PTZ-induced seizures and the effects were dose-dependent. The drug significantly delayed the onset of myoclonic jerks ($F_{3,16}$=18.18, P<0.0001, fig. 3.1) as well as the onset of tonic convulsions ($F_{3,16}$ =5.10, P=0.0115, fig. 3.2c). Also, diazepam caused significant reduction of the frequency ($F_{3,16}$ =6.03, P=0.006, fig. 3.2c) and duration of tonic convulsions ($F_{3,16}$ =21.34, P<0.0001, fig. 3.2d).

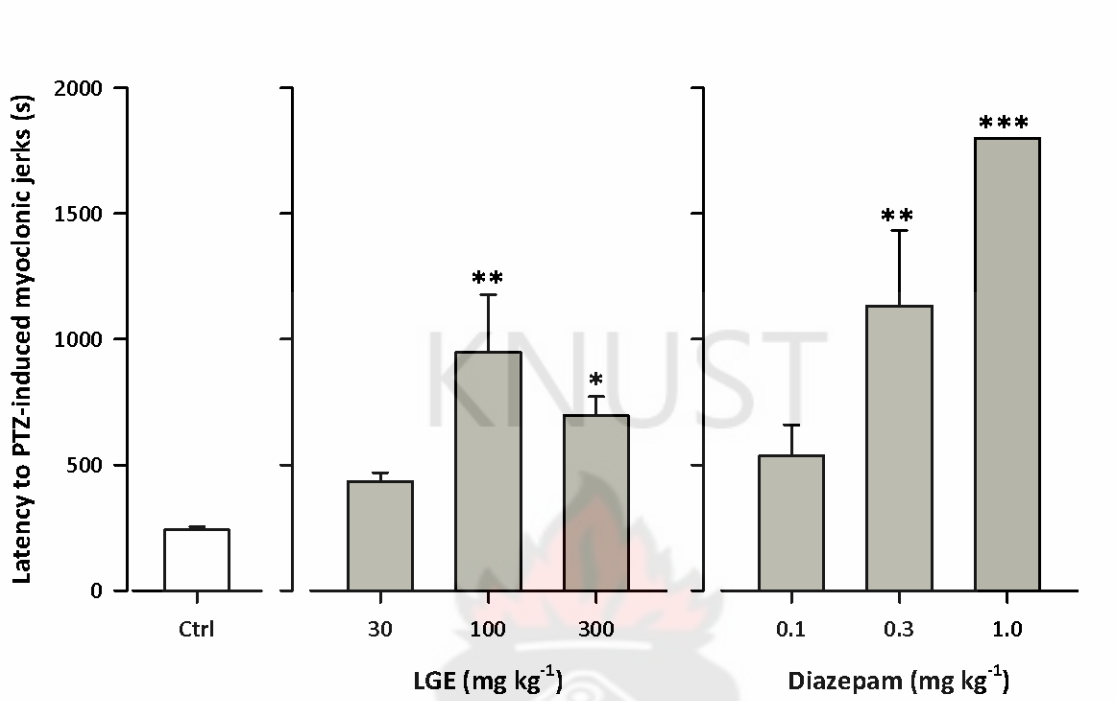

Fig. 3.1: Effect of LGE (30-300 mg/kg, *p.o.*) and diazepam (0.1-1.0mg/kg, *i.p.*) on the latency to PTZ-induced myoclonic jerks. Each column represents the mean ± S.E.M. n=5, ****P*<0.001; ***P*<0.01 and **P*<0.05, one-way ANOVA followed by Newman-Keuls test.

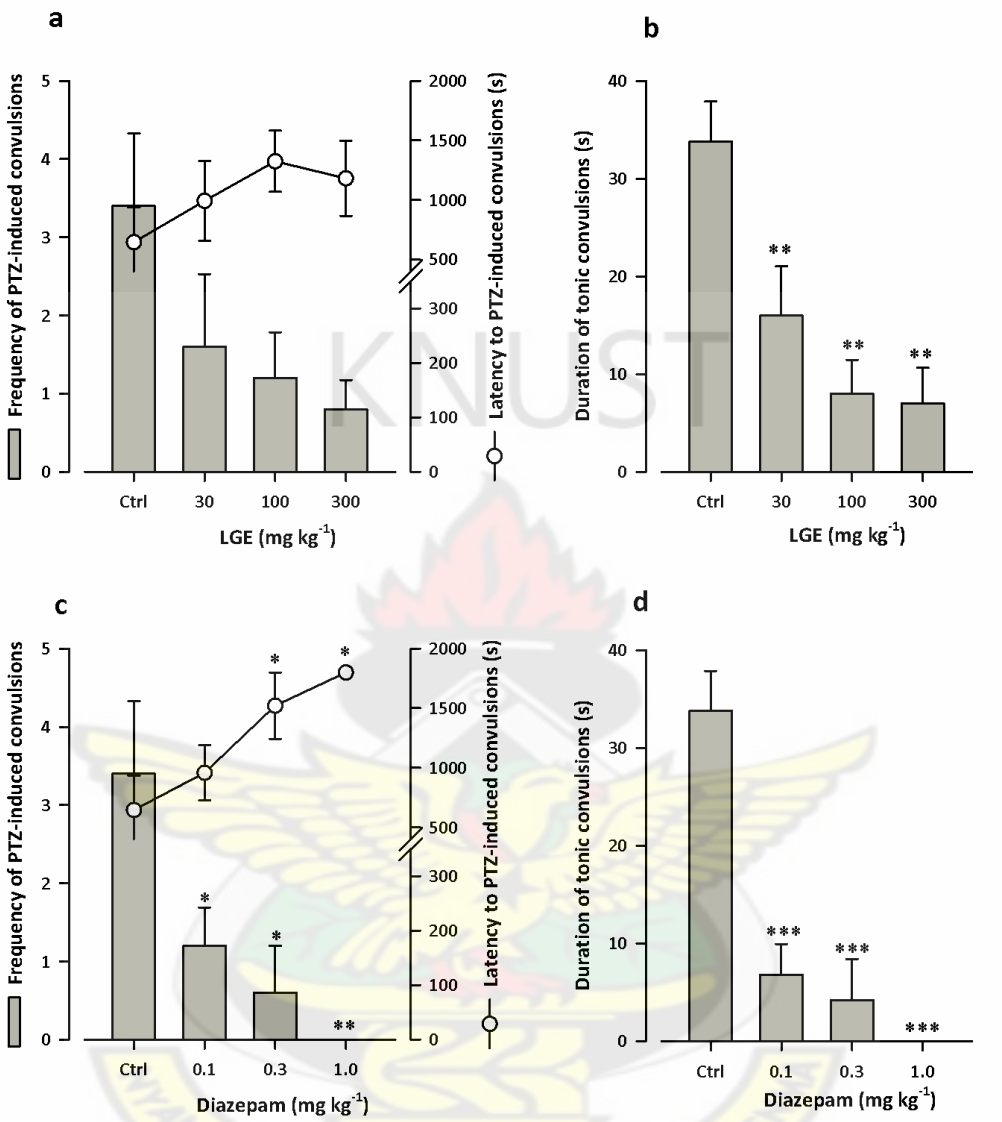

Fig. 3.2: Effect of LGE (30-300 mg/kg, *p.o.*) and diazepam (0.1-1.0 mg/kg, *i.p.*) on PTZ-induced seizures. (a) Effect of LGE on frequency and latency to tonic convulsions. (b) Effect of LGE on duration of tonic convulsions. (c) Effect of diazepam on the frequency and latency to tonic convulsions. (d) Effect of diazepam on duration of tonic convulsions. Each point and column represents the mean ± S.E.M. n=5, ***P<0.001; **P<0.01 and *P<0.05, one-way ANOVA followed by Newman-Keuls test.

3.2.2 Effect of extract on picrotoxin-induced seizures

The extract exhibited profound anticonvulsant effect on picrotoxin-induced seizures. It significantly decreased the frequency ($F_{3,16}$ =7.28, P=0.0027, fig. 3.4a) and duration of tonic convulsions ($F_{3,16}$=6.60, P=0.0041, fig. 3.4b). Also there was significant delay in the onset of tonic convulsions ($F_{3,16}$ =9.72, P=0.0007, fig. 3.4a). Though the extract produced a dose-dependent delay in the onset to myoclonic jerks, this effect was not statistically significant ($F_{3,16}$ =0.53, P=0.6682, fig. 3.3). The anticonvulsant effects of the extract on picrotoxin-induced seizures were decreasing with increasing dose. The frequency of convulsions decreased with increasing dose (p<0.01 at 30 $mgkg^{-1}$ and p<0.05 at 100-300 mg/kg, fig. 3.4a) as well as the duration of tonic convulsions (p<0.01 at 30 mg/kg and p<0.05 at 100-300 mg/kg, fig. 3.4b).

Diazepam produced effects analogous to the extract in the picrotoxin-induced seizure test and the effects increased with increasing dose. It significantly delayed the onset of myoclonic jerks ($F_{3,16}$ =13.45, P=0.0001, shown in fig. 3.4) and tonic convulsions ($F_{3,16}$ =25.02, P<0.0001, fig. 3.4c). The drug also significantly decreased the frequency ($F_{3,16}$ =12.81, P=0.001, fig. 3.4c) and duration of tonic convulsions ($F_{3,16}$ =15.96, P<0.0001, fig. 3.4d).

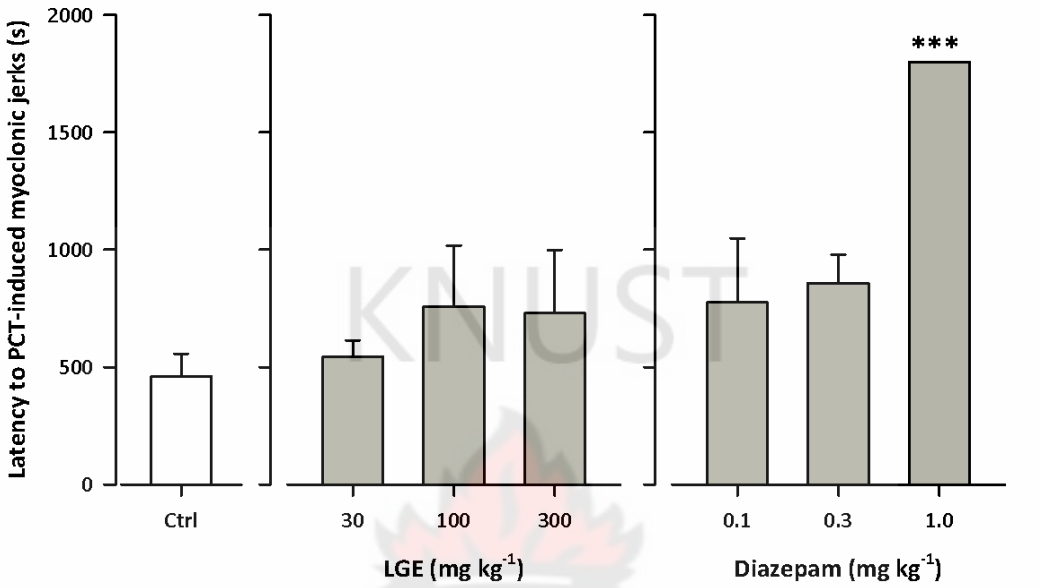

Fig. 3.3: Effect of LGE (30-300 mg/kg) and diazepam (0.1-1.0 mg/kg) on the latency to PTX-induced myoclonic jerks. Each column represents the mean ± S.E.M. n=5. ****P*<0.001; ***P*<0.01 and **P*<0.05, one-way ANOVA followed by Newman-Keuls test.

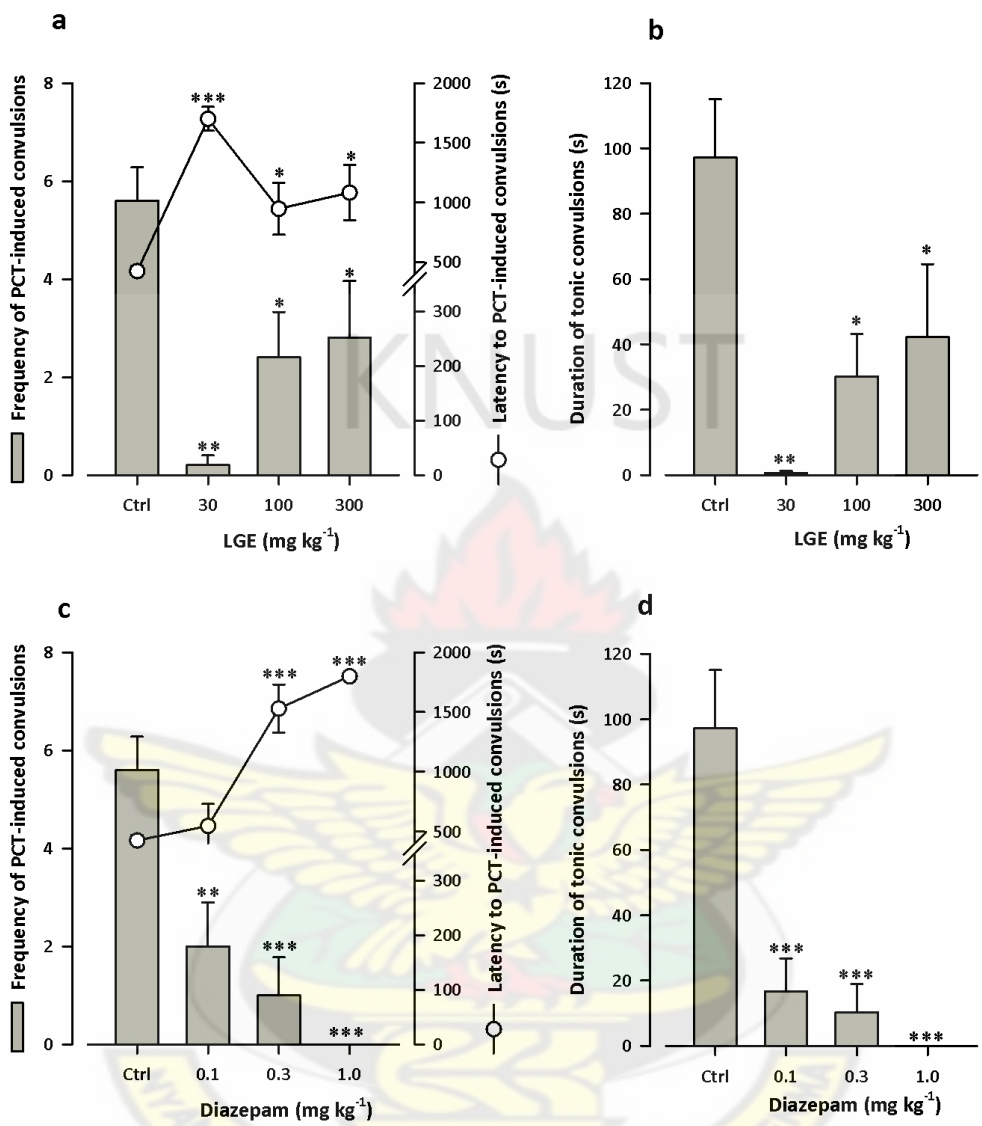

Fig. 3.4: Effect of LGE (30-300 mg/kg) and diazepam (0.1-1.0mg/kg) on PTZ-induced convulsions. (a) Effect of LGE on frequency and latency to tonic convulsion. (b) Effect of LGE on duration of tonic convulsions. (c) Effect of diazepam on the frequency and latency to tonic convulsions. (d) Effect of diazepam on duration of tonic convulsions. Each point and column represents the mean ± S.E.M. n=5. ***P<0.001; **P<0.01 and *P<0.05, one-way ANOVA followed by Newman-Keuls test.

3.2.3 Effect of extract in maximal electroshock seizures

The extract caused significant decrease in the duration of tonic hind limb extension (THLE) induced by maximal electroshock ($F_{3,35}$ =5.08, P=0.0050, fig. 3.5) but was unable to completely prevent its occurrence. LGE at 100-300 mg/kg produced significant reduction of the duration of THLE ($p<0.05$) however, 30 mg/kg did not cause significant effect ($p>0.05$). Carbamazepine (3-30 mg/kg *p.o.*) significantly reduced the duration of MES-induced THLE ($F_{3,36}$=7.35, P=0.0006, fig. 3.5) and completely prevented the occurrence of this behaviour at 30 mg /kg. CBZ at 3 mg/kg did not produce significant effect compared to the control group ($p>0.05$).

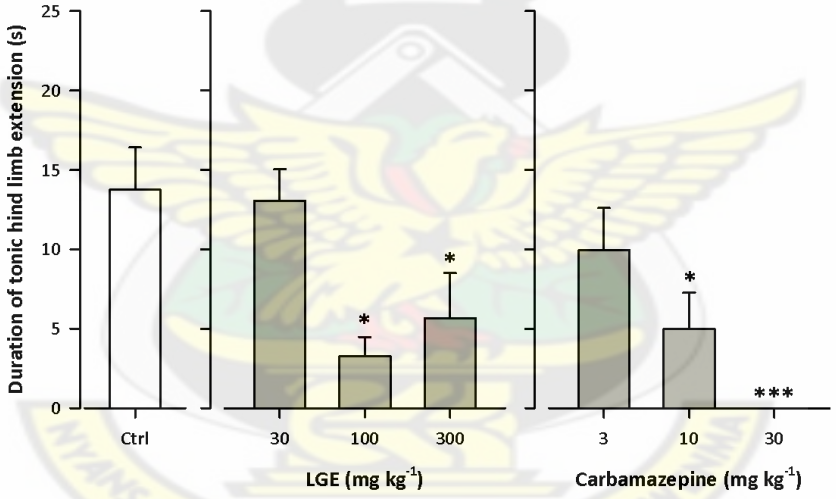

Fig. 3.5: Effect of LGE (30-300 mg/kg) and carbamazepine (3-30 mg/kg) on the duration of MES-induced tonic hind limb extension. Each column represents Mean ± S.E.M. n=10 ****P*<0.05; ***P*<0.01 and **P*<0.001 ANOVA followed by Newman-Keuls test.

3.3 ANXIOLYTIC EFFECTS

3.3.1 *The elevated plus-maze test*

Administration of LGE significantly decreased anxiety in mice. A one-way ANOVA showed that LGE caused significant increase in the number of open arm entries and the amount of time spent in the open arms in mice compared to vehicle-treated mice as shown in fig. 3.6 and 3.7 ($F_{3,16}$=5.21, P=0.0106 and $F_{3,16}$=4.18, P=0.0230, respectively). The increase was dose-dependent and was statistically significant (p<0.05) at doses 100 and 300 mg/kg for the number of open arm entries and at only 300 mg/kg for the open arm time. When entries into the arms are expressed as percentage open arm entries, and the time in the arms expressed as percentage open arm time, the extract caused a significant increase in these parameters ($F_{3,16}$=7.65, P=0.0022 and $F_{3,16}$=4.18, P=0.0230, respectively, fig. 3.6d and 3.7f). The increase is dose-dependent and significant at doses of 100 and 300 mg/kg (p<0.01) for the percentage open arm entries and only at 300 mg/kg (p<0.05, 3.7d) for the percentage open arm time. Two-way ANOVA (treatment group X arm type, i.e. open or closed) revealed a significant arm type effect where the number of open arm entries and open arm time are increased ($F_{1,24}$=6.09, P=0.0388 and $F_{3,16}$=7.18, P=0.0280; fig. 3.6a and 3.7d, respectively). However, *post hoc* analysis (Bonferroni's test) revealed that all treatment groups did not show significant more open arm entries and open arm time compared with the closed arm entries and time. In comparison, treatment of mice with the anxiolytic drug, diazepam produced effects that were similar to those produced by LGE both in the spatiotemporal and ethological measures of anxiety. Administration of diazepam caused significant and dose-dependent increase in the amount of time spent in the open arms ($F_{3,16}$=5.13, P=0.0113, fig 3.7b) and the percentage of open arm time

($F_{3,16}$=5.13, P=0.0113, fig 3.7d). Diazepam also increased the number of open arm entries and the percentage of arm entries, but these were not statistically significant as revealed by one-way ANOVA ($F_{3,16}$=2.19, P=0.1296, fig 3.6b and $F_{3,16}$=3.21, P=0.0511, fig 3.6b). However, two-way ANOVA (treatment group X arm type, i.e. open or closed) revealed a significant arm type effect ($F_{1,24}$=51.37, P<0.0001, fig 3.6b). In contrast to LGE and diazepam, pentylenetetrazole increased anxiety in mice. Pentylenetetrazole caused significant decrease in the percentage of open arm entries ($F_{3,16}$ =3.60, P=0.0367, fig 3.6f) and the percentage of time in the open arms ($F_{3,16}$ =5.34, P=0.0097, fig 3.7f). Only the highest dose of the drug caused significant decrease in the percentage of open arm entries and the percentage open arm time (p<0.05 and 0.01at 30 mg/kg for % open arm entries and % open arm time, respectively). At the lower doses, pentylenetetrazole did not cause significant decrease in these parameters compared to the control group (p>0.05 at 3-10 mg/kg). Also the drug decreased the number of open arm entries as well as the time spent in the open arms but not statistically significant (p>0.05 at 3-30 mg/kg). However, upon two-way ANOVA, treatment group X arm type (open or closed) showed significant arm type effect where the number of open arm entries ($F_{1,24}$=960.04, P<0.0001, fig 3.6c) and the open arm time ($F_{1,24}$=255.61, P<0.0001, fig 3.7c) increased significantly compared to the closed arm entries and closed arm time.

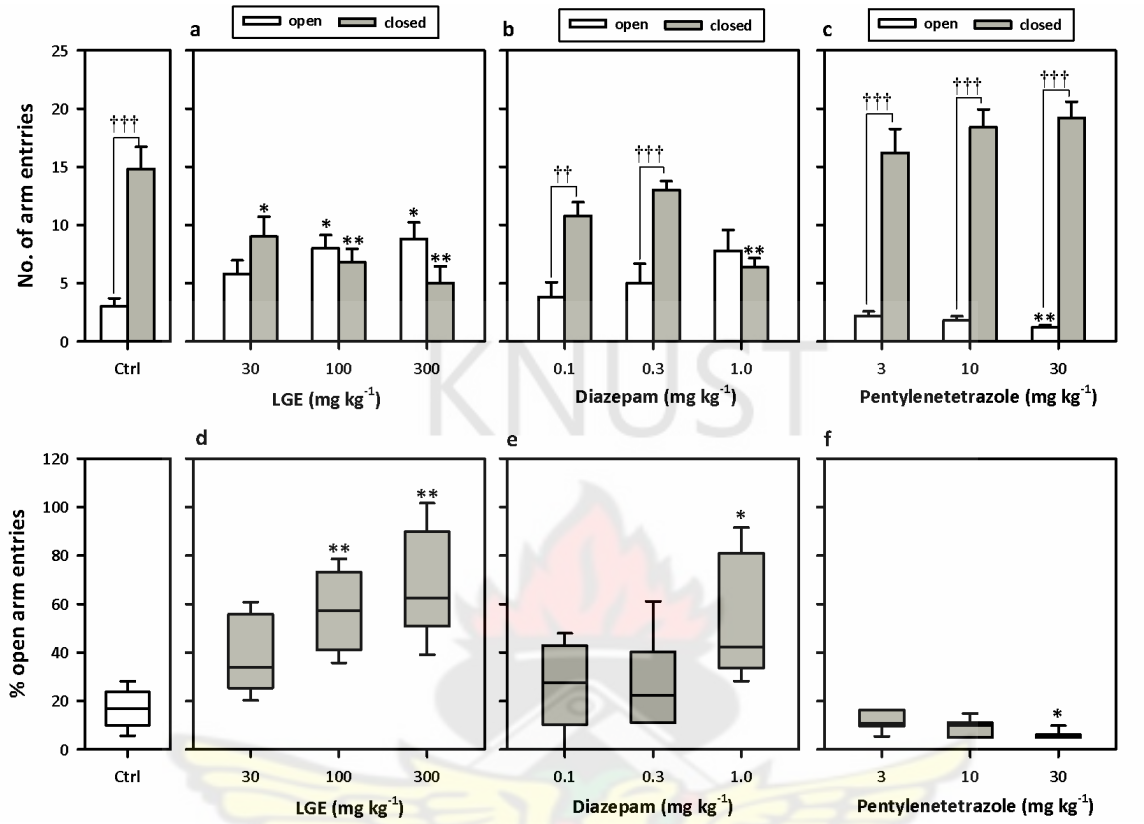

Fig. 3.6: Effects of LGE (30-300 mg/kg), diazepam (0.1-1.0 mg/kg) and pentylenetetrazole (3-30 mg/kg) on mice behaviour on the EPM. Data are presented as group mean ± SEM, n=5. The lower and upper margins of the boxes (d, e and f) represent the 25th and 75th percentiles, with the extended arms representing the 10th and 90th percentiles, respectively. The median is shown as the horizontal line within the box. Significantly different from control: **P*<0.05, ***P*<0.01, ***P<0.001(one-way ANOVA followed by Newman-Keuls test) and significant difference when open arm and closed arm were compared: †*P*<0.05, ††*P*<0.01, †††*P*<0.001 (two-way repeated measures ANOVA followed by Bonferroni's *post hoc*).

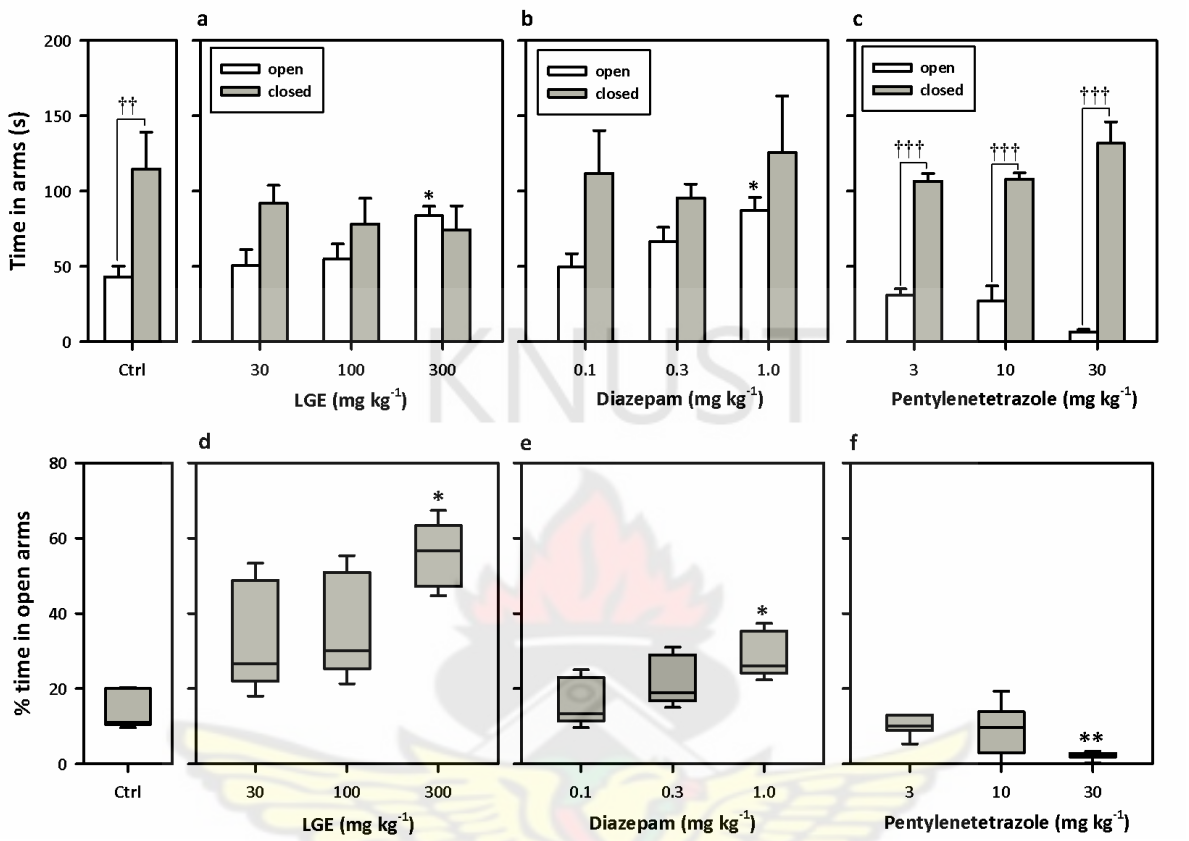

Fig. 3.7: Effects of LGE (30-300 mg/kg), diazepam, (0.1-1.0 mg/kg) and pentylenetetrazole, (3-30 mg/kg) on mice behaviour on the elevated plus-maze. Data are expressed as group mean ± SEM n=5. The lower and upper margins of the boxes (d, e and f) represent the 25th and 75th percentiles, with the extended arms representing the 10th and 90th percentiles, respectively. The median is shown as the horizontal line within the box. Significant difference from control: **P*<0.05, ***P*<0.01, ****P*<0.001 (one-way ANOVA followed by Newman-Keuls test) and significant difference: $^{\dagger}P$<0.05, $^{\dagger\dagger}P$<0.01, $^{\dagger\dagger\dagger}P$<0.001(two-way ANOVA followed by Bonferroni's *post hoc* test) when closed arms and open arms are compared.

For ethological measures, LGE caused significant increase in frequency and duration of unprotected stretch-attend postures (USAPs) and total head dips (HDs). The extract also increased grooming and rearing behaviours of mice. One-way ANOVA showed that pretreatment with LGE caused significant increase in frequency and duration of USAPs ($F_{3,16}$=5.39, P=0.0093 in fig. 3.8a and $F_{3,16}$=3.47, P=0.0411, table 3.2, respectively). The effect was dose-dependent and statistically significant at all the doses used (p<0.05 at 30-300 mg/kg, fig. 3.8). When the USAPs are expressed as percentage of stretch-attend postures (SAPs), a significant increase is achieved only at 300 mg/kg, (p<0.05, fig. 3.8d). Also, two-way ANOVA, treatment group X SAP type (protected or unprotected SAP) showed significant SAP type effect whereas the USAPs by mice increased significantly compared to the protected stretch-attend postures (PSAPs) ($F_{1,24}$=15.06, P=0.0047; fig. 3.8a). *Post hoc* analysis (Bonferroni's test) revealed that all treatment groups except 30 mg/kg, made significant increase in USAPs when compared with PSAPs (p<0.05 at 100 mg/kg and p<0.001 at 300 mg/kg, fig 3.8a). Again, one-way ANOVA showed that LGE caused profound increase in the frequency and duration of total HDs ($F_{3,16}$=5.50, P=0.0087 and $F_{3,16}$=6.60, P=0.0041, shown in table 3.2). LGE significantly increased the frequency and duration of unprotected head dips (UHDs) ($F_{3,16}$=3.95, P=0.0277 and $F_{3,16}$=5.15, P=0.0111, respectively; table 3.2) and decreased the frequency and duration of protected head dips (PHDs) ($F_{3,16}$=4.28, P=0.0214 and $F_{3,16}$=4.51, P=0.0179 respectively; table 3.2). The extract did not have profound effects on grooming and rearing behaviours as shown in table 3.2. Ethological measures of anxiety and risk assessment (RA) behaviours were also increased by diazepam. It caused an increased frequency ($F_{3,16}$ =3.20, P=0.0516, fig 3.8b) and duration ($F_{3,16}$ =4.08, P=0.0250, table 3.3) of USAPs. The increase in the frequency of USAPs was only significant at 1.0 mg/kg (p<0.05) whereas the increase caused by 0.1-0.3 mg/kg

were not significant compared to the control. Diazepam dose-dependently and significantly decreased the frequency ($F_{3,16}$ =7.45, P=0.0024, fig 3.8b) and duration ($F_{3,16}$ =11.67, P=0.0003, table 3.3) of PSAPs. The percentage of PSAPs was also significantly decreased by diazepam ($F_{3,16}$ =10.63, P=0.0004, table 3.3). Diazepam caused a profound increase in the frequency ($F_{3,16}$ =3.89, P=0.02291, table 3.3) and duration ($F_{3,16}$ =6.05, P=0.0059, table 3.3) of UHDs. Diazepam did not have any effect on the frequency and duration of both grooming and rearing as shown in table 3.3. The effect of pentylenetetrazole on ethological measures was contrary to that of LGE and diazepam. A two-way ANOVA showed significant SAP type effect where performance of USAPs significantly decreased ($F_{1,24}$=67.25, P<0.0001, fig 3.8c) compared to the performance of PSAPs. One-way ANOVA revealed that pentylenetetrazole decreased frequency and duration of total HDs, SAPs, grooming and rearing, but these were not statistically significant compared to the vehicle-treated group as shown in table 3.4.

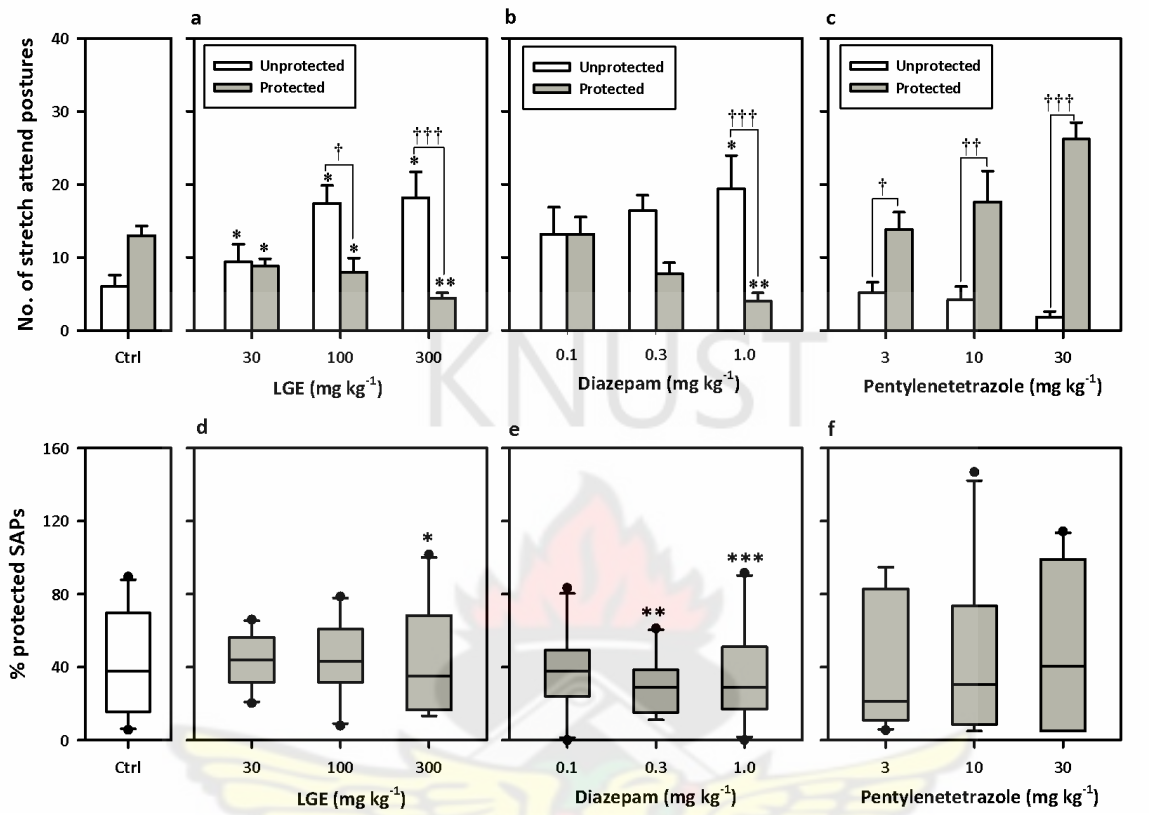

Fig. 3.8: Effects of LGE (30-300 mg/kg,), diazepam (0.1-1.0 mg/kg) and pentylenetetrazole (3-30 mg/kg) on risk assessment behaviours (protected and unprotected stretch-attend postures) in mice on the EPM. Data are expressed as group mean ± SEM, n=5. The lower and upper margins of the boxes (d, e and f) represent the 25th and 75th percentiles, with the extended arms representing the 10th and 90th percentiles, respectively. The median is shown as the horizontal line within the box. Outlying points are shown individually. Significant difference: **P*<0.05, ***P*<0.01, ****P*<0.001 compared to control group (one-way ANOVA followed by Newman-Keuls test) and †*P*<0.05, ††*P*<0.01, †††*P*<0.001 when protected and unprotected stretch-attend postures are compared (two-way ANOVA followed by Bonferroni's *post hoc* test).

Table 3.2 Effect of LGE (30-300 mg/kg) on mice behaviour in the elevated plus-maze.

		LEEA GUINEENSIS (mg/kg, *p.o*)			
PARAMETERS	**CONTROL**	**30**	**100**	**300**	**P VALUES**
Freq. of PSAPs	13.00±1.30	8.80±1.30*	8.00±1.92*	4.40±0.75**	F=7.12, P=0.0030
PSAPs time	11.00±1.70	5.20±0.80**	3.20±0.80***	1.60±0.40***	F=15.54,P<0.0001
Freq. of USAPs	6.00±1.58	9.40±2.40	17.40±2.46*	18.20±3.53*	F=5.39, P=0.0093
USAPs time	2.60±0.93	6.40±1.94	10.60±2.62*	8.60±1.44	F=3.47, P=0.0411
Freq. of PHDs	1.60±0.51	3.20±0.58	1.00±0.45	0.60±0.60	F=4.28, P=0.0214
PHDs time	3.80±0.97	5.20±0.73	1.8±0.58	2.00±0.77	F=4.51, P=0.0179
Freq. of UHDs	3.20±1.32	15.80±2.22*	11.60±2.23	9.60±1.69	F=3.95, P=0.0214
UHDs time	1.40±0.75	15.00±4.16**	10.40±2.50	7.2±1.11	F=5.15, P=0.0111
Total HDs	7.00±1.48	21.00±3.54**	13.40±2.62	11.60±1.78	F=5.50, P=0.0087
Freq. of rearing	6.60±1.75	9.80±2.13	6.00±1.60	3.60±1.40	F=2.15, P=0.1346
Rearing time	3.40±1.03	7.20±1.59	3.00±1.23	1.40±0.68	F=4.34, P=0.0203
Freq. of grooming	0.20±0.20	1.00±0.45	1.60±0.40	1.60±0.40	F=3.14, P=0.0543
Grooming time	0.00±0.00	0.40±0.25	1.20±0.49	1.40±0.75	F=2.03, P=0.1501

Key: Freq. = Frequency

Table 3.3 Effect of DZP (0.1-1.0 mg/kg, *i.p.*) on mice behaviour in the EPM

PARAMETERS	CONTROL	DIAZEPAM (mg/kg, *i.p.*)			P VALUE
		0.1	0.3	1	
Freq. of PSAPs	13.00±1.30	13.20±2.33	7.80±1.46	4.00±1.14**	F=7.45,P=0.0024
PSAPs time	11.00±1.70	5.40±1.12**	3.00±0.84***	2.00±0.84***	F=11.67,P=0.0003
Freq. of USAPs	6.00±1.58	13.20±3.68	6.40±2.11	19.40±4.57*	F=3.20, P=0.0516
USAPs time	2.60±0.93	8.60±2.58	7.40±0.93	13.20±3.20*	F=4.08, P=0.0250
Freq. of PHDs	3.80±0.97	2.60±0.93	3.40±1.03	2.40±0.75	F=0.51, P=0.6806
PHDs time	1.60±0.51	1.00±0.55	1.40±0.51	1.20±0.58	F=0.23, P=0.8742
Freq. of UHDs	3.20±1.32	7.60±2.60	8.60±1.81*	13.40±2.50	F=3.89, P=0.0241
UHDs time	1.40±0.75	4.60±1.60	5.80±0.97**	10.60±2.36	F=6.05, P=0.0059
Total HDs	7.00±1.48	10.20±2.56	12.00±2.76	15.00±2.40**	F=5.45, P=0.0090
Freq. of rearing	6.60±1.75	4.80±2.04	4.80±1.16	3.40±0.81	F=0.75, P=0.5393
Rearing time	3.40±1.03	2.60±1.12	2.40±0.75	1.20±0.37	F=1.10, P=0.3799
Freq. of grooming	0.20±0.20	0.80±0.37	1.20±0.37	0.20±0.20	F=2.67, P=0.0829
Grooming time	0.00±0.00	0.80±0.58	0.80±0.37	0.20±0.20	F=1.31, P=0.3063

Key: Freq. = Frequency

Table 3.4 Effect of PTZ (30-300 mg/kg) on behaviour of mice in the EPM.

PARAMETERS	CONTROL	PENTYLENETETRAZOLE (mg/kg, *i.p.*)			P VALUES
		3	10	30	
Freq. of PSAPs	13.00±1.30	13.80±2.40	17.60±4.21	26.20±2.29*	F=4.80,P=0.0143
PSAPs time	11.00±1.70	8.80±4.13	7.80±1.66	13.60±1.69	F=1.04,P=0.4025
Freq. of USAPs	6.00±1.58	5.20±1.39	4.20±1.80	1.80±0.80	F=1.60,P=0.2295
USAPs time	2.60±0.93	3.60±0.81	1.40±0.60	0.80±0.49	F=2.94,P=0.0647
Freq. of PHDs	3.80±0.97	8.00±2.30	5.40±1.29	7.40±1.91	F=1.28,P=0.3160
PHDs time	1.60±0.51	4.80±1.77	2.80±0.86	4.20±1.43	F=1.34,P=0.2978
Freq. of UHDs	3.20±1.32	4.60±1.78	2.00±1.14	1.00±0.63	F=1.46,P=0.2622
UHDs time	1.40±0.74	2.40±1.17	0.80±0.58	0.60±0.40	F=1.08,P=0.3857
Total HDs	7.00±1.48	12.60±3.67	7.40±2.27	8.40±2.38	F=1.00,P=0.4197
Freq. of rearing	6.60±1.75	6.00±1.14	5.40±1.91	2.80±0.73	F=1.31,P=0.3061
Rearing time	3.40±1.03	4.00±1.14	3.60±1.60	1.80±0.37	F=0.73,P=0.5447
Freq. of grooming	0.20±0.20	1.20±0.58	0.80±0.37	0.00±0.00	F=2.33,P=0.5847
Grooming time	0.00±0.00	0.20±0.20	0.00±0.00	0.00±0.00	F=0.67,P=0.5847

With regard to horizontal exploration, LGE and diazepam caused significant increases in locomotor activities in a dose-dependent manner, as reflected in the total distance travelled on the maze. The increase in total distance travelled by LGE was significant at higher doses (p<0.01 at 100-300 mg/kg, and p>0.05 at 30 mg/kg, fig 3.9a). Diazepam caused significant increase in total distance travelled by mice at all the doses used (p<0.05 at 0.1-1.0 mg/kg, fig 3.9b). Inversely, pentylenetetrazole dose-dependently and significantly caused decreased total distance travelled on the maze at 10-30 mg/kg (p<0.001, fig 3.9c). The reduction in total distance travelled was not significant at 3mg/kg.

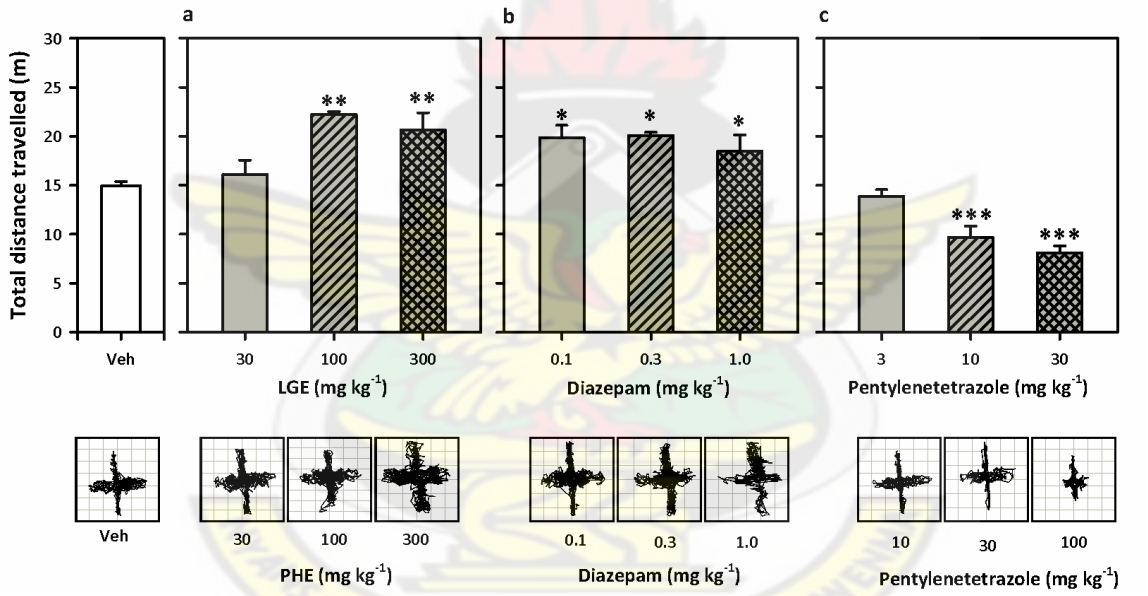

Figure 3.9: Effects of LGE (30-300 mg/kg), diazepam (0.1-1.0 mg/kg) and pentylenetetrazole (3-30 mg/kg) on total distance travelled on the EPM. Data are presented as group Mean ± SEM, n=5. *P < 0.05, **P < 0.01, ***P < 0.001 compared to control group (one-way ANOVA followed by Newman-Keuls test). Line plots (lower panels) 3D plots were generated from the time and XY data obtained using SigmaPlot Version (Systat Software Inc., Point Richmond, CA, USA).

3.3.2 The light/dark box test

In the light/dark box test, the emergence latency of mice from the dark box into the light box, the amount of time spent in each compartment and the frequency of transitions between the compartments were used as indices of anxiety.

The extract dose-dependently and significantly decreased the latency to emerge from the dark box into the light box ($F_{3,16}$ =7.50, P=0.0024, fig. 3.10a). Newman-Keuls *post hoc* test showed that only 100-300 mg/kg produced a profound decrease in the emergence latency (p<0.01). Diazepam, an anxiolytic significantly decreased the emergence latency ($F_{3,16}$ =7.95, P=0.0018, fig. 3.10c). All the doses of diazepam used produced significant decrease in the latency to emerge from the dark box into the lit box. However, Newman-Keuls *post hoc* test revealed that 0.1 and 1.0 mg/kg produced very significant decreases in the emergence latency than 0.3 mg/kg (thus p<0.01 at 0.1 and 1.0 mg/kg and p<0.05 at 0.3 mg/kg as shown in fig. 3.10c). In contrast to LGE and diazepam, pentylenetetrazole, although not statistically significant, caused dose-dependent increase in the emergence latency into the lit box ($F_{3,16}$ =1.03, P=0.4059, fig. 3.10e).

The extract caused significant increase in the amount of time mice spent in the lit box ($F_{3,16}$ =4.99, P=0.0124, fig. 3.10b) while significantly decreased the amount of time spent in the dark box ($F_{3,16}$ =3.49, P=0.0403, fig 3.10b). The extract did not produce significant increase in the time spent in the lit box at the 30-100 mg/kg but did only at 300 mg/kg (p>0.05 at 30-100 mg/kg and p<0.01 at 300 mg/kg, Newman-Keuls test). Furthermore, two-way ANOVA (treatment X box type, i.e. lit or dark) revealed significant box type effect ($F_{1,24}$=16.70, P=0.0035, fig. 3.10b) where mice spent more time in the lit box compared to the dark box. *Post hoc* analysis indicate that only 300 mg/kg treated group spent significantly more time in the lit box

(p<0.001, Bonferroni's test, fig. 3.10b) compared to the time spent in the dark box. Diazepam's effect on the proportion of time spent in either of the boxes was not statistically significant at the doses used (p>0.05 at 0.1-1.0 mg/kg, fig. 3.10d) however, there was a steady increase as the doses increased. On the other hand, pentylenetetrazole caused significant decrease in the amount of time spent in the lit box ($F_{3,16}$ =4.12, P=0.0241, fig. 3.10f) while it significantly increased the amount of time spent in the dark box ($F_{3,16}$ =8.38, P=0.0014, fig. 3.10f). In addition, two-way analysis of the effects of PTZ revealed significant box type effect ($F_{1,24}$=48.99, P=0.0001, fig. 3.10f) with the *post hoc* analysis (Bonferroni's test) revealing that all treatment groups spent significantly less time in the lit box compared to the time spent in the dark box.

LGE did not have significant changes in the total number of transitions between compartments (lit or dark box) ($F_{3,16}$=0.48, P=0.7028, fig. 3.10a). Diazepam however, significantly decreased the total transitions ($F_{3,16}$=5.15, P=0.0111, fig. 3.10c) between the lit box and box. Newman-Keuls *post hoc* test revealed that only 1.0 mg/kg diazepam produced significant decrease in the total transitions between the compartments. Likewise DZP, PTZ caused significant decrease in the total transitions between the compartments ($F_{3,16}$=5.49, P=0.0087, fig. 3.10e).

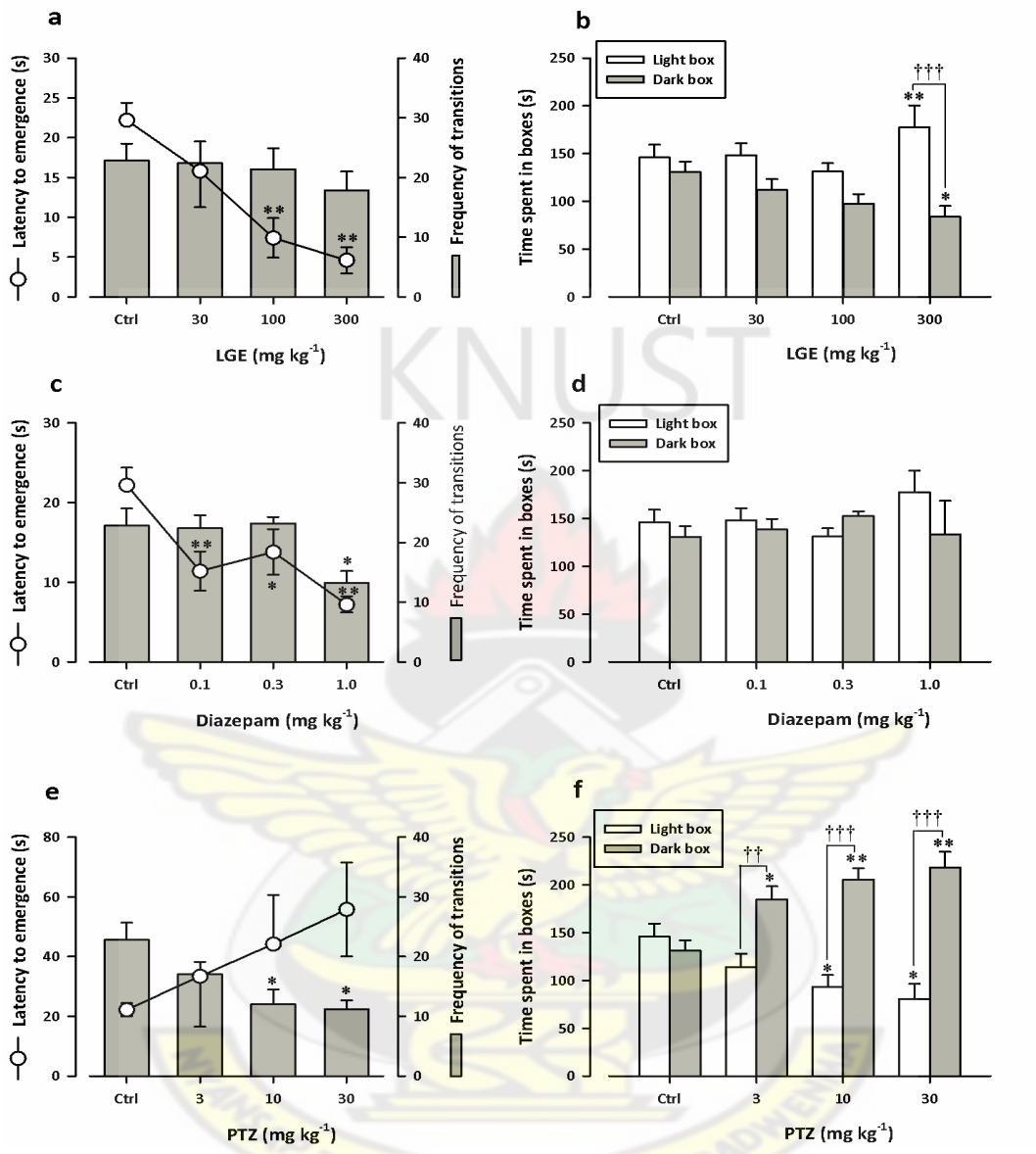

Figure 3.10 Effects of LGE, diazepam and pentylenetetrazole on mice behaviour in the light/dark box. Data are expressed as group Mean ± SEM, n=5. **P*<0.05, ***P*<0.01, ****P*<0.001 compared to control group (one-way ANOVA followed by Newman-Keuls test). †*P*<0.05, ††*P*<0.01, †††*P*<0.001 when light compartment was compared with dark compartment (two-way ANOVA followed by Bonferroni's *post hoc* test).

3.4 THE FORMALIN TEST

The extract caused significant antinociceptive effect in both the first phase ($F_{3,16}$ =17.18, P<0.0001, fig 3.11) and second phase ($F_{3,16}$ =40.17, P<0.0001, fig. 3.11) of formalin-induced pain. It was approximately equally potent in both the first phase ($ED_{50;}$ 16.37±4.57) and the second phase (ED_{50}; 14.06±4.39). Morphine also significantly reduced nociceptive scores in both the first phase ($F_{3,16}$=33.24, P<0.0001, fig. 3.11) and the second phase ($F_{3,\ 16}$=40.42, P<0.0001, fig. 3.11) of formalin-induced pain. Also, it was approximately equipotent in both the first phase (ED_{50}; 0.09±0.03, table 3.2) and the second phase (ED_{50}; 0.11±0.04, table 3.2). LGE was approximately 181.88 less potent than morphine in the first phase and about 127.82 less potent than morphine in the second phase.

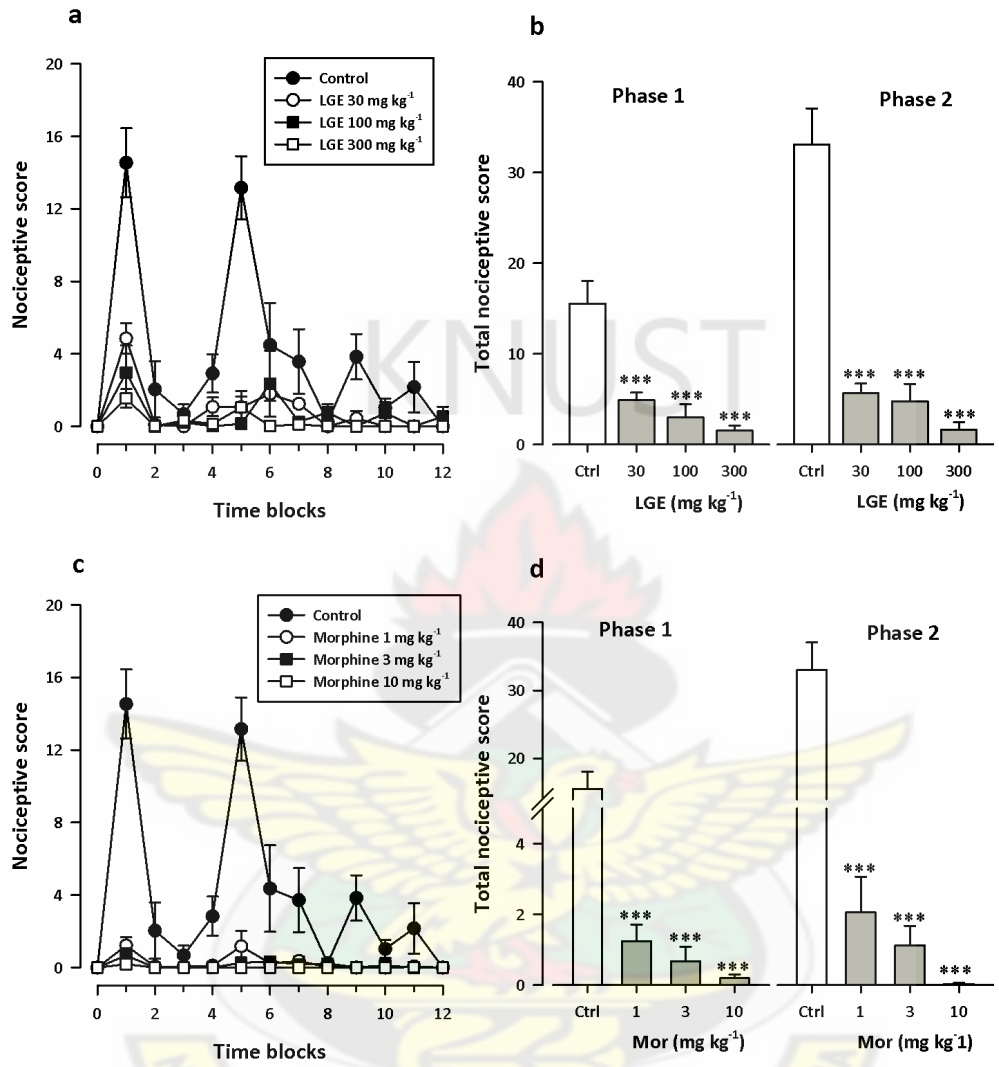

Figure 3.11 Effect of LGE and morphine on phase 1 and 2 of formalin induced pain. (a)Time course effects of LGE (30-300 mg/kg) and its total nociceptive score in (b) phase 1 and (c) phase 2. (c) Time course effects of morphine (1-10 mg/kg) and its total nociceptive score in (d) phase 1 and (e) phase 2. Nociceptive scores are shown in 5 min time blocks up to 60 min for the time course curves. Data are presented as mean ± SEM (n=5).). ***P<0.001, ** P < 0.01; *P < 0.05 compared to vehicle-treated group (one-way ANOVA followed by Newman-Keuls' *post hoc* test).

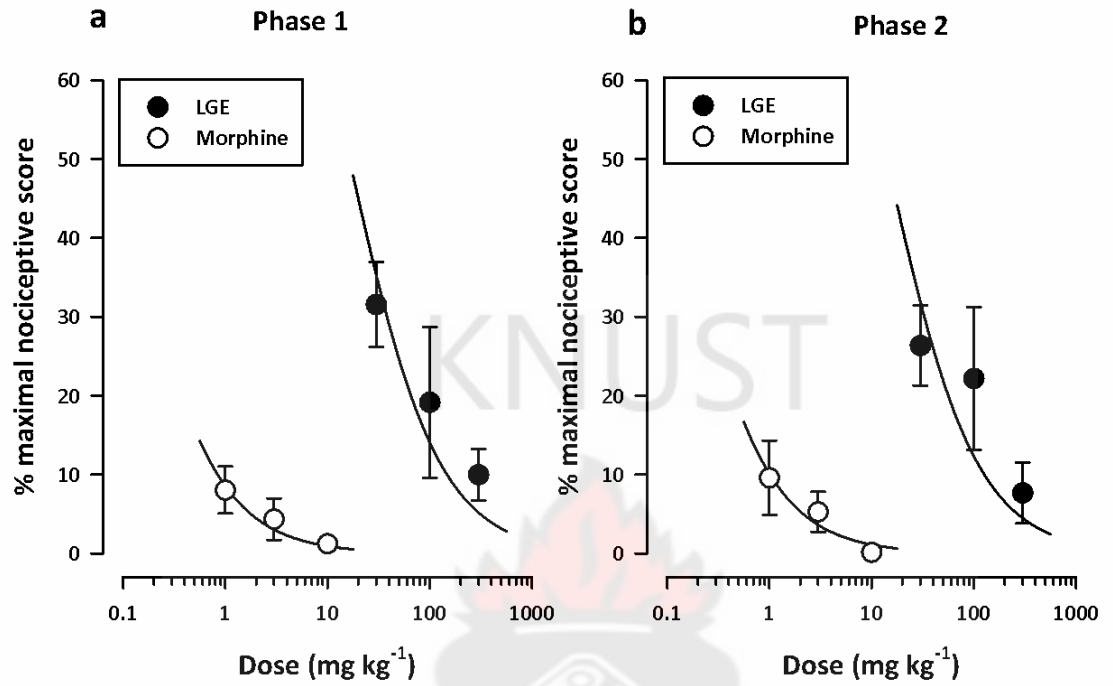

Figure 3.12 Dose response curves of LGE (30-300 mg/kg, *p.o.*) and morphine effects on the total nociceptive score for the first phase (a) and second phase (b) of the formalin-induced pain in mice. Each point represents mean ± S.E.M (n=5).

Table 3.5 ED_{50} values of LGE and morphine in the formalin test

Drug	**ED_{50} (mg kg^{-1})**	
	Phase 1	***Phase 2***
LGE	16.37±4.57	14.06±4.39
Morphine	0.09±0.03	0.11±0.04

Both naloxone and theophylline caused significant blockade of the antinociceptive activity of LGE in phase 1 of the formalin-induced pain. The nociceptive scores for 100 mg/kg LGE + 2 mg/kg NLX and 100 mg/kg LGE +10 mg/kg TH were significantly reduced ($p<0.05$, fig. 3.13b) in the first phase compared to the nociceptive score for only 100 mg/kg LGE in the first phase. However, NLX and TH were not able to antagonize the antinociceptive activity of LGE in the second phase and hence the antinociceptive scores for 100 mg/kg LGE + 2 mg/kg NLX and 100 mg/kg LGE +10 mg/kg TH were not affected significantly in the second phase compared to that for only 100 mg/kg LGE in the second phase ($p>0.05$, fig. 3.13b). The antinociceptive effect of morphine, a nonselective opioid agonist, was significantly blocked by NLX both in the first phase and the second phase whereas TH did not cause significant attenuation of morphine antinociceptive activity in both phases (fig. 3.13 d).

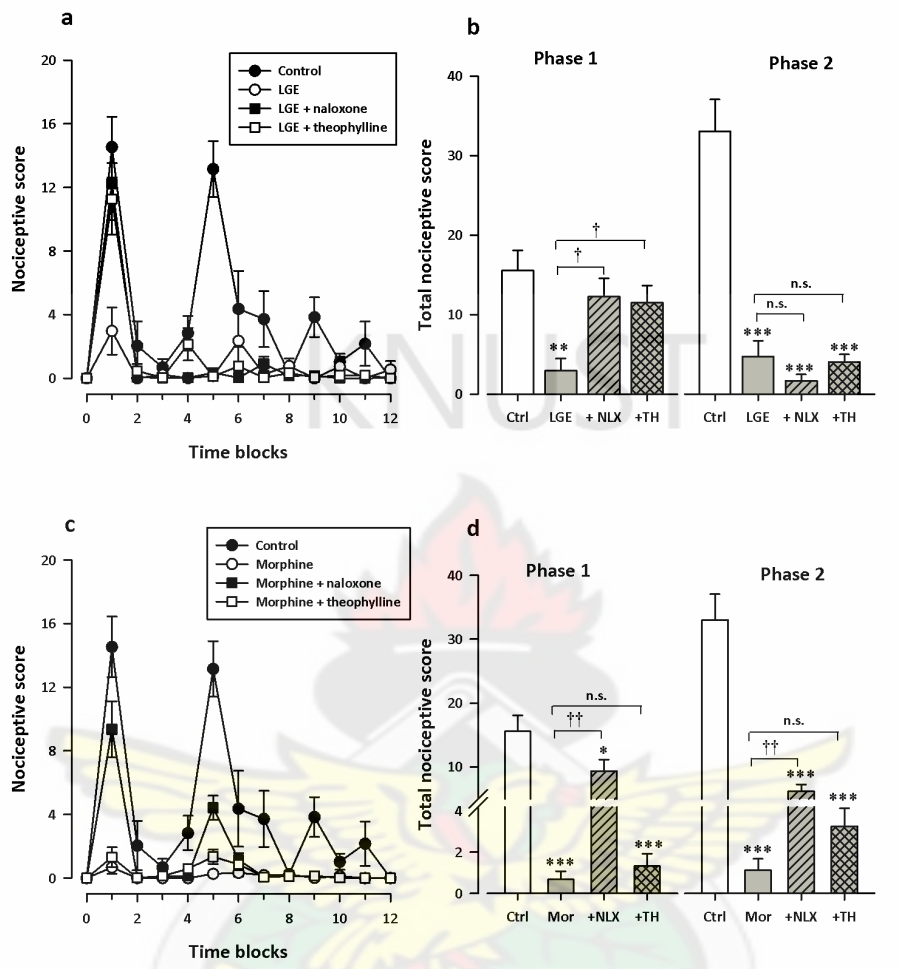

Figure 3.13 Effect of naloxone and theophylline on antinociceptive effects of LGE and morphine. Nociceptive scores are shown in 5 min time blocks up to 60 min for time course curves. Data are presented as mean ±S .E.M (n=5). ***P<0.001, **P<0.01, *P<0.05 compared to vehicle-treated group (one-way ANOVA followed by Newman-Keul's post hoc). $^{\dagger\dagger\dagger}P$<0.001, $^{\dagger\dagger}P$<0.01, $^{\dagger}P$<0.05 (two-way ANOVA followed by Bonferroni's *post hoc* test)

3.5 EFFECT ON MOTOR FUNCTION NEUROMUSCULAR EFFECT *IN VITRO*

3.5.1 The beam traversal test

LGE (30-300 mg/kg) did not alter the time taken by mice to traverse the beam in any significant way compared to the control group ($p>0.05$ at 30-300 mg/kg, fig. 3.14). No stepping errors were observed in groups treated with the extract and there was no effect on the total steps ($p>0.05$). Diazepam did not also have significant effect on the time taken to cross the beam at 0.1-0.3 mg/kg ($p>0.05$) except 1.0 mg/kg which caused significant delay in the time to traverse the beam compared to vehicle-treated group ($p<0.05$). Diazepam did not also affect significantly the number of steps to cross the beam at 0.1-0.3 mg/kg ($p>0.05$) except 1.0 mg/kg which caused significant increase in the number of footsteps ($p<0.05$). No stepping errors were observed in the diazepam treated groups.

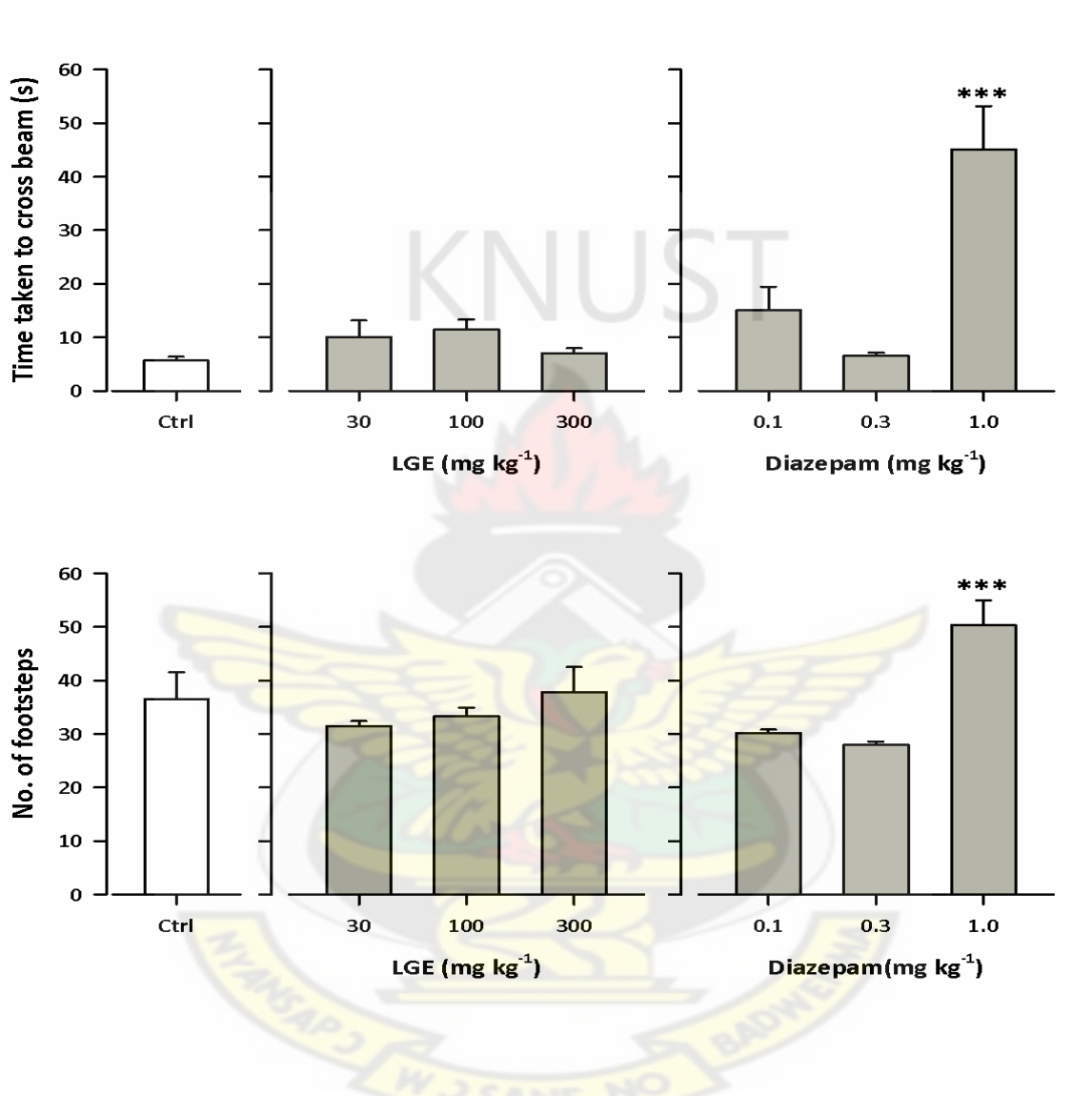

Figure 3.14 Effect of LGE and diazepam on the latency and number of footsteps to traverse beam in the beam traversal task. Each column represents the mean ± S.E.M. **P*<0.05, ***P*<0.01, *P*<0.001. n=5. (One-way ANOVA followed by Newman-Keuls test).

3.5.2 The rotarod test

The extract caused no significant effect on the time taken by mice to fall off the rotarod compared to the control at all the doses used (p>0.05 at 30-300 mgkg^{-1}). Similar results were observed with diazepam except 1.0 mg/kg which caused significant decrease in the latency to fall off the rotating rod (p<0.05).

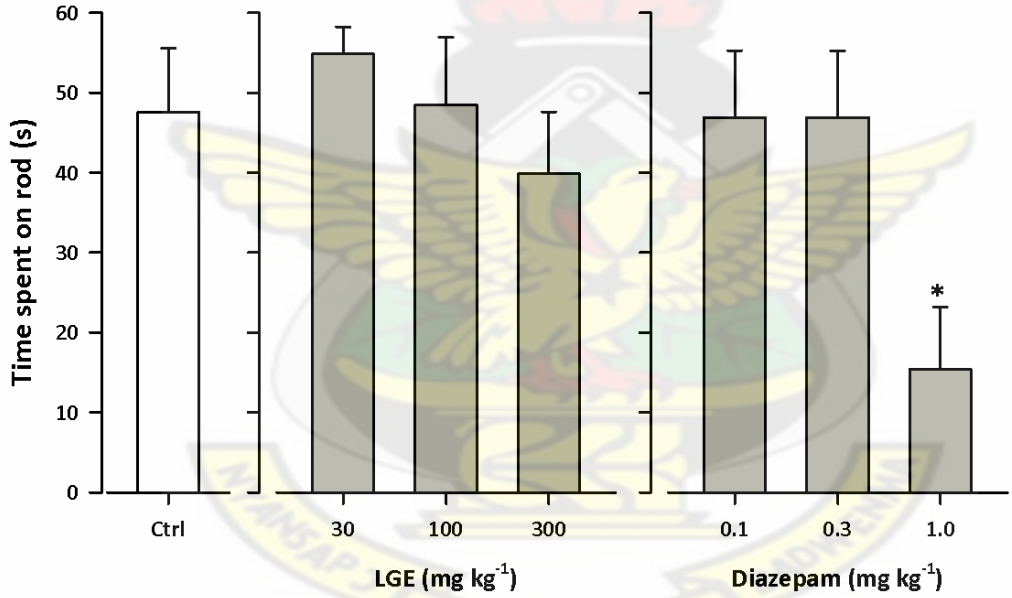

Figure 3.15 Effect of LGE and diazepam on mice on the rotarod. Data are expressed as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001 compared to control groups (one-way ANOVA followed by Newman Keuls test)

3.5.3 Effect on toad rectus abdominis muscle

The effects of the extract on the rectus abdominis muscle show that LGE is a partial neuromuscular agonist. It caused concentraction-dependent contractile responses similar to Ach but did not cause a maximum response compared to ACh. Its effects as well as that of ACh were dose-dependently blocked by tubocurarine however, the blockade in LGE was irreversible whilst that of ACh was reversible and the EC_{50} increased with increasing concentration of tubocurarine as shown in table 3.4. Also, LGE enhanced the concentration response curves of Ach by reducing the EC_{50} of Ach but at higher concentrations, LGE antagonsied Ach causing lesser maximal respones and high $EC_{50.}$

These effects are shown in fig. 3.16 and table 3.6.

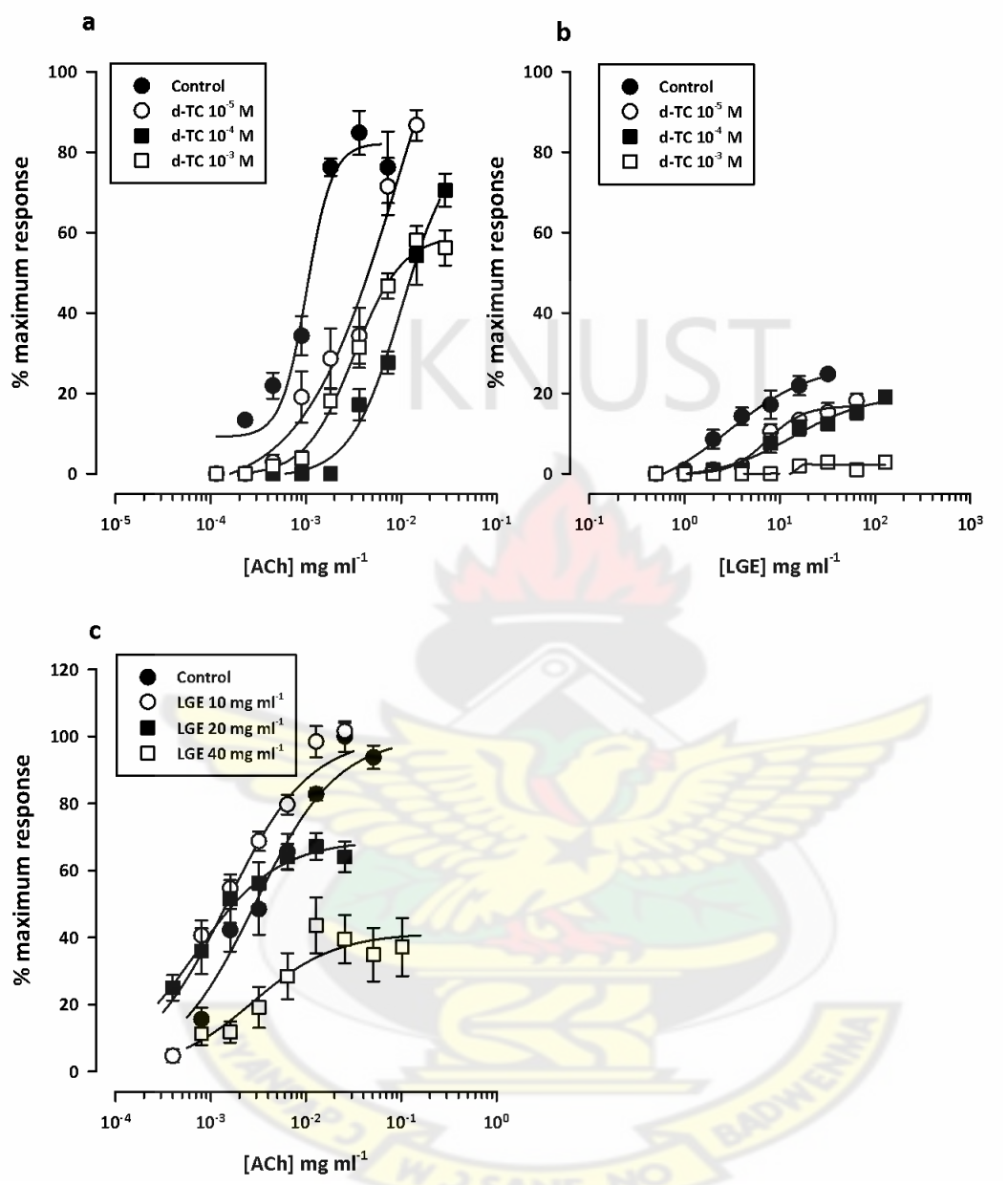

Figure 3.16 Concentration response curves for ACh and LGE on toad rectus abdominis muscle (a) Effect of tubocurarine on ACh-induced contractions (b) effect of tubocurarine on LGE-induced contractions (c) effect of LGE on ACh-induced contractions. Each point on each curve is the mean of at least 5 separate experiments.

Table 3.6 EC_{50} values for LGE and Ach on the rectus abdominis muscle.

DRUG TREATMENT (mg/ml)	**EC_{50} (mg/ml)**
LGE alone	2.9168±1.851
LGE + 10^{-5}M tubocurarine	7.3570±1.189
LGE + 10^{-4}M tubocurarine	13.7404±5.323
LGE + 10^{-3}M tubocurarine	∞
ACh alone	0.0010±0.000
ACh + 10^{-5}M tubocurarine	0.0095±0.031
ACh + 10^{-4}M tubocurarine	0.0101±0.002
ACh + 10^{-3}M tubocurarine	0.0032±0.000
Ach alone	0.0029±0.00
Ach + 10 mg/ml LGE	0.0015±0.001
Ach + 20 mg/ml LGE	0.0007±0.000
Ach + 40 mg/ml LGE	0.0028±0.002

Chapter 4

DISCUSSION

4.1 ANTICONVULSANT ACTIVITY

4.1.1 Effect of extract on pentylenetetrazole-induced seizures

The ability of an agent to prevent or delay the onset of tonic and tonic-clonic convulsion induced by PTZ in animals is an indication of anticonvulsant activity (Amabeoku *et al.*, 1993; Vellucci *et al.*, 1984). In this study, the extract, LGE, caused significant dose-dependent anticonvulsant effect against PTZ-induced seizures by delaying the onset of myoclonic jerks and tonic convulsions in mice. It also caused profound decrease in the duration of the tonic convulsions. Anticonvulsant activity in PTZ-induced seizures identifies compounds that can raise seizure threshold in brain (Raza *et al.*, 2001). AEDs effective in the therapy of generalised seizures of petit mal type (absence of myoclonic) i.e. phenobarbitone, valproate, ethosuximide and benzodiazepines suppress PTZ-induced seizures in a dose-dependent manner (Loscher *et al.*, 1991). Diazepam which was used in this study as a reference anticonvulsant agent showed significant activity by delaying the onset of myoclonic jerks and tonic convulsions and decreasing the frequency and duration of tonic convulsions.

According to De Sarro *et al.*, (1999), PTZ may be exerting its convulsant effect by inhibiting the activity of GABA at $GABA_A$ receptors. GABA is the major inhibitory neurotransmitter which is implicated in epilepsy. The enhancement and inhibition of the neurotransmission of GABA attenuates and enhances convulsion, respectively (Meldrum, 1981). Standard antiepileptic drugs such as diazepam and phenobarbitone are thought to produce their effects by enhancing GABA-mediated

inhibition in the brain (Macdonald *et al.*, 1994) and in this study with diazepam showed anticonvulsant activity against PTZ seizures. Seizures induced by PTZ are also blocked by drugs such as ethosuximide, by reducing T-type Ca^{2+} currents (Meldrum, 1996). Activation of N-methyl-D-aspartate (NMDA) (Meldrum, 1996) receptor system is also involved in the initiation and propagation of PTZ-induced seizures (Yudkoff *et al.*, 2006). In this regard, drugs such as Felbamate that block glutamatergic excitation mediated by NMDA receptor have demonstrated anticonvulsant activity against PTZ-induced seizures. Since the extract delayed the occurrence and decreased the duration of convulsions induced by PTZ, it is possible that the anticonvulsant effects might be due to enhancement of GABA-mediated inhibition and/or inhibition of Ca^{2+} currents or blockade of glutamatergic neurotransmission mediated by NMDA receptor; which is not tested in this study.

4.1.2 Effect of extract on picrotoxin-induced seizures

The extract showed remarkable dose-dependent anticonvulsant activity against PTX-induced seizures. The extract significantly delayed the onset of tonic convulsions and potently reduced the duration and frequency of the tonic convulsions induced by picrotoxin. Picrotoxin exerts its convulsant effect by blocking the $GABA_A$ receptor-linked chloride ion conductance into the brain cell following the activation of GABA receptors by GABA (Meldrum *et al.*, 2007).

In affirmation to the anticonvulsant activity of the extract, the clinically used and well validated anticonvulsant; diazepam, caused significant delay to the onset of myoclonic jerks and tonic convulsions and as well reduced the frequency and duration of tonic convulsions induced by PTX. It is therefore, possible that LGE attenuated PTX convulsion by enhancing GABA neurotransmission. This further

corroborates the hypothesis that LGE may be affecting GABAergic mechanism(s) to exert its anticonvulsant activity against PTZ-induced seizures.

4.1.3 Effect of extract in maximal electroshock seizures

The extract, LGE, was not able to abolish tonic hind limb extension at all the doses used in this study but significantly reduced the duration of the tonic hind limb extension.

Tonic hind limb extension is the universal feature of maximal electroshock in mice, rats, rabbits, cats, monkeys and humans (Raza *et al.*, 2001). Abolishing tonic hind limb extension in MEST predicts the ability of testing material to prevent the spread of seizure discharge from the epileptic focus and its effectiveness in MEST correlates well in suppressing generalized tonic-clonic seizures (Krall *et al.*, 1978; Porter *et al.*, 1984).

Also, abolishing hind limb extension indicates the ability of testing material to inhibit or prevent seizure discharge within brainstem seizure substrate (Raza *et al.*, 2001). All the currently available drugs that are clinically effective in the treatment of generalised tonic seizures (phenytoin, carbamazepine, phenobarbitone, valproate, lamotrigine, oxycarbamazepine, etc) are effective in MEST (Macdonald *et al.*, 1995). LGE in this study was not able to abolish tonic hind limb extension but significantly reduced its duration. Carbamazepine in this experiment caused significant reduction of the tonic hind limb extension phase and completely abolished this behaviour at 30 mg/kg. This validates the activity of the extract in this model.

Reduction in the duration of tonic hind limb extension but inability to completely abolish it by LGE indicated weak anticonvulsant activity in MEST but suggested strongly the presence of anticonvulsant compounds in the extract.

4.2 ANXIOLYTIC ACTIVITY

4.2.1 The elevated plus-maze test

The EPM is one of the most widely used animal models for screening putative anxiolytics (Wei *et al.*, 2007). In this test, rodents display an avoidance of exposed open areas of the maze, which is presumed to be the most aversive, and a preference for sections enclosed by protective walls (Weiss *et al.*, 1998). In fact, the primary indices of EPM anxiety comprise spatiotemporal measures of open arm avoidance (% of entries and time spent in open arms) (Carobrez *et al.*, 2005). However, risk assessment (RA) is a significant behavioural dimension closely related to fear/anxiety (Carobrez *et al.*, 2005; Rodgers *et al.*, 1997) and anxiolytic agents increase the performance of unprotected behaviours (Dawson *et al.*, 1995). The anxiolytic effectiveness of a drug can be demonstrated by an increase in rodent activity in the open arms (Han *et al.*, 2009). This could be the percentage of open arm entries and time spent in open arms or risk assessment behaviours in the open arms (unprotected RA behaviours such as stretch attend postures and head dipping). In this study, the extract caused statistically significant increase in the percentage of open arm entries and time spent in there and also significantly increased the frequency and duration of RA behaviours in the open arms (unprotected RA behaviours) including unprotected stretch-attend postures and unprotected head dips. At the same time the extract decreased the % of closed arm entries and decreased the performance of RA behaviours in the closed arms (protected RA behaviours including protected stretch-attend postures and protected head dipping).

Other explorative behaviours that indicate less anxiety such as grooming ant rearing were increased by the extract. To validate this test, diazepam and pentylenetetrazole which are clinically used anxiolytic and anxiogenic agents, respectively produced results similar and opposite respectively, to the extract.

Rodents display enhanced RA behaviours even after ceasing to avoid unprotected areas (open arms), suggesting that this defensive pattern may even be more sensitive to anxiety modulating drugs than avoidance-related measures (Griebel *et al.*, 1997; Rodgers *et al.*, 1994; Rodgers *et al.*, 1997; Setem *et al.*, 1999). Thus the anxiolytic effects of the extract were demonstrated not only by the increased open arm entries and percentage of open arm time, but also by unprotected stretch-attend postures, unprotected head dips, grooming and rearing. The fact that diazepam which is a clinically used anxiolytic agent and produced similar effects to that of the extract whereas PTZ which is a known anxiogenic agent caused anxiogenic effects opposite to that of the extract and diazepam attests to the anxiolytic activity of the extract.

4.2.2 The light/dark box test

The light/dark box test exploits rodents' natural aversion to bright areas compared to dark areas and their innate exploratory behaviour (File *et al.*, 2004). When mice are initially placed in the dark compartment, clinically effective anxiolytics have been shown to cause reduction of the latency to emerge from the small, dark compartment into the large, brightly lit and open area whereas stress and anxiogenic treatments increase emergence latency and time spent in the dark (Crawley, 1985; Onaivi *et al.*, 1989; Shimada *et al.*, 1995). In the present study, the extract showed significant anxiolytic activity by decreasing the emergence latency of mice into the lit compartment and also greatly increasing the amount of time spent in the lit box especially at 300 mg/kg. Diazepam which is a classical anxiolytic caused similar

effects to the extract on mice in this test whereas pentylenetetrazole which is a known anxiogenic agent caused opposite effects to that of the extract in this study. This affirms that the extract possibly contains compounds that have anti-anxiety activity.

4.3 ANALGESIC EFFECTS

Administration of the extract demonstrated significant antinociceptive/analgesic effects by inhibiting licking responses in both the early and late phases of the formalin test. Similarly, morphine produced marked inhibition in both the early phase and the late phase of this test. The formalin test is a widely accepted method for the rapid and easy screening and evaluation of pharmacological drugs (Saddi *et al.*, 2000; Vissers *et al.*, 2003). This test may involve sensory C-fibres in early phase and a combined process generated by peripheral inflammatory tissue and functional changes in the dorsal horn in the late phase (Dalal *et al.*, 1999; Dickenson *et al.*, 1987). Centrally-acting drugs such as morphine inhibit both phases equally (Shibata *et al.*, 1989). The functional changes involved in the late phase involves inflammatory components with the release of different pain-mediating substances responsive to NSAIDs (Le Bars *et al.*, 2001)), corticosteroids (Vasconcelos *et al.*, 2003) as well as analgesics with central effects. Inhibition of both phases of pain observed with the extract in this study demonstrates that the extract may contain compounds that have central analgesic effects as well as anti-inflammatory analgesic activity. Morphine which is a clinically used opioid known to block both phases inhibited both phases of nociceptive response (almost equally) in this study and this attests to the antinociceptive activity of the extract. To investigate the possible involvement of the opioid and/or the adenosinergic systems in the antinociceptive effect of the extract, animals were pretreated with naloxone (a non-

selective opioid antagonist) and theophylline (a non-selective adenosine receptor antagonist), respectively before administration of the extract and morphine. Both naloxone and theophylline significantly reversed the antinociceptive effect of the extract in the early phase but were unable to significantly attenuate its analgesic effect in the late phase. Regarding this finding, the antinociceptive effect caused by LGE is likely to be related with modulation of the opioid and adenosinergic systems. This clearly demonstrates why central mechanisms may be involved in the antinociceptive effect of the extract in the first phase as already stipulated. The analgesic effect of morphine was significantly antagonized by naloxone in both phases whereas theophylline did not have any significant impact. This is not surprising because morphine produces its analgesic effects by activation of opioid receptors and not adenosine receptors.

4.4 EFFECT ON MOTOR FUNCTION AND NEUROMUSCULAR EFFECT *IN VITRO*

4.4.1 The beam traversal test

This test poses challenges to the subject's fine motor balance and coordination skills (Carter *et al.,* 1999; Meredith *et al.,* 2006). The test measures skilled walking and increased errors in experimental groups reflect impaired coordination (Meredith *et al.*, 2006). In this study, LGE did not show any significant influence on the number of steps or the time to traverse the beam and did not cause stepping errors compared to the control group. Diazepam at 1.0 mg/kg caused significant increase in the time to traverse the beam. This is not surprising because benzodiazepines at high doses have sedative effects and cause ataxia (Charney *et al.*, 2001; Helton *et al.*, 1996; Woods *et al.*, 1995).

4.4.2 The rotarod test

The rotarod is a gold standard test of motor coordination and balance in rats and mice. Latency, the measure of how long the animal can stay on the rotating rod before falling off, reflects the animal's gross motor capacity. The plant extract did not cause any alteration in the amount of time mice spent on the rotating rod suggesting absence of impaired motor coordination. Diazepam caused significant impaired motor coordination at a higher dose as reflected by the decreased time spent on the rotating rod in animals pretreated with diazepam. Benzodiazepines such as diazepam, act as anxiolytics (at low doses) and anticonvulsants, producing also myorelaxant effect at higher doses (Onaivi *et al.*, 1992; Wolffgramm *et al.*, 1994). Hence it is not surprising that diazepam produced motor impairment at a higher dose.

4.4.3 Effect on toad rectus abdominis muscle

The isolated rectus abdominis muscle is an example of a skeletal muscle preparation. It is innervated by somatic nerves, which travel directly from the CNS to the skeletal muscle without being interrupted by ganglia. The synapse between the axon terminal of a somatic motor neuron and the skeletal muscle is the neuromuscular junction. Acetylcholine binds to muscular nicotinic (N_M) receptors on the motor end plate of the skeletal muscle fibre causing depolarization of the muscle and contraction (Rang *et al.*, 2003). The extract caused concentration-response contractions similar to ACh and was also blocked by tubocurarine (a neuromuscular nicotinic receptor antagonist) as in the case of ACh. However, LGE could not recover from the blockade to restore the maximal response unlike ACh. LGE also enhanced the contractile responses to ACh and blocked the responses to ACh as its concentration became higher. This implies that the extract is a partial

neuromuscular agonist probably affecting the N_M receptors to produce its contractile responses on the tissue. This also implies that the anticonvulsant effects of LGE are not mediated through peripheral neuromuscular blockade but rather evoked its action through central mechanisms.

4.5 GENERAL DISCUSION

This present study demonstrates that LGE has anticonvulsant, anxiolytic and antinociceptive activities but does not cause neuromuscular impairment.

The extract inhibited seizures induced by pentylenetetrazole, picrotoxin and maximal electroshocks. Inhibition of seizures induced by pentylenetetrazole and maximal electroshock in laboratory animals is the most common predictive screening tests used for characterizing potential anticonvulsant drugs (Krall *et al.*, 1978; Loscher *et al.*, 1988; Raza *et al.*, 2001). The maximal electroshock-induced seizure test is considered to be a predictor of likely therapeutic efficacy against generalised tonic-clonic seizures. By contrast, the pentylenetetrazole-induced seizure test represents a valid model for human generalised myoclonic and absence seizures (Loscher *et al.*, 1988). *L. guineensis* may therefore, contain compounds that have activity against generalised tonic-clonic seizures as well as generalised myoclonic and absence seizures.

It was not surprising when the extract showed anxiolytic activity in both the elevated plus-maze and the light/dark box tests because pentylenetetrazole and picrotoxin are known $GABA_A$ receptor antagonists (De Sarro *et al.*, 1999; Kasture *et al.*, 2000) and the fact that the extract inhibited convulsions induced by these agents may suggest that enhancement of GABA neurotransmission may be responsible for its anticonvulsant activity. The benzodiazepine-like anticonvulsants

such as diazepam, which enhance GABA neurotransmission act as anxiolytics at low doses and have anticonvulsant and myorelaxant or neurotoxic effects at higher doses (De Sarro *et al.*, 1999; Onaivi *et al.*, 1992; Wolffgramm *et al.*, 1994). It was therefore, expected that the extract may have anxiolytic effects. However, unlike centrally acting anticonvulsants such as diazepam, which have skeletal muscle relaxant effects, the extract did not cause defective motor coordination or balance but caused *in vitro* skeletal muscle contractions. The extract is not a pure compound and contains a variety of secondary metabolites including alkaloids, saponins, cardiac glycosides, reducing sugars and flavonoids and any of these could have skeletal muscle contractile effects.

Another finding from the study that is not surprising is the antinociceptive effect shown by the extract. It is now accepted that many anticonvulsants have analgesic effect in human neuropathic pain (McCleane *et al.*, 2003; McQuay *et al.*, 1995). The process of pain transduction, gating and modulation involves neurotransmitters including GABA, L-glutamate, NMDA, sodium and calcium ion channels and neuropeptides (Okuse, 2007). These mechanisms make anticonvulsants useful in the treatment of many neuropathic and even acute pain conditions (Lopes *et al.*, 2009; Thienel *et al.*, 2004). Some anticonvulsants such as topiramate have been found to have antinociceptive activity in acute pain models (Lopes *et al.*, 2009). Secondary metabolites including alkaloids, saponins, reducing sugars, cardiac glycosides and flavonoids were found to be present in the extract and these could have a wide range of compounds with pharmacological activities including those found in this study. For instance, Chauhan *et al.*, (1988) evaluated triterpenoids for their anticonvulsant activity against PTZ-induced seizures in mice and 10-40% of the animals were protected. Saponins of the triterpenoid type could be present in the plant extract in this study.

Chapter 5

CONCLUSIONS

The results obtained in this study indicate that the aqueous leaf extract of *L. guineensis* possesses bioactive principles that have anticonvulsant, anxiolytic, antinociceptive activities and that LGE is a partial neuromuscular agonist. Enhancement of GABAergic neurotransmission and/or calcium ion channel mechanisms may be involved in the anticonvulsant activity of the extract. Also, this GABAergic mechanism may account for its anxiolytic effect while neuromuscular nicotinic receptors mediation similar to acetylcholine may accounts for its contractile effects on the frog's rectus abdominis muscle. Then again, central mechanisms including the opioid and adenosinergic systems and an anti-inflammatory action may be responsible for the antinociceptive activity of the extract. In conclusion, the extract exhibited neurobehavioural activities that do not involve impairment of motor coordination and balance

RECOMMENDATIONS

By way of recommendation, further studies including chronic models of epilepsy should be carried out on the extract. Also, structural elucidation of the chemical constituents of the extract as well as neurochemical analysis should be done to elucidate the precise mechanism of action of the extract in epilepsy and analgesia. Finally but not the least, toxicological studies can be done to establish the safety level of the plant.

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