ANTICONVULSANT AND RELATED CENTRAL NERVOUS SYSTEM EFFECTS OF *ANTIARIS TOXICARIA* PERS. LESCH. (MORACEAE)

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DECLARATION

I hereby declare that I am the sole author of this thesis. 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. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

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ABSTRACT

Antiaris toxicaria is a plant traditionally used in Ghana for the treatment of various neurological conditions such as epilepsy and pain. This present study therefore seeks to screen for the anticonvulsant and neuropharmacological activities of the aqueous extract of *Antiaris toxicaria* (AAE) stem bark.

The extract was first taken through a preliminary screening. Analgesia and Straub tail effect were observed at 300-3000 mg kg^{-1} in the Irwin test suggestive of a morphine-like action. These effects were absent after 24 h. No deaths were recorded in the test estimating the LD_{50} to be above 3000 mg kg^{-1}. Spontaneous locomotor activity of the mice in the activity meter test was increased at 300-3000 mg kg^{-1}. It however showed no impairment on motor coordination in the beam traversal test. The extract potentiated duration of sleeping time in the pentobarbitone interaction test and showed susceptibility to metabolism by hepatic enzymes. Analgesic properties were also further confirmed in the tail withdrawal test while it inhibited PTZ-induced convulsions.

Based on the preliminary screening, the extract was further evaluated for anticonvulsant activity in rodents. The extract was relatively more effective in all models used except the maximal electroshock test and strychnine-induced convulsions. AAE (200-800 mg kg^{-1}) produced a significant dose–dependent increase in the onset to clonic seizures in the PTZ and PCT-seizure tests. The frequency of seizures was also decreased significantly. AAE in addition produced significant decrease in the total duration of convulsions in all pretreated animals. The extract significantly delayed onset of 4-aminopyridine convulsions and improved survival of the animals. Flumazenil, a $GABA_A$ receptor antagonist, significantly reversed the anticonvulsant effect of AAE strongly suggesting that *Antiaris toxicaria* may be acting by enhancing the effects of the GABAergic system

For chronic convulsion models, administration of AAE suppressed PTZ-induced kindling significantly. Pilocarpine and kainic acid-induced *status epilepticus* were reduced significantly by the extract as well. AAE, nonetheless, showed no protective effect against damage to hippocampal cells.

Drugs acting on GABA receptors find use as anxiolytics. Anxiolytic activity was assessed using the elevated plus maze (EPM), light/dark box (LDB) and social interaction tests. *A.*

toxicaria (200-800 mg kg^{-1}) decreased anxiety in mice by producing significant increases in open arm spatiotemporal and ethological parameters such as rearing in the EPM. Time spent in the light area of the LDB was also significantly increased. The extract had no effect on social interaction in mice, on the other hand.

Antidepressant property was assessed using the forced swimming (FST) and tail suspension tests (TST). Mobility time was significantly increased with a corresponding immobility time decrease in both tests. Swinging duration showed significant increase at the highest dose. A significant decrease in both pedalling frequency and duration was obtained implicating opioidergic mechanisms. Pretreatment with α-methyldopa (α-MD) reversed the antidepressant property of AAE as did ρ-chlorophenylalanine (PCPA), reserpine and reserpine combined with α-MD. The extract significantly increased the number of head twitches produced by 5-hydroxytryptophan in a manner to similar to fluoxetine, confirming possible serotoninergic mechanisms. The immobility time for the extract was not increased in the presence of D –serine ruling out glycine/NMDA mediated effects. Propranolol (10^{-7} -10^{-5} M) produced a non-parallel rightward shift of increasing concentrations of AAE on the isolated rat uterus indicating β_2- adrenoceptor activity. Rotarod test results show that AAE at the doses employed has no significant effect on motor coordination.

Anticonvulsants are very effective in some painful conditions. Hence, the extract was tested for possible analgesic effect. Treatment of mice with AAE (200-800 mg kg^{-1}, *p.o.*) produced a marked inhibition of both phases of formalin-induced nociception and similarly reduced the number of writhes induced by acetic acid in mice by 56.89 %.

Acute and sub-acute toxicity testing recorded no deaths over the 14-day period. Animals did not exhibit any sign of decreased mobility, respiratory depression or convulsions. Haemoglobin level was significantly decreased as well as the mean platelet volume in rats. All other parameters remained unaffected. Significant increases in Aspartate and Alanine Transaminases were also obtained in the rats. The extract decreased significantly urea and serum creatinine. None of these effects were, however, recorded in mice.

In summary, the aqueous stem bark extract of *Antiaris toxicaria* possesses anticonvulsant, anxiolytic, antidepressant and analgesic properties. Thus, *Antiaris* may be a potential source for novel drug discovery in the field of neuropsychiatric research.

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ABBREVIATIONS

5-HT	5-Hydroxytryptamine
5-HTP	5-Hydroxytryptophan
AEDs	Antiepileptic drugs
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AMPA	Alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
AMPT	Alpha-methyl-para-tyrosine
ANOVA	Analysis of variance
AST	Aspartate aminotransferase
BUN	Blood urea nitrogen
CNS	Central nervous system
DOPA	3,4-dihydroxyphenylalanine
EPM	Elevated plus maze
GABA	Gamma amino butyric acid
GAD	General anxiety disorder
GAT-1	GABA transporter 1
GGT	Gamma-glutamyltranspeptidase
HCT	Haematocrit
HLEs	Hind limb tonic extensions
I.P.	Intraperitoneal

ICH	International Committee for Harmonization
ICR	Imprinting control region
iGuR	ionotropic glutamate receptors
ILAE	International League Against Epilepsy
KA	Kainic acid
KNUST	Kwame Nkrumah University of Science and Technology
LDB	Light/dark box
MCH	Mean corpuscular haemoglobin
MCHC	Mean corpuscular haemoglobin concentration
MCV	Mean corpuscular volume
MEST	Maximal electroshock test
mGuR	Metabotropic glutamate receptors
MPE	Maximal possible effect
MPV	Mean platelet volume
NET	Norepinephrine transporter
NMDA	N-methyl-D -aspartate
P.O.	*Per os*
PCPA	Para-chlorophenylalanine
PD	Panic disorder

PLT	Platelets
PSTD	Post-traumatic stress disorder
PTZ	Pentylenetetrazole
QS	Quantity sufficient
RBC	Red blood cells
RDW	Red cell distribution width
S.C.	Subcutaneous
SE	Status epilepticus
SERT	Serotonin transporter
SSRI	Selective serotonin reuptake inhibitors
TCAs	Tricyclic antidepressants
WBC	white blood cells

Chapter 1

INTRODUCTION

1.1 GENERAL INTRODUCTION

Complementary and alternative medicines are often resorted to when chronic illnesses fail to respond to conventional therapy. Neurological disorders such as epilepsy, pain and anxiety, are a few examples of such chronic illnesses (Spinella, 2001). In Ghana, it is estimated that one traditional doctor cares for approximately four hundred (400) people as opposed to an orthodox doctor to every twelve thousand (12,000) people. A study revealed 52% of epilepsy patients in Nigeria rely on medicinal herbs (Danesi and Adetunji, 1994). This gives an indication of how greatly a majority of patients in the developing world rely on medicinal plants.

Natural products are in particular being explored because of the belief that they possess minimal side effects and are less expensive as compared to synthetic drugs (Fabricant and Farnsworth, 2001; Rates, 2001). Quite a number of medicinal plants used for the management of chronic illnesses such as epilepsy have been shown to possess promising activities (Stafford *et al*., 2008). This confirms that scientific validation of the claims about medicinal plants already in use by traditional medicine practitioners is important in order to enhance their safety and efficacy. Medicinal plants have also been an important source of bioactive compounds as they have served as lead compounds for drug development (Cragg *et al*., 1997). Drugs such as aspirin, scopolamine, theophylline and vincristine are important examples of such discoveries (Cox, 1994; Cox and Balick, 1994).

For these reasons, this study focuses on *Antiaris toxicaria*, a plant used in Ghana for management of many conditions including epilepsy and convulsions (Mshana *et al*., 2001).

1.2 THE PLANT ANTIARIS TOXICARIA

Botanical Name: *Antiaris toxicaria* (Pers.) Lesch.

Family: Moraceae

Local Name: Ofo, kyenkyen in Akan.

Synonyms: *Antiaris africana.*

1.2.1 **Description**

The plant *Antiaris toxicaria* (family Moraceae) is an indigenous tree common in the wetter parts of the Ghanaian forests. The tree has low blunt buttresses; smooth grey bark; finely hairy leaves that soon become glabrous and orange pear–shaped fruit (Mshana *et al.*, 2001).

Figure 1.1 *Antiaris toxicaria* tree (Courtesy: www.pngplants.org)

1.2.2 **Ecological and geographical distribution**

The specie can be found throughout the tropics from West Africa to Madagascar, and in Sri Lanka, India, Indo-China and southern China; also in Thailand, the Malesian region, the Pacific (east to Fiji and Tonga) and northern Australia. *Antiaris toxicaria* is

found from the wettest to dry forest types and often common in secondary forests (Bosu and Krampah, 2005).

1.2.3 Traditional uses

The bark of this plant is used as an antiepileptic traditionally and seeds as an antipyretic and for management of pain (Mshana *et al.*, 2001). In Africa, the latex produced by the bark of *A. toxicaria* is applied to cuts, wounds and skin conditions such as eczema and leprosy. It is also taken internally as a purgative (Bosu and Krampah, 2005). The latex serves as a component of most dart and arrow poisons in South East Asia.

1.2.4 Previous work

Antiaris is known to produce mainly prenylphenols (Hano *et al.*, 1990; Hano *et al.*, 1991). Cardiac glycosides have also been isolated from the latex (Kiliani, 1910; Mühlradt *et al.*, 1964; Carter *et al.*, 1997a; Carter *et al.*, 1997b). Cardenolides such as toxicariosides A-M, convallatoxin, convallatoxol, convalloside, 3-O-ß-D-xylopyranosylstrophanthidi, glucostrophanthidin, strophanthidin,α- and β-antiarins have been isolated (Levrier *et al.*, 2012). Examples of prenylphenols isolated from the root bark are antiarones A-K (Hano *et al.*, 1990). Isolates from the specie such as toxicariosides A and M have been previously investigated and shown to be active against various cancer cell lines (Levrier *et al.*, 2012; Li *et al.*, 2012). The growth inhibitory effect of the cardenolides on cancerous cells may possibly be due to induction of apoptosis (Jiang *et al.*, 2008). Isolation of compounds and acute toxicity studies on the leaf extracts have also been carried out (Kang *et al.*, 2008-09).

1.3 EPILEPSY

Epilepsy is a common neurological disorder associated with an interruption in psychological and social aspects of life. Epileptic seizure attacks are episodic; patients may live normal lives between seizures. Thus clinical diagnosis relies heavily on eyewitness account (Dhillon and Sander, 2003). Epileptic seizures may be due to

abnormal hypersynchronous discharges of neurones that may be caused by any pathological process that affects the brain (Dhillon and Sander, 2003).

Epilepsy has received a great deal of interest in medical research because epileptic seizures are common but not sufficiently understood (Dhillon and Sander, 2003). Prevalence of the condition in developing countries is generally higher than in the developed countries (Stafford *et al.*, 2008). It is the second most common neurological disorder after stroke and is estimated that approximately 0.8 % of the population is affected by some form of epilepsy (Pitkanen and Lukasiuk, 2009).

In African communities, epilepsy is seen as a shameful disorder. It carries with it a stigma and has severe social implications. Sufferers are often shunned and discriminated against with respect to employment, marriage, etc (Baskind and Birbeck, 2005).

1.3.1 Pathophysiology of epilepsy

The recurrent spontaneous seizures arising in epilepsy may be due to a variety of different electrical or chemical stimuli in a normal brain. The epileptic seizure always reflects abnormal hypersynchronous electrical activity of neurons caused by an imbalance between excitation and inhibition in the brain (Treiman, 2001; Bienvenu *et al.*, 2002). Neurons are interconnected in a complex network in which each individual neuron is linked through synapses with hundreds of others (Dhillon and Sander, 2003). Normal neurons usually discharge repetitively at a low baseline frequency. However, after damage or electrical or metabolic insult, a change in the pattern of discharge may develop. A single neuron, discharging abnormally is inadequate to produce sufficient excitation and hence a clinical seizure. This occurs only with large neuronal networks. More than hundred neurotransmitters or modulators 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) (Schwartz, 1988; Olsen and Li, 2012). An increase in excitatory processes or a decrease in inhibitory processes can result in a seizure. Mutations in several genes that code for protein subunits of voltage-gated and ligand-gated ion channels have been associated with

epilepsy. These genes may code for sodium ion channel proteins; defective sodium channels stay open for too long resulting in neuron hyper-excitability.

1.3.2 Role of GABA and glutamate in the pathogenesis of epilepsy

Generation of seizures has been attributed to an imbalance between excitatory and inhibitory neurotransmission in the brain. Hence, it is important to highlight the role of neurotransmitters especially γ-amino butyric acid (GABA) and glutamate in epileptogenesis, since they are the major inhibitory and excitatory neurotransmitters in the central nervous system, respectively.

GABA is located primarily in short-axon interneurons that synapse on cell bodies and proximal axons, and serves to counterbalance neuronal excitation. A perturbation of this balance causes seizures (Treiman, 2001). Compounds that interfere with GABA-mediated inhibition have been shown to be convulsants (Treiman, 2001). GABA exerts its major inhibitory effect via activation of $GABA_A$ receptor (which is a ligand-gated ion channel). The receptor is directly linked to chloride ion channels. Consequently, activation increases neuronal membrane conductance of chloride ions causing membrane hyperpolarisation, reduced neuronal excitability and most rapid inhibition in the brain (Sieghart, 1992). $GABA_A$ receptor has become a target for many neuroactive drugs such as benzodiazepines and barbiturates (Scholze *et al.*, 1996). $GABA_A$ receptor consists of five subunits that form the chloride ion channel (Macdonald and Olsen, 1994). The subunits consist of various subtypes and studies have shown that individual subunits and subtypes confer different sensitivities to agents acting on $GABA_A$ receptors (Neelands *et al.*, 1998).

L-Glutamate is the most important excitatory neurotransmitter of the central nervous system. It acts via two types of receptors— ionotropic glutamate receptors (iGuR) 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), AMPA (alpha- amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) and kainate. The role played by metabotropic glutamate receptors depends on the type of receptors: activation of type I is convulsant whereas activation of types II and III is

anticonvulsant (Moldrich et al., 2003). Activation of these receptors is responsible for basal excitatory synaptic transmission and many forms of synaptic plasticity such as long-term potentiation (LTP) and long-term depression (LTD) (Arai and Lynch, 1992; Arai *et al.*, 2004).

Epilepsy may result from exaggerated glutamate neurotransmission. As such generally blocking glutamate-mediated excitatory neurotransmission protects against seizures in both *in vitro* and *in vivo* models of epilepsy (Cunningham *et al.*, 2000; Cunningham *et al.*, 2004). Limbic seizures in experimental animals, for instance, result in brain damage that resembles that due to glutamate toxicity and is similar to changes as seen at autopsy in patients with intractable epilepsy (Leonard, 2004). Reduction of excitatory glutamatergic neurotransmission is therefore potentially important. AMPA receptor blockade has been shown probably contributes to the antiepileptic effect of drugs such as lamotrigine (Lee *et al.*, 2008).

1.3.3 **Types of epilepsy**

The International League Against Epilepsy (ILAE) recognizes two main categories: focal seizures and generalised seizures (Engel, 2006). However, some may have common characteristics or may vary and individuals can be affected by one or more types of seizures. Focal (partial) seizures are those in which the discharge begins locally and remains localised, producing relatively simple symptoms such as involuntarily muscle contractions or more complex effects on mood and behaviour, without loss of consciousness (Dwivedi and Smar, 1994; Rang *et al.*, 2003). Generalised seizures involve the production of unusual electrical activity throughout both hemispheres of the brain. Immediate loss of consciousness is typical of generalised seizures (Dwivedi and Smar, 1994). Generalised tonic-clonic seizures (grand mal) and absences seizures (petit mal) are the main categories of generalized seizures (Dwivedi and Smar, 1994; Rang *et al.*, 2003). Absence seizures are characterized by a partial loss of consciousness; the individual briefly appears unresponsive with involuntary muscle twitches, particularly in the face. Myoclonic seizures involve very brief and irregular arrhythmic movements (Rang *et al.*, 2003). Tonic-clonic seizures begin with the tonic phase. This characterized by abrupt stiffening movements with symptoms

such as loss of orofacial motor control resulting in tongue biting and/or urinary incontinence. The clonic phase follows with rhythmic body movements. Individuals may be confused or sleepy after recovery (Rang *et al.*, 2003). *Status epilepticus* describes a life-threatening condition where an individual experiences prolonged or successive seizures with no recovery time. Seizure activity can be considered *status epilepticus* if it lasts a minimum of 5 minutes up to about 30 minutes (Rang *et al.*, 2003).

Patients with focal epilepsy may have focal sensory or focal motor seizures or even secondarily generalised tonic-clonic seizures (e.g. focal seizures with secondary generalization) (Engel, 2006).

Generalized epilepsies are never associated with focal seizures, but some localization-related epilepsies may be associated with generalized seizures if they are secondarily generalized (Engel and Schwartzkroin, 2006).

1.3.4 Causes of epilepsy

Several mechanisms have been proposed for the cause of epilepsy such as biochemical insults to the brain e.g. hypoglycaemia and even sudden withdrawal of certain drugs such as barbiturates (Loscher, 2002b). Epilepsy may also be caused by previous head trauma or by infections like meningitis (Loscher, 2002b). An imbalance between excitatory and inhibitory neurotransmitters resulting from structural pathology, genetic factors or stress may as well lead to epilepsy (Dwivedi and Smar, 1994). There is, however, no single unifying explanation to the exact cause of epilepsy though it is now possible to investigate the physiological events contributing to the onset of epilepsy.

1.3.5 Mechanism of action of antiepileptic drugs

The three major mechanisms of action of antiepileptic drugs (AEDs) recognised are modulation of ion channels, enhancement of inhibitory neurotransmission, and attenuation of excitatory transmission (Kwan *et al.*, 2001).

1.3.5.1 *Modulation of ion channels*

The voltage-gated sodium ion channel is of principal importance and responsible for depolarisation of the cell membranes as well as the upstroke of the neuronal action

potential (Porter and Rogawski, 1992; Deckers *et al.*, 2003). Quite a number of established AEDs act by blocking sodium ion channels e.g. phenytoin and carbamazepine as well as newer drugs like topiramate and lamotrigine (Deckers *et al.*, 2003). These drugs largely inhibit sustained neuronal firing of action potentials by binding to the inactive channel, without an effect on amplitude or duration (McLean and Macdonald, 1986; Kwan *et al.*, 2001). Voltage-gated calcium channels are also major targets for AEDs. Many of these drugs are believed to be acting by blockade of various subtypes of this receptor (Stefani *et al.*, 1997). The T-type channel is believed to mediate the efficacy of ethosuximide against generalised absence seizures (Kwan *et al.*, 2001; Deckers *et al.*, 2003). Lamotrigine as well has been reported to act by blocking N- and P-subtypes of voltage-sensitive calcium channel hence limiting the release of neurotransmitters (Deckers *et al.*, 2003).

1.3.5.2 *Enhancement of inhibitory neurotransmission*

GABA is the major inhibitory neurotransmitter in the mammalian central nervous system (Olsen and Avoli, 1997). GABA acts at $GABA_A$, $GABA_B$ and $GABA_C$ when released post-synaptically (Kwan *et al.*, 2001; Deckers *et al.*, 2003). $GABA_A$ receptors are ligand-gated ion channels and responds to GABA by an increase in chloride ion conductance to cause neuronal hyperpolarisation. After release, GABA is rapidly removed by uptake into glia and presynaptic nerve terminals and then broken down by GABA-transaminase (Treiman, 2001). Barbiturates and benzodiazepines bind to distinct sites on the $GABA_A$ receptor. Barbiturates prolong the duration of channel opening, while benzodiazepines increase frequency of channel opening (Deckers *et al.*, 2003). GABA-transaminase is irreversibly inhibited by vigabatrin whiles tiagabin inhibits GABA transporter (GAT-1) (Deckers *et al.*, 2003).

1.3.5.3 *Attenuation of excitatory neurotransmission*

Glutamate is the most important excitatory neurotransmitter in the brain. It acts on both ionotropic and metabotropic receptor types. The ionotropic glutamate receptors have been classified into three subtypes kainate, AMPA and NMDA. The AMPA and kainate subtypes are concerned with fast excitatory neurotransmission, whereas the NMDA receptor is activated during periods of delayed depolarization (Meldrum, 2000). Some AEDs have been reported to reduce glutamate release. This effect may,

however, be more related to action on calcium channels rather than direct action on the glutamatergic system (Stefani *et al.*, 1997). Topiramate for instance, is well-known for its inhibitory action on AMPA/kainate subtypes (Kwan *et al.*, 2001).

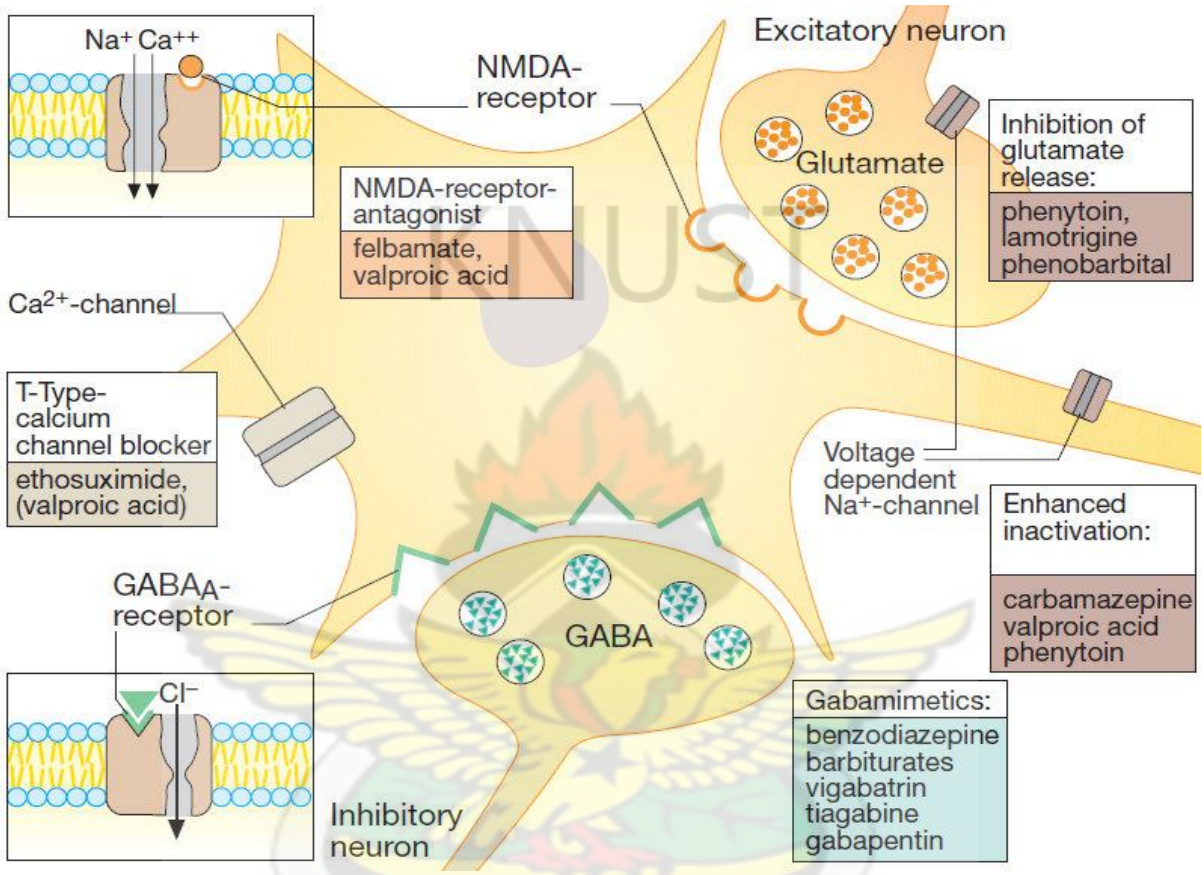

Figure 1.2 A summary of the various mechanisms of action of antiepileptic drugs (Courtesy of www.pharma-munvar.co.cc).

1.4 ANTICONVULSANT SCREENING

1.4.1 Acute Models

The maximal electroshock test (MEST) in mice, introduced by Putnam and Merritt is the most widely used animal model because seizure induction is simple and of predictive value for potentially clinically-effective AEDs (Löscher, 1999). This test identifies agents that are effective against generalised tonic-clonic and partial seizures (White, 1997; Loscher, 2002a). The Pentylenetetrazole (PTZ) test is another important

acute model used to discover agents that are effective in the treatment of generalized myoclonic and absence seizures (Loscher and Schmidt, 1988; White, 1997). Administration of bicuculline (BlC), picrotoxin (PCT) and strychnine (STN) are also important tests used to induce seizures and evaluate the efficacy along with mechanisms of anticonvulsants (Raza *et al.*, 2001). Several widely used drugs such as valproate and ethosuximide have been discovered by these acute models. However, these models might not detect all potential compounds when used alone. The MEST is known to preselect drugs with particular mechanisms, but may miss others (Meldrum, 1997). Drugs that block sodium channels are particularly effective in this model though it is considered a mechanism-independent model (Kupferberg, 2001). The PTZ test might not be able to detect AEDs that act against non-convulsive seizures (Loscher, 2002a). Lamotrigine which is very efficacious against non-convulsive seizures in patients is ineffective in the PTZ test, whereas vigabatrin, which is effective in the PTZ test, is ineffective in patients and may even exacerbate non- convulsive seizures (Loscher, 2002b).

1.4.2 Chronic Models

Kindling is a chronic model of epilepsy and epileptogenesis. Kindling has been well studied as an epilepsy model of complex partial seizures with secondary generalization. Traditionally, kindling has been induced by application of brief, low-intensity electrical stimulation. Since its first description by Goddard in 1967, several studies have been published describing its validity in studying the underlying course of epilepsy and testing of antiepileptics. Kindled seizures in animals after daily electrical stimulation of the amygdaloid complex have been described (Takei *et al.*, 1999). Chemical kindling is similar to electrical kindling but in this case a chemical rather than an electrical stimulus is used. As with electrical kindling, the main features of chemical kindling are the progressive development of behavioural seizures, a reduction in seizure threshold, and a maintained heightened sensitivity to the seizure-inducing stimulus. The most common chemical kindling agent is PTZ, although other drugs that interfere with GABAergic function are also effective kindling agents. PTZ is a selective blocker of the chloride channel coupled to the $GABA_A$ receptor and is

associated with a reduction in GABA-mediated neurotransmission in the central nervous system (Nutt *et al.*, 1982; Corda *et al.*, 1990).

Sustained status epilepticus (SE) can be induced by administration of chemical convulsants such as pilocarpine and kainic acid (Löscher, 1999). Acute administration of a high dose of pilocarpine in rodents is widely used to study the pathophysiology of seizures. It was first described by Turski *et al.*in 1983. Pilocarpine-induced seizures reveal behavioural and electroencephalographic features that are similar to those of human temporal lobe epilepsy.

Kainic acid will induce seizure activity when administered systemically or directly into the brain. Kainic acid-induced SE is similar to human SE (Treiman *et al.*, 1990). The behavioural activity during the first hour after kainic acid injection includes staring episodes, head nodding and frequent Wet Dog Shakes (WDS). These are followed by isolated limbic motor seizures which increase in frequency and eventually lead to SE (Jeffrey, 1998).

1.5 ANTICONVULSANTS AND SOME CENTRAL NERVOUS SYSTEM EFFECTS

1.5.1 Convulsion and anxiety

Anxiety can be a normal emotional state or in some instances a psychiatric disorder (Nutt, 2005). Anxiety may be defined as a feeling of apprehension or fear, combined with symptoms of sympathetic activity (Rang *et al.,* 2003). It is a usual response to stress and becomes a clinical problem only if it becomes severe or persistent, causing disability and/or is present in absence of a stressor (Nutt, 2005). It has a lifetime prevalence of over 15% of the population (Nutt, 2005). Available evidence strongly suggests that epilepsy sufferers have a higher prevalence of anxiety disorders than controls in both hospital and community samples (Vazquez and Devinsky, 2003). Community studies of epilepsy patients have estimated the prevalence of anxiety to be between 14.8 and 25% (Vazquez and Devinsky, 2003).

Some pharmacological theories have suggested that anxiety is caused by either inhibitory or excitatory amino acid dysfunction. The $GABA_A$ receptor is the brain's main inhibitory receptor regulating activity of many types of systems including dopaminergic, noradrenergic and serotoninergic (Sinclair and Nutt, 2007). Studies have therefore shown that the down-regulation of $GABA_A$ function may underlie some anxiety forms (File and Lister, 1984; Malizia *et al.,* 1998). Anxiolytics have therefore been developed to target specific brain neurotransmitter systems.

Since a number of anticonvulsant drugs are known to act via GABA and glutamate neurotransmission, this offers promise for anxiolytic pharmacotherapy. For instance, benzodiazepines are effective in panic disorder (PD) and general anxiety disorder (GAD) (Malizia *et al.,* 1998). The anticonvulsant properties of lamotrigine are mediated via NMDA glutamate receptor antagonism and its efficacy has been shown in post-traumatic stress disorder (PTSD) (Sinclair and Nutt, 2007).

1.5.2 Animal models used for screening anxiolytics

1.5.2.1 Introduction

Animal models of anxiety are bidirectionally sensitive and may be able to screen for substances with both anxiolytic and anxiogenic activity (Pellow *et al.*, 1985; Pellow and File, 1986).

A range of animal tests of anxiety presently exist. They involve exposure of animals to stimuli (exteroceptive or interoceptive) that are capable of causing anxiety in humans. Animal models of anxiety are classified according to the nature of the aversive stimulus and of the response elicited. This suggests that the neuronal control of anxiety may vary according to whether the interpretation of an aversive stimulus is innate or learned and whether it results in the production of a response or on the other hand, inhibits an ongoing, rewarded behaviour (Bourin *et al.*, 2007). Two major categories involving either conditioned (e.g. potentiated startle) or unconditioned (elevated plus maze and light/dark exploration tests) responses have been established (Rodgers *et al.*, 1997). Conditioning models require extensive training of subjects to adapt to situations such food or water deprivation. Unconditioned responses require no prior training and so allow for a very much more complete rodent behavioural characterization of the effects of experimental manipulations (Rodgers *et al.*, 1997).

An ideal model should have predictive, face and construct validity Predictive validity requires that animals display reduced anxiety when treated with anxiolytics. A model with face validity should show that the response of an animal to a threatening stimulus is similar to the human response. Construct validity exhibits the mechanisms underlying anxiety (McKinney and Bunney, 1969). One may require the use of more than one model in order to achieve to all these parameters.

Animal models of anxiety cannot model every aspect of human anxiety but permit detailed exploration of neurobiological and psychological processes in states in which fear might be inferred, such as responses to acute and repeated aversive stressors. The heterogeneity of anxiety disorder has been accepted clinically suggesting that there are peculiar neurobiological substrates for each type. It is therefore necessary to examine whether different animal models might reflect those differences (Bourin *et al.*, 2007). Various animal models may be more appropriate for one type of anxiety

disorder than for another; it is inappropriate to assume that any one model may be able to detect compounds for a condition that is mediated through multiple and diverse mechanisms (Bourin *et al.*, 2007).

1.5.2.2 The elevated plus-maze (EPM) test

The elevated plus-maze (EPM) is the most popular animal model of anxiety (Carobrez and Bertoglio, 2005). The model was described first by Pellow and co-workers (Pellow *et al.*, 1985). It is made up of four arms (two open and two enclosed) arranged to form a plus shape. The maze is raised off the ground in order that the open arms may combine elements of unfamiliarity, openness and elevation (Bourin *et al.*, 2007). This model is based on the natural aversion of rodents for open spaces using the conflict existing between exploration and aversion to elevated open places (Bourin *et al.*, 2007). Assessment of anxiety behaviour of rodents is done by the use of the ratio between time spent in the open arms and the time spent in the enclosed arms. Other ethological parameters (i.e. head dips and stretch-attend postures) reflect anxiolytic behaviour (Walf and Frye, 2007). Behavioural assessment is devoid of the use of noxious stimuli (e.g. use of electric shock) that is typical of a conditioned response (Walf and Frye, 2007).

Figure 1.3 The elevated plus maze apparatus (courtesy of www.lafayetteneuroscience.com).

1.5.2.3 The light /dark box (LDB) test

The light/dark box also provides unconditioned anxiety-like behavioural response. The test is based on the natural avoidance of rodents to brightly lit areas in addition to the natural exploratory behaviour of rodents in response novel environments (Crawley and Goodwin, 1980). This model permits mice to freely explore two inter-connected compartments that vary in size (2:1), colour (white : black) and illumination (bright : dim) (Bourin *et al.*, 2007). Mice placed into the box will naturally show preference for the dark area. After anxiolytic drug treatment, the apparent apprehension of the lit area is abolished (Bourin *et al.*, 2007). Therefore, increase in behaviours in the lit compartment of a two-compartment box, suggests anxiolytic activity. In addition, an increase in transitions without an increase in spontaneous locomotion is considered as anxiolytic activity. Transitions are reported to be a

measure of activity exploration since habituation occurs over time while time spent in each compartment is a reflection of aversion (Bourin and Hascoet, 2003).

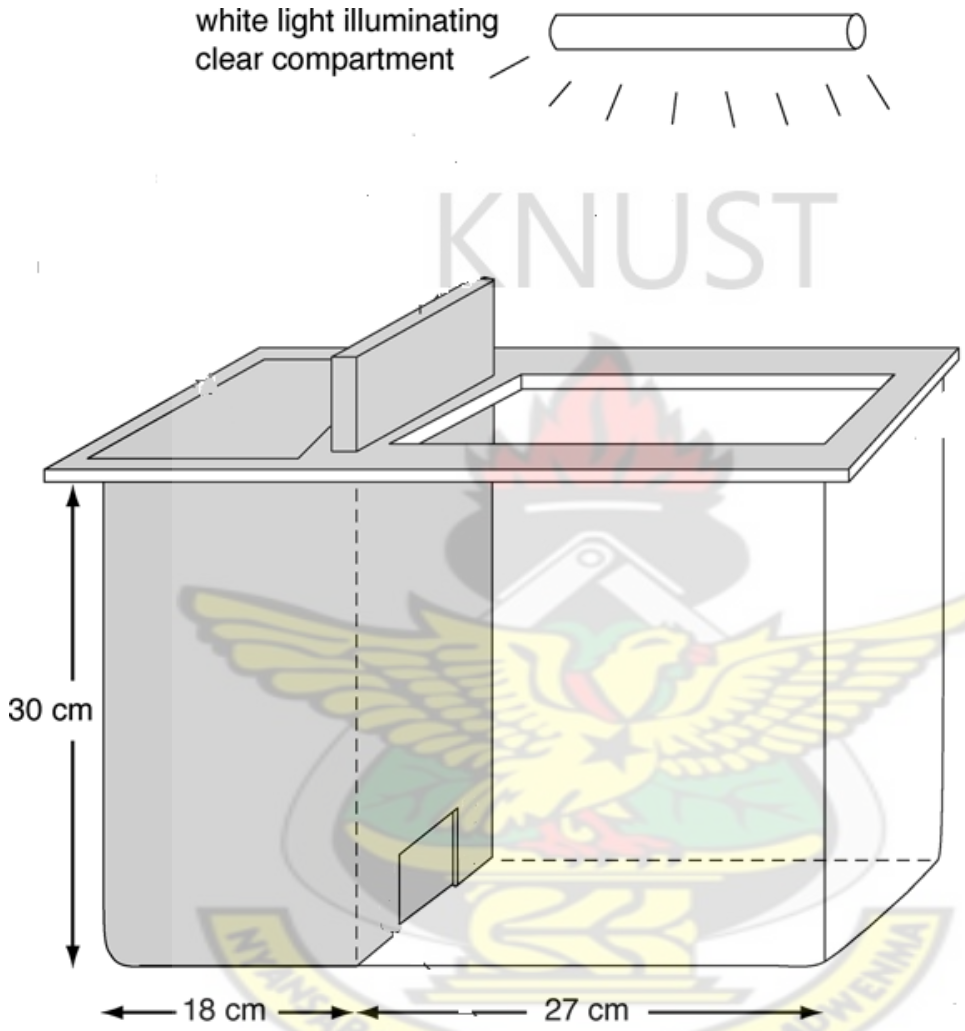

Figure 1.4 The light/dark box apparatus (courtesy: Current protocols in pharmacology, supplement 27)

1.5.2.4 Social Interaction Test

Time spent in social interaction decreases when rodents are anxious (Crawley, 2004; Moy *et al.*, 2004). Test conditions involve varying the light intensity and familiarity of the testing area. Bright light and unfamiliarity are used to generate anxiety. Benzodiazepines and barbiturates have been shown to significantly increase social interaction (File *et al.*, 2001). Anxiolytic action can be best detected in a condition that generates low levels of interaction (e.g. high light, unfamiliar testing area); anxiogenic action is best detected in the low light and familiar testing areas (File *et al.*, 1987).

1.6 EPILEPSY AND DEPRESSION

1.6.1 Background

Depression is the most frequent comorbid condition among people with epilepsy (Dias *et al.*, 2010). Researchers have reported a threefold increase in the diagnosis of depression in addition to a fourfold increase in suicide rate among patients with epilepsy compared with the general population (Christensen *et al.*, 2007). One review article for instance reported that 181 of 2196 deaths (8%) among patients with epilepsy resulted from suicide (Wolfersdorf and Froscher, 1987). Antiepileptic drugs (AEDs) contributing to the increased rates of depression and suicide among epilepsy patients remains controversial (Hesdorffer and Kanner, 2009). The U.S. Food and Drug Administration, however, on December 16, 2008 issued a caution about an increased risk of suicidal ideation and behaviour among people using AEDs (Food and Drug Administration, 2008). 119 clinical trials of 11 antiepileptic drugs were reviewed and it was concluded that patients taking AEDs have a 1.8-fold higher possibility of suicidal behaviour or ideation compared with patients taking a placebo (Food and Drug Administration, 2008). It has since been the opinion of some neurologists that the FDA's warning may unduly frighten patients, increase noncompliance rates, and eventually increase mortality among patients with epilepsy (Roc, 2009)

1.6.2 Theories of Depression

Over the years, the major theories of depression have been centred on function or amount of monoamines. In recent times, other factors such as neurotrophic and endocrine factors have been found to affect the pathogenesis of clinical depression.

1.6.2.1 Monoamine Theory of depression

This theory suggests that depression is due to a deficiency in the amount and/ or function of cortical and limbic monoamines namely serotonin (5-HT), norepinephrine (NE), and dopamine (DA) (Hirschfeld, 2000). Besides the fact that most antidepressants potentiate activity of monoamines, there has been a lot of evidence to support this theory from numerous sources (Elhwuegi, 2004). Patients with clinical depression who respond to serotonergic antidepressants such as fluoxetine have been known to quickly suffer relapse when given tryptophan-free diets; tryptophan is a precursor of serotonin synthesis. On the other hand, patients on noradrenergic antidepressants such as desipramine are less likely to relapse on a tryptophan-free diet (Hirschfeld, 2000; Yamada and Higuchi, 2002). Reserpine treatment, which depletes monoamines, is known to be coupled with depression in some of patients. This implies that not only can monoamine agonists decrease depression, but monoamine antagonists such as reserpine can induce depression (Yamashita *et al.*, 1998; Heslop and Curzon, 1999). In addition, levels of 5-HT, as measured by its metabolites, seem to be correlated with depression (Jotaro *et al.*, 1994). For example, patients with low levels of a 5-HT metabolite were found to be more likely to have committed suicide (Marazziti *et al.*, 1995).

1.6.2.2 Neurotrophic Hypothesis

This hypothesis proposes that a deficiency in neurotrophic support may result in depression (Nestler *et al.*, 2002). Evidence indicates that nerve growth factors such as brain-derived neurotrophic factor (BDNF) are crucial in the regulation of neural plasticity, resilience, as well as neurogenesis (Groves, 2007; Hasler, 2010). This evidence further suggests that antidepressant pharmacotherapy increases neurogenesis and synaptic connectivity in cortical areas such as the hippocampus.

BDNF promotes neuronal survival and growth effects by activating the tyrosine kinase receptor B in both neurons and glia cells (Groves, 2007; Hasler, 2010). In addition, animal and clinical studies point out that depression and stress is associated with a fall in BDNF levels and decreased neurogenesis (Karege *et al.*, 2002; Aydemir *et al.*, 2006). This in turn results in atrophic structural changes in the hippocampus and medial frontal cortex which are important in clinical depression (Duman, 2004). Besides, all known classes of antidepressants have been associated with an increase in BDNF levels and neurogenesis in animal models with chronic administration (Duman and Li, 2012; Voleti and Duman, 2012).

1.6.2.3 Neuroendocrine factors involved clinical depression

Abnormalities in the hypothalamic-pituitary-adrenal (HPA) axis in man has been identified in some depressed patients (Gotlib et al., 2008). Major depressive disorder (MDD) is associated with elevated cortisol levels, non-suppression of adrenocorticotropic hormone (ACTH) release in the dexamethasone suppression test, and chronically elevated levels of corticotropin-releasing hormone (Gotlib et al., 2008; Pariante and Lightman, 2008). Though, the significance of these HPA abnormalities is uncertain, they are considered to signify a dysregulation of the stress hormone axis. Markopoulou *et al.,* in 2009 have stated that both exogenous glucocorticoids and endogenous elevation of cortisol are associated with mood symptoms and cognitive deficits similar to those seen in MDD. High cortisol levels may produce hippocampal damage impairing hippocampal function. This might be expected to contribute to some of the cognitive abnormalities of depression. Antidepressant treatments would therefore, work, to reverse these abnormalities, although the molecular and cellular mechanisms are not known (Nestler *et al.*, 2002).

Sex steroids have also been implicated in the pathophysiology of clinical depression (Kessler, 2003; Almeida *et al.,* 2004). Oestrogen deficiency states occurring in the postpartum and postmenopausal periods are believed to play a role in the aetiology of depression in some women. Severe testosterone deficiency in men is also similarly associated with some depressive symptoms (Kessler, 2003). Hormone replacement therapy in hypogonadal men and women may be linked with an improvement in

depressive symptoms (Almeida *et al.,* 2004). As much as 25% of depressed patients are reported to have abnormal thyroid function. Clinical hypothyroidism often coexists with depressive symptoms, which subside with thyroid hormone supplementation (Cole *et al.,* 2002; Engum *et al.,* 2002).

1.6.3 Antidepressant pharmacotherapy

1.6.3.1 Monoamine Oxidase Inhibitors (MAO-I)

Monoamine oxidase inhibitors were among the first clinical antidepressants to be introduced but were largely superseded by TCAs and other antidepressants with clinical profiles that were considered more efficacious and safer (Rang *et al.*, 2003). Iproniazid, a hydrazine derivative, was the first antidepressant used clinically but was discontinued due to hepatotoxicity (Nelson *et al.,* 1978; Timbrell, 1979). Later, two other hydrazine-derivative inhibitors of MAO, phenelzine and isocarboxazid were introduced into clinical practice. Tranylcypromine, structurally related to amphetamine, was the first MAO inhibitor unrelated to hydrazine to be discovered (Cesura and Pletscher, 1992). Subsequently, reversible, selective MAO inhibitors with potentially broad applications (e.g. selegiline) were developed. Selegiline, a propargylamine, is fairly specific for MAO-B (Cesura and Pletscher, 1992). The non-selective MAO inhibitors in clinical use are either reactive hydrazines (e.g. phenelzine) or amphetamine derivatives (e.g. tranylcypromine) (Rang *et al.*, 2003). These drugs irreversibly and nonselectively block both MAO-A and MAO-B, which metabolize dopamine, norepinephrine, and serotonin in neuronal tissues (Rang *et al.*, 2003).

1.6.3.2 Tricyclic Antidepressants (TCAs)

Tricyclic antidepressants (TCAs) were initially synthesized in 1949 for use as antipsychotic drugs (Rang *et al.*, 2003). They are closely related in structure to the phenothiazines (Gallanosa *et al.*, 1981; Rang *et al.*, 2003). They act by inhibiting amine reuptake but most TCAs affect other types of neurotransmitter receptors, including muscarinic acetylcholine receptors, histamine receptors and 5-HT receptors (Gallanosa *et al.*, 1981). Antimuscarinic effects of TCAs do not contribute to their antidepressant effects but are however responsible for their various side effects (Rang

et al., 2003). Older TCAs with a tertiary amine side chain (including amitriptyline and imipramine) block neuronal uptake of both serotonin and norepinephrine (Beasley *et al.*, 1992). Clomipramine is on the other hand relatively selective against serotonin (Gallanosa *et al.*, 1981). TCAs with secondary amine side chains or the N-demethylated metabolites of agents with tertiary amine moieties (e.g. desipramine, nordoxepin, and nortriptyline) are relatively selective inhibitors of the norepinephrine transport system (Rang *et al.*, 2003).

1.6.3.3 Selective Serotonin Reuptake Inhibitors (SSRIs)

Selective serotonin reuptake inhibitors (SSRIs) are the dominant class of antidepressants prescribed. They are not more effective than other classes of antidepressants but most patients show optimal benefit from one daily dose of any SSRI and they have a relatively benign side effect profile (Gitlin, 2007). SSRIs commonly block the serotonin transporter, responsible for reuptake of serotonin back into the presynaptic neuron. The functional consequence of blocking serotonin reuptake is an increase in serotonin levels and enhancement of serotonin receptor activity and neurotransmission. The strength of serotonin activity at serotonin receptors is inversely proportional to the number of serotonin transporter molecules present at the presynaptic membrane (Gitlin, 2007). Even though the mechanism by which SSRIs ameliorate depression is far more complex than this, the initial enhancement of serotoninergic function by reuptake blockade is generally thought to be the first step in a cascade of biological effects. The resultant increase in synaptic concentrations of serotonin stimulates a great number of postsynaptic 5-HT receptor types (Azmitia *et al.*, 1995). Stimulation of 5-HT_3 receptors is suspected to contribute to common adverse effects including gastrointestinal effects (nausea and vomiting). This improves with reduction in dose, taking of drug after food and use of 5-HT3 receptor blockers such as cisapride (Vaswani *et al.*, 2003). Stimulation of 5-HT_{2C} receptors may result in agitation or restlessness (Azmitia *et al.*, 1995). These may resolve with dose reduction (Vaswani *et al.*, 2003). Repeated administration of serotonin reuptake inhibitors results in some complex late adaptations. These may include indirect enhancement of norepinephrine output by reduction of tonic inhibitory effects of 5-

HT_{2A} heteroceptors (Azmitia *et al.*, 1995). The most important advantage of the SSRIs is the absence of severe adverse effects and death from accidental overdose (Vaswani *et al.*, 2003).

Fluoxetine and its major metabolite norfluoxetine are examples serotonin transport inhibitors. Others include citalopram and sertraline (Azmitia *et al.*, 1995).

1.6.3.4 Atypical antidepressants

Atypical antidepressants represent a group with diverse mechanisms of action. They may interfere only weakly or not at all with monoamine reuptake (e.g.trazodone and bupropion), specifically block reuptake of norepinephrine (reboxetine), or dually inhibit 5-HT and norepinephrine reuptake (venlafaxine) (Horst and Preskorn, 1998). Venlafaxine is probably as effective as tricyclic antidepressants in severe depression. Mirtazapine and mianserin are structural analogs of serotonin and potently antagonize effects at several postsynaptic serotonin receptor types (including 5-HT_{2A}, 5-HT_{2C}, and 5-HT_3 receptors) (Golden *et al.*, 1998).

1.6.3.5 Herbal antidepressants

The most common and accepted herbal antidepressant is St John's wort (*Hypericum perforatum*) (Butterweck, 2003). Several early *in vitro* experiments with St John's wort have focused on pathways that alter monoamine neurotransmission in the CNS. Initial reports have suggested its main mechanism of action involves inhibition of monoamine oxidase (MAO)–the enzyme that is responsible for the catabolism of biogenic amines. The inhibitory effect on MAO by the extract has been confirmed (Sparenberg *et al.*, 1993). Other authors have shown that St John's wort inhibits serotonin and catecholamine reuptake (Muller and Rossol, 1994; Perovic and Muller, 1995; Butterweck *et al.*, 2002). St John's wort has also been shown to influence activity of the HPA-axis; delayed changes in the CRH gene transcription have been studied (Bath and Butterworth, 1996; Butterweck *et al.*, 2001).

1.6.4 Animal models used to screen antidepressants

1.6.4.1 Forced Swimming Test (FST)

When rodents are forced to swim in a narrow space from which there is no escape, they adopt, after an initial period of vigorous activity— an immobile state, moving only when necessary in order to keep their heads above the water. This immobility posture has been hypothesized showing that they had learned that escape was impossible and therefore given up hope. Immobility is referred to as "behavioural despair". A wide range of antidepressant drugs have been found to reduce this immobile state making this simple behavioural procedure a useful test for screening novel antidepressants (Borsini and Meli, 1988; Porsolt *et al.*, 2001a).

1.6.4.2 Tail Suspension Test (TST)

Suspending the animal by the tail produces an immobile state, similar to that in the FST, after previous vigorous movements in an attempt to escape. Like the forced swimming test, immobility is reduced by a wide variety of antidepressants (Steru *et al.*, 1985). However, this procedure has some advantages over the forced swimming procedure. For instance, it is less stressful to the experimental animal in that no hypothermia is induced and the animals, once removed from the experiment, resume normal spontaneous activity immediately. Therefore, no special post-experimental treatment (rubbing down or maintenance in a warmed environment) is required. Simultaneous testing of several animals is possible when the system is automated. Another characteristic of the tail suspension procedure is that it shows a different spectrum of pharmacological sensitivity from the forced swimming test, consequently providing a complementary approach to the behavioural screening of antidepressant and other psychotropic activity (Porsolt *et al.*, 1977; Porsolt *et al.*, 1991; Porsolt *et al.*, 2001a).

1.7 EPILEPSY AND PAIN

1.7.1 Background

The International Association for the Study of Pain (IASP) considers pain to be an unpleasant sensory and emotional experience associated with potential or actual tissue damage, or described in terms of such damage, or both (Merskey, 1994; Evans, 2007). Pain often may be the only symptom for the diagnosis of several diseases and hence has a protective function. Pain is highly subjective as patients are the only ones able to describe the intensity and quality of the pain (Clancy and McVicar, 1992; Evans, 2007). Pain can be disabling thus its control is one of the most important therapeutic priorities (Rang *et al.*, 2003). Pain differs from nociception. Nociception involves the encoding processes of noxious stimuli by the central nervous system.

Pain can be described as nociceptive if the pain is due to tissue injury which activates the nociceptive system. Nociceptive pain apparently occurs as a result of the activation of the sensory system by persistent noxious stimuli, involving processes of transduction, transmission, modulation as well as perception (Evans, 2007).

Nociceptive pain can be acute or chronic. Acute pain is also referred to as adaptive pain, since it helps in protecting the individual from further injury. It is usually self-limiting. It is associated with injury or surgery and is of short duration, lasting less than 3 to 6 months. It is chronic when it lasts longer than 6 months. Pain does not necessarily reflect tissue damage. It may persists beyond the point at which healing would be expected to be complete (DeLeo, 2006). Chronic pain might also result from malignancy including the pain of cancer, multiple sclerosis, sickle cell disease or of a non-malignant nature which includes the pain associated with various neuropathic and musculoskeletal disorders such as fibromyalgia and osteoarthritis (DeLeo, 2006).

Pathophysiological pain occurs due to inflamed or injured tissue appearing as hyperalgesia or allodynia. This may be effectively treated with non-steroidal anti-inflammatory drugs (NSAIDS) and opiates (Schaible and Richter, 2004). Pain as a result of neuronal injury or neuropathic pain in the peripheral or central nervous system is better managed by anticonvulsants or tricyclic antidepressants (Schaible and Richter, 2004). Even though neuropathic pain could be strongly influenced by ongoing

tissue injury, there is an assumption that the primary mechanisms sustaining the pain are independent of any tissue injury (Evans, 2007).

1.7.2 Animal models of pain

Many models of pain are available for investigating the antinociceptive activity of drugs. These include chemical and thermal methods in rodents. The formalin test is the most commonly used and involves subcutaneous injection of formalin solution. It is the most predictive of acute pain (Le Bars *et al.*, 2001).

Subcutaneous, unilateral intraplantar administration of formalin into the rodent paw generates a biphasic nocifensive behavioural response (Ellis *et al.*, 1998). The early phase consists of intense flinching, licking and biting of the injected paw and lasts up to 10 minutes. It is considered to be the neurogenic phase. The late phase of licking and biting however occurs from 11 to 60 minutes after injection (Duboisson and Dennis, 1977). The late phase is thought to be a state of facilitated pain response (hyperalgesia) associated with inflammation. The formalin behavioural test involves sensitization of sensory neurons of the spinal dorsal horn as a result of injury or intense artificial activation of C-fibre afferents (Woolf and Wall, 1986).

Acetic acid writhing test is considered as a model of visceral pain and involves intraperitoneal administration of 0.9% acetic acid into rodents. This induces an abdominal constriction response (writhing). Frequency and duration of this response are counted within a 30 minute period (Ito *et al.*, 2001).

1.8 JUSTIFICATION OF WORK

Epilepsy affects about 20-50 million people worldwide and is more common in children than adults. Epilepsy is the second most common neurological disorder, after stroke with 0.5 % prevalence, and a 2–3 % life time risk of being given a diagnosis of epilepsy (Browne and Holmes, 2001; Pitkanen and Lukasiuk, 2009). Considerable progress in the pharmacotherapy of epilepsy has been made in recent years with the introduction of newer and more effective AEDs (McCabe, 2000). However, about one

third of patients with epilepsy are still resistant to current AEDS and this proportion is quite high in the developing countries. AEDs do not seem to affect the progression or underlying natural history of epilepsy (Loscher, 2002a).

Furthermore, a significant number of epilepsy cases in Africa are managed with older generations of antiepileptic drugs due to high cost of newer and more effective ones. Quite a number of patients continue to resort to the use of traditional medicine. Interestingly, the use of some of these remedies may be based solely on community knowledge of existence and application but not necessarily as a result of scientific validation (Zhang, 2000). There is therefore, a need for continual research into the development of newer and more effective agents for the management of this disorder. Plant sources have often served as effective means of obtaining lead compounds. In Ghana, the aqueous extract the stem bark *of Antiaris toxicaria* is used to manage epilepsy (Mshana *et al.*, 2001) and may possess great potential in epilepsy pharmacotherapy. As a result, this study aims to investigate the possible anticonvulsant and other related neuropharmacological effects of the aqueous stem bark extract of *Antiaris toxicaria.* These findings would in turn provide pharmacological evidence for the traditional use of the plant in the management of epilepsy.

1.9 AIMS OF THE STUDY

The aim of the study is to investigate the anticonvulsant and related neuropharmacological effects of the aqueous stem bark extract of *Antiaris toxicaria.*

1.9.1 SPECIFIC OBJECTIVES

The specific objectives of the thesis include to:

- Carry out preliminary screening on the aqueous extract of the stem bark using
    - Phytochemical screening (Harborne, 1998; Trease and Evans, 2002)
    - Irwin Test (Irwin, 1968; Williams *et al.*, 2007)

- Activity Meter Test (Anon, 2000)
- Beam Traversal Test (Carter *et al.*, 2001; Meredith and Kang, 2006)
- Pentobarbitone Interaction Test (Paton and Pertwee, 1972)
- Tail Withdrawal Test (Janssen *et al.*, 1963; Steinmiller and Young, 2008)
- Convulsive Threshold Test (PTZ-induced seizures) (Vellucci and Webster, 1984)

- Evaluate the anticonvulsant activity of the aqueous stem bark extract in
  - acute animal models of epilepsy
    - Pentylenetetrazole-induced seizure model (Vellucci and Webster, 1984)
    - Picrotoxin -induced seizure model (Vellucci and Webster, 1984)
    - Maximal electroshock test (Loscher *et al.*, 1991)
    - Strychnine -induced seizure model (Bogdanov *et al.*, 1997)
    - 4-aminopyridine-induced seizure model (Morales-Villagran *et al.*, 1999)
  - chronic animal models of epilepsy
    - Pentylenetetrazole –induced kindling (Malhotra and Gupta, 1997)
    - Pilocarpine status epilepticus (Cavalheiro *et al.*, 1991; Loscher, 2002a)
    - Kainate status epilepticus (Cilio *et al.*, 2001)
- Evaluate central effects of the extract using
  - Anxiety related models including
    - Elevated plus–maze test (Pellow *et al.*, 1985)
    - Light–dark box test (Crawley, 1981; Belzung *et al.*, 1987; Belzung and Le Pape, 1994)
    - Social interaction test (de Angelis and File, 1979)

- Depression related models including
    - Forced swimming Test (Porsolt *et al.*, 1977)
    - Tail Suspension Test (Steru *et al.*, 1985)
    - Rotarod Test (Dunham and Miya, 1957)

- Evaluate the extract for analgesic activity using
    - Formalin Test (Malmberg and Yaksh, 1995)
    - Acetic acid induced writhing assay (Amresh *et al.*, 2007)

- Carry out general toxicity studies (OECD, 2008).
    - Acute and sub acute toxicity studies; evaluation of effect of extract on body weight, haematological indices and biochemical parameters.
    - Histopathology and relative weights of the liver, stomach, kidney, spleen, brain and spinal cord.

Chapter 2

PLANT COLLECTION AND EXTRACTION

2.1 PLANT COLLECTION AND EXTRACTION

2.1.1 Plant material

The bark of *Antiaris toxicaria* was collected from the KNUST campus, Kumasi (6 º 41'6.4"N, 1º 33'42.8"W), Ghana in March, 2010. Authentication was done at the Department of Herbal Medicine, KNUST where a voucher specimen (KNUST/HM1/011/S007) has been retained.

2.1.2 Preparation of aqueous extracts

The *Antiaris* bark was air-dried at room temperature (28ºC) and powdered. Four hundred and thirty-one (431) gram powder was macerated with cold distilled water for five days. The filtrate was concentrated under reduced temperature (60 ºC) and pressure in a rotary evaporator. It was then oven-dried at 60ºC to obtain *Antiaris* Aqueous Extract (AAE). A yield of 23.40% $^{w}/_{w}$ was obtained.

2.2 PHYTOCHEMICAL TESTS

Antiaris toxicaria was tested for the presence of tannins, alkaloids, triterpenoids, flavonoids, general test for glycosides (reducing sugars), anthracene glycosides, steroids and saponins by simple quantitative and qualitative methods.

2.2.1 Tannins

About 0.5 g of the powdered material or extract was boiled with 25 ml of water for 5 minutes. It was cooled, filtered and the volume adjusted to 25 ml. 10ml of water was added to 1 ml of the filtrate and then 5 drops of 1% lead acetate. The colour and amount of precipitate was noted and recorded. The procedure was repeated using 5 drops of 1% ferric chloride (Odebiyi and Sofowora, 1978).

2.2.2 Alkaloids

The powdered material or extract was extracted with 30 ml of ammoniacal alcohol (ammonia/alcohol 1:9) and filtered. The filtrate was then evaporated to dryness and the residue extracted with 1% H_2SO_4. This was then filtered and the filtrate rendered alkaline with dilute ammonia solution. The alkaline solution of the extract was then put in a separating funnel and partitioned with chloroform. The chloroformic layer was then separated and evaporated to dryness. The residue was again dissolved in 1% H_2SO_4 and few drops of dragendorff's reagent added. An orange precipitate indicated the presence of alkaloid (Harborne, 1998).

2.2.3 Triterpenoids (Salkowski test)

The powdered material or extract was warmed in 5 ml chloroform. The chloroform solution was then treated with a small volume of concentrated sulphuric acid and mixed properly. A reddish brown coloration of the interface showed a positive result for the presence of triterpenoids (Harborne, 1998).

2.2.4 Flavonoids

The powdered material or extract was dissolved in 15ml of ethanol (98%).To the ethanolic extract, a small piece of zinc metal was added, this was followed by drop wise addition of concentrated hydrochloric acid. Colours ranging from orange to red indicated flavones, red to crimson indicated flavonols, crimson to magenta indicated flavonones (Odebiyi and Sofowora, 1978).

2.2.5 General test for glycosides

About 200 mg of the powdered material or extract was warmed with 5 ml dilute H_2SO_4 on a water bath for 2 minutes. It was then filtered and the filtrate rendered distinctly alkaline with 2 to 5 drops of 20% NaOH. 1 ml each of fehlings solution A and B was then added to the filtrate and heated on the water bath for 2 minutes. A brick red precipitate indicated the presence of glycosides (Harborne, 1998).

2.2.6 Anthracene glycosides

The powdered material or extract was boiled with dilute H_2SO_4 for five minutes and filtered whilst still hot and the filtrate allowed to cool. The filtrate was then mixed with an equal volume of chloroform in a separation funnel. The chloroformic layer was separated and dilute NH_3 added and observed (Sofowora, 1993; Harborne, 1998).

2.2.7 Saponins

A small amount (0.2 g) of the powdered material or extract was shaken with a few ml of water in a test tube and the mixture observed for the presence of a froth which does not break readily upon standing (Trease and Evans, 1983; Trease and Evans, 2002).

2.2.8 Steroids (Liebermann-Burchard test)

2 ml of acetic acid was added to 0.2 g of the powdered material or extract. The solution was cooled well in ice followed by the addition of conc. H_2SO_4 carefully. Colour development from violet to blue or bluish-green indicated the presence of a steroidal ring i.e. aglycone portion of cardiac glycoside (Sofowora, 1993).

2.3 RESULTS

2.3.1 Phytochemical Tests

Phytochemical analysis of the stem bark of *Antiaris toxicaria* powder and extract revealed the presence of the anthracene glycosides, tannins, flavonoids, alkaloids, saponins, reducing sugars and triterpenoids.

Table 2.1 Phytochemical analysis of *A. toxicaria* powder and extract

TEST	***RESULTS***	
	POWDER	***EXTRACT***
Anthracene glycosides	+	+
Tannins	+	+
Alkaloids	+	+

Triterpenoids	+	+
Flavonoids	+	+
Saponins	+	+
Steroids	-	-

+/- indicates constituent is present or absent respectively

2.4 DISCUSSION

Phytochemical analysis of the powdered stem bark and extract of *Antiaris toxicaria* revealed the presence of the anthracene glycosides, tannins, flavonoids, alkaloids, saponins, reducing sugars and triterpenoids. Steroids were absent. Many authors have shown that the presence of biologically active secondary metabolites is responsible for the therapeutic effects of certain plant extracts (Singh *et al.*, 2002; Agarwal and Rangari, 2003). For instance constituents of *Picralima nitida* (family: Apocynaceae) have been shown to have hypotensive, skeletal muscle relaxant, antiplasmodial activity, anti-inflammatory and analgesic activity (Duwiejua *et al.*, 2002; Woode *et al.*, 2006; Okokon *et al.*, 2007). Cardiac glycosides were absent in this extract though many authors have isolated and identified various steroidal glycosides in the latex of *A. toxicaria*.

Of special interest was the presence of triterpenoids. Chauhan *et al.* in 1988 evaluated some triterpenoids for anticonvulsant activity and showed that they are able to confer up to 40 % protection against seizures in the PTZ-induced seizure test. As the test for steroids came out negative, it may be concluded that the saponins present have a pentacyclic nucleus and are therefore of the triterpenoid type.

2.5 CONCLUSION

The aqueous extract of the stem bark of *Antiaris toxicaria* contains anthracene glycosides, tannins, flavonoids, alkaloids, pentacyclic saponins and reducing sugars. The biological activities reported in this thesis are therefore due to the total effect of all constituents of the crude extract.

Chapter 3

PRELIMINARY SCREENING FOR NEUROPHARMACOLOGICAL EFFECTS

3.1 INTRODUCTION

The present study was undertaken to investigate the CNS activity of *Antiaris toxicaria* extract in rodents. Methods employed in this study were adapted from the core battery of assessment of the central nervous system as proposed by the International Conference on Harmonization (ICH) S7A Guideline for Safety Pharmacology (Anon, 2000). The guideline recommends the testing of novel compounds on the central and peripheral nervous system and on the cardiovascular system as part of the "core battery" of assessment (Williams *et al.*, 2007). Rodents are mainly the species of choice for detecting behavioural and neurological effects. The mouse shares many anatomical, cellular, biochemical and molecular features with man. Other functions, such as memory, sexual behaviour and emotional responses are also similar. Based on these similarities, murine models are therefore employed to approximate human behavioural responses in disease states (Van Meer and Raber, 2005). Hence, this study involved the use of *in vivo* methods in freely moving conscious animals.

General behavioural observation, measures of spontaneous activity, locomotor activity, pain and convulsive thresholds in addition to interaction with hypnotics were assessed.

3.2 MATERIALS AND METHODS

3.2.1 Animals

ICR mice (20–25 g) were obtained from the Noguchi Memorial Institute for Medical Research. Animals were kept in the Departmental Animal House and allowed to acclimatize to laboratory conditions before the study. All animals were treated according to the Guide for the Care and Use of Laboratory Animals (NRC, 1996) and experiments were approved by the Faculty Ethics Committee.

3.2.2 Drugs and chemicals

Caffeine (CFN), diazepam (DZP), pentobarbitone (PBT), pentylenetetrazole (PTZ) and phenobarbitone (PHE) were purchased from Sigma-Aldrich Inc., St. Louis, MO, USA. Morphine hydrochloride (MOR) was obtained from Phyto-Riker Pharmaceuticals Limited, Accra, Ghana.

3.2.3 Irwin Test

AAE was administered orally to male ICR mice at doses of 100-3000 mg kg^{-1} body weight in groups of seven. Behavioural, neurological and autonomic statuses were evaluated in each animal at 0, 15, 30, 60, 120 and 180 min, up to 48 hours after treatment (Irwin, 1968; Williams *et al.*, 2007).

3.2.4 Activity Meter Test

The mouse activity cage (model 7401, Ugo Basile, Comerio, *VA*, Italy) was used in this test. Animals were first pretreated with either AAE (100-3000 mg kg^{-1}, *p.o*) or diazepam (6 mg kg^{-1}, *p.o*) or caffeine (18 mg kg^{-1}, *p.o.*). ICR mice were placed individually in the activity cage 60 min after AAE, diazepam or caffeine treatment. Activity was observed every 5 minutes for 30 minutes.

3.2.5 Beam Traversal Test

Animals were randomly divided into seven groups consisting of five mice each and treated with extract (300-3000 mg kg^{-1}, *p.o.*) or diazepam (0.1, 0.3 and 1.0 mg kg^{-1}, i.p). Animals were trained to traverse the beam (three consecutive trials each day for three days) to the goal box in less than 30 seconds. Mice that could not achieve the goal were excluded from the study. During the test, mice were placed at the start end of the beam and allowed sixty seconds to traverse the beam. Test sessions were recorded with a video camera and analysed for total number of steps, time to traverse and stepping errors. A score of sixty seconds was awarded to animals which could not cross the beam or fell off (Carter *et al.*, 2001; Meredith and Kang, 2006).

3.2.6 **Pentobarbitone Interaction Test**

Animals were randomly divided into eighteen groups comprising five mice each and treated with extract (300-3000 mg kg^{-1}, *p.o*), diazepam (8 mg kg^{-1}, i.p) or caffeine (16 mg kg^{-1}, i.p). Thirty minutes later, pentobarbitone (50 mg kg^{-1}, i.p) was administered to each mouse to induce sleep. Mice were observed for the latency to sleep (time between pentobarbitone administration to loss of righting reflex) and duration of sleep (time between loss and recovery of righting reflex). Other groups of animals were pretreated with phenobarbitone (25 mg kg^{-1}, i.p) for two days prior to the testing day to investigate the effect of hepatic enzyme induction on sleeping time. Those animals were treated in the same manner as the naïve animals during testing (Paton and Pertwee, 1972).

3.2.7 **Tail Withdrawal Test**

The test was carried out according to the method described by Janssen *et al.*, 1963 and Steinmiller and Young, 2008 with slight modifications. Tail withdrawal latency was defined as the time (in seconds) to withdraw the tail from hot water maintained at 50.0± 1.0 °C. A cut-off latency of 10 s was set to avoid tissue damage. Increase in tail withdrawal latency was the measure of anti-nociception. It was calculated as

$$\%\ Maximal\ Possible\ Effect\ (MPE) = \frac{[(T_1 - T_0)]}{[(T_2 - T_0)]} \times 100$$

where T_0 and T_1 are defined as the latencies obtained before and after drug treatment respectively, and T_2 is the cut-off latency.

The maximum possible anti-nociceptive effect was awarded to animals that did not show a tail withdrawal reaction within 10 s. Animals were tested at single time points of 60 min after administration of AAE (100–1000 mg kg^{-1}, *p.o.*) and morphine (32-128 mg kg^{-1}, *p.o*).

3.2.8 **Convulsive Threshold Test (PTZ-induced seizures)**

Mice were divided into 7 groups (n=7). AAE was administered orally at 30-3000 mg kg^{-1}. Other mice received diazepam (0.3-3.0 mg kg^{-1}, i.p.) while the control group received distilled water (10 ml kg^{-1}). Seizures were induced with pentylenetetrazole

(85 mg kg^{-1}, s.c.) 30 min after distilled water or diazepam and 1 h after AAE. The mice were then observed via video recording for the frequency, duration of and latency to clonic convulsions for 1 h.

3.2.9 **Analysis of data**

Data was presented as mean±S.E.M and significant differences between means were determined by one-way analysis of variance (ANOVA) with Newman-Keuls' *post hoc* test. In the pentobarbitone interaction test, effect of treatment was determined by two-way ANOVA (Dose × treatment) followed by Bonferroni test. Graph Pad Prism® Version 5.0 (GraphPad Software, San Diego, CA, USA) was used for all statistical analyses. In all cases, $P < 0.05$ was considered significant.

3.3 **RESULTS**

3.3.1 **Irwin Test**

Acute dosing between 300-3000 mg kg^{-1} produced Straub tail effect in the mice. Only the 1000 and 3000 mg kg^{-1} doses produced analgesia to tail pinch test. These effects were not present, however, after 24 h of treatment. All other parameters showed no changes and there were no deaths recorded in the Irwin test (Table 3.1).

Table 3.1 Effects of *Antiaris toxicaria* aqueous extract in the Irwin Test

	MORTALITY	EFFECTS
DOSE (mg kg^{-1})	D/T	
0	0/7	No change
100	0/7	No change
300	0/7	Straub tail at 15′→120′
1000	0/7	Straub tail and Analgesia at 15′→120′
3000	0/7	Straub tail and Analgesia at 15′→120′

D=Deaths recorded; T=Total number of animals observed.

3.3.2 **Activity Meter Test**

AAE showed significant (*P< 0.01, $F_{4, 20}$= 26.61*; Figure 3.1 b) decrease in locomotor activity at 100 mg kg^{-1}, and an increase at all other doses used mimicking effects of both diazepam and caffeine. Diazepam (6 mg kg^{-1}, *p.o.*) significantly *(P<0.001, $F_{2, 12}$= 136.3*; Figure 3.1 b*)* decreased spontaneous activity while caffeine (18 mg kg^{-1}, *p.o.*) increased it.

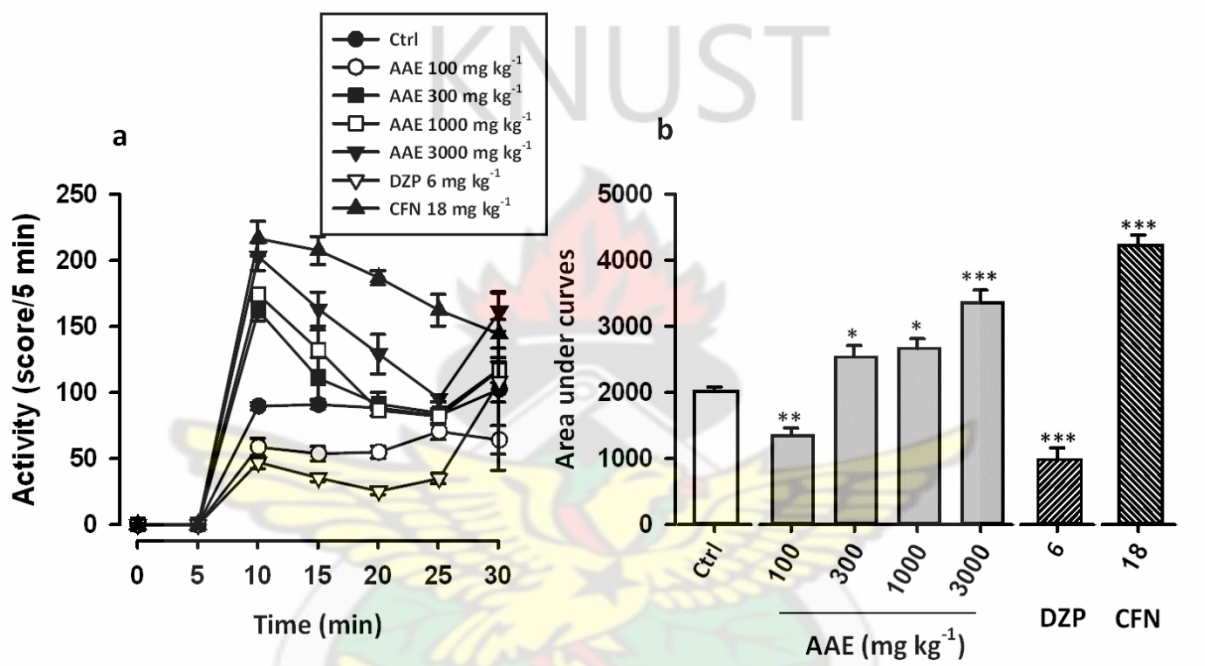

Figure 3.1 Effects of acute AAE (100-3000 mg kg^{-1}, *p.o*), diazepam (6 mg kg^{-1}, *p.o.*) and caffeine (18 mg kg^{-1}, *p.o.*) treatment in the activity meter test. Data are presented as group mean±SEM (n=8). **P<0.05, **P<0.01, ***P<0.001* compared with vehicle-treated group (One-way ANOVA followed by Newman-Keuls' *post hoc* test).

3.3.3 **Beam Traversal Test**

No stepping errors were observed for all treatment groups in this test. The extract exhibited no significant change in the time taken to traverse the beam as well as the total number of steps as compared to the control. Diazepam however at dose of 1 mg kg^{-1} showed a significant increase in both the time it took animals to traverse the beam (*P =0.0123, $F_{3, 16}$=5.009*, Figure 3.2 a) and the total number of steps taken (*P =0.0048, $F_{3, 16}$=6.350*, Figure 3.2 b*).*

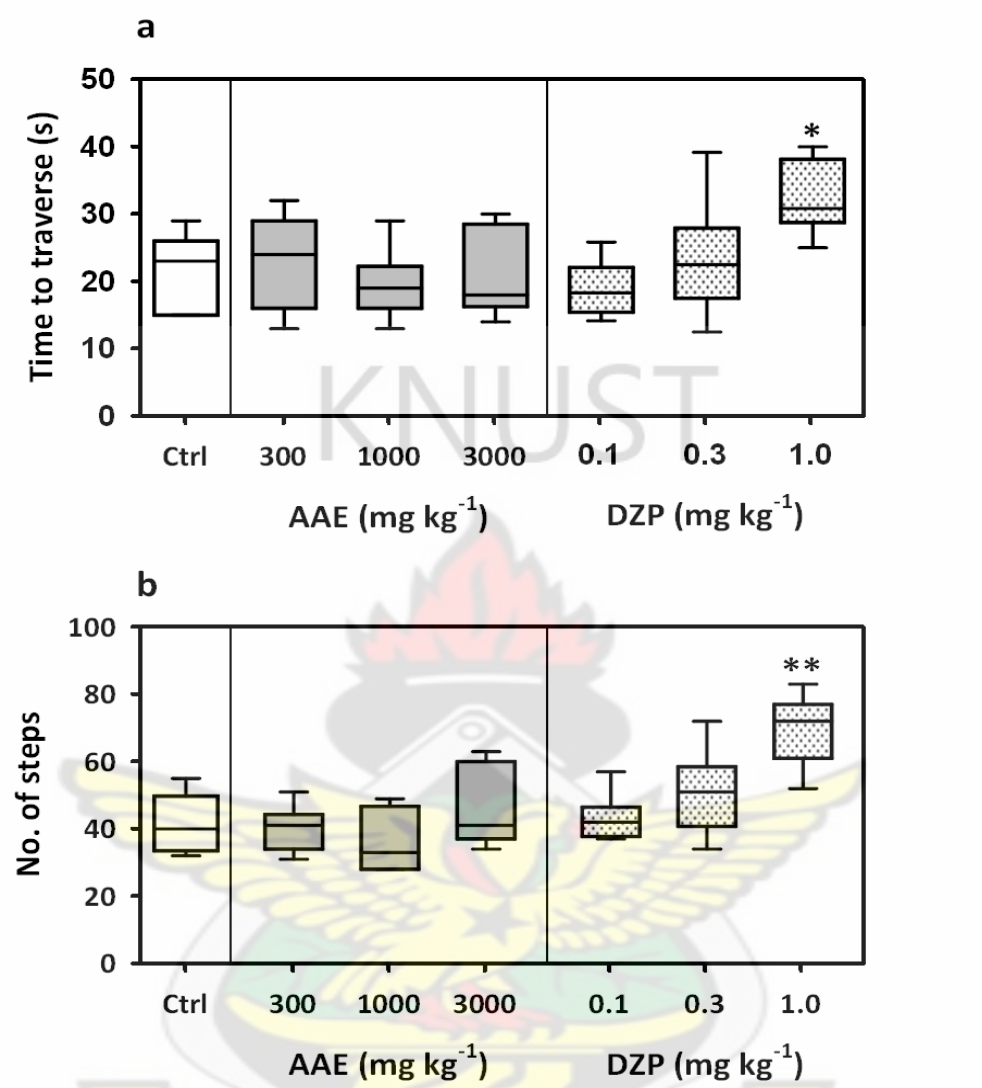

Figure 3.2 Effects of AAE (300, 1000 and 3000 mg kg^{-1}, *p.o*) and diazepam (0.1, 0.3 and 1.0 mg kg^{-1}, i.p) on the traversal time (a) and total number of steps taken (b) in the beam traversal test. Data are presented as group mean±SEM (n=5).The lower and upper margins of the boxes represent the 25th and 75th percentiles, with the extended arms representing the 10th and 90th percentiles respectively. The median is shown as a horizontal line within the box. *$P<0.05$, **$P<0.01$ compared to vehicle-treated group (One-way analysis of variance followed by Newman-Keuls' *post hoc* Test).

3.3.4 **Pentobarbitone Interaction Test**

In the pentobarbitone interaction test, AAE significantly ($P<0.001$, $F_{1,8}=211.97$ [Two-way ANOVA]; Figure 3.3 a) prolonged the duration of sleeping time in test animals as compared to control. The onset of sleep was also significantly affected as it was

reduced by 43.59% compared to saline-treated animals. Pretreatment with phenobarbitone resulted in significant ($P < 0.0001$, $F_{1,\ 8} = 39.8$ [Two-way ANOVA]; Figure 3.3 b) decreases in the duration of sleep at dose levels 1000 and 3000 mg kg^{-1}. Onset of sleep was not affected significantly by pretreatment with phenobarbitone. Sleep induced by diazepam was not significantly affected by either pentobarbitone or phenobarbitone. Sleep onset of caffeine was significantly ($P<0.01$) decreased by pentobarbitone administration but phenobarbitone pretreatment had no effect.

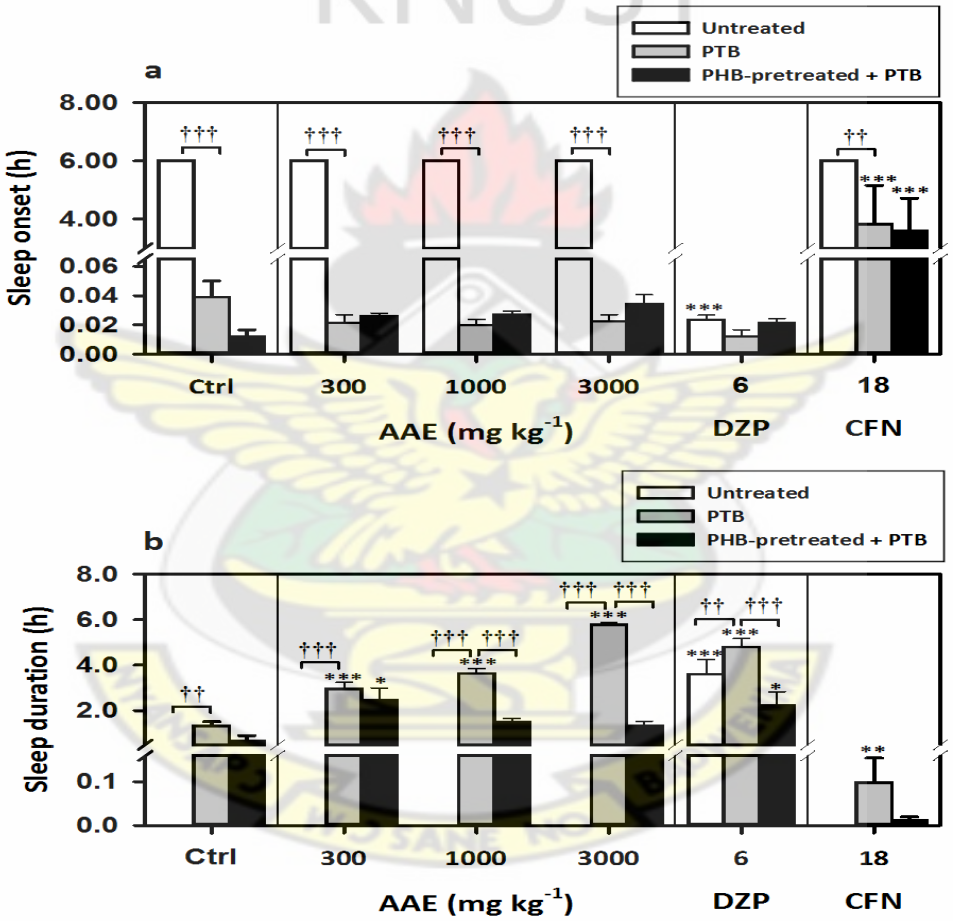

Figure 3.3 Effects of acute AAE (300, 1000 and 3000 mg kg^{-1}, *p.o*), diazepam (8 mg kg^{-1}, i.p) and caffeine (16 mg kg^{-1}, i.p) in the Pentobarbital Interaction Test. Data are presented as group mean±SEM (n=5). **P<0.05, **P<0.01 ***P<0.001* compared with control (One-way analysis of variance followed by Newman-Keuls' *post hoc* Test) and ††*P<0.01*, ††† *P<0.001* (two-way ANOVA followed by Bonferroni test).

3.3.5 **Tail Withdrawal Test**

AAE increased tail withdrawal latency significantly (*P=0.0045, $F_{3, 28}$= 5.440*) at all doses in a non-dose dependent manner. Similarly, morphine also showed marked increases (*P<0.001, $F_{3, 28}$= 38.38*) in latency at all doses; effects which were greater than that of the extract (Table 3.2).

Table 3.2 Effect of *Antiaris toxicaria* aqueous extract in the Tail withdrawal test.

	Dose (mg kg^{-1})	% Maximal Possible Effect
Control		-20.27±15.29
AAE	100	48.52±14.53*
	300	48.80±15.49**
	1000	44.27±12.44**
Morphine	32	31.89±10.81***
	64	99.40±00.60***
	128	99.39±00.61***

Data presented as mean±S.E.M (n=10). *P<0.05, **P<0.01, *** P<0.001 compared to control (Newman-Keuls' *post hoc* test).

3.3.6 **Convulsive Threshold Test (PTZ-induced seizures)**

Onset of clonic convulsions were delayed by AAE significantly (*P<0.0001, $F_{5, 36}$= 7.664*; Figure 3.4a.) in mice. The frequency of convulsions likewise was reduced significantly (*P<0.0001, $F_{5, 36}$=11.46*; Figure 3.4 a.) in addition to the duration of clonic convulsions (*P<0.0001, $F_{5, 36}$=8.043*; Figure 3.4 b). The standard drug diazepam (0.3-3.0 mg kg^{-1}) also significantly reduced and abolished convulsions.

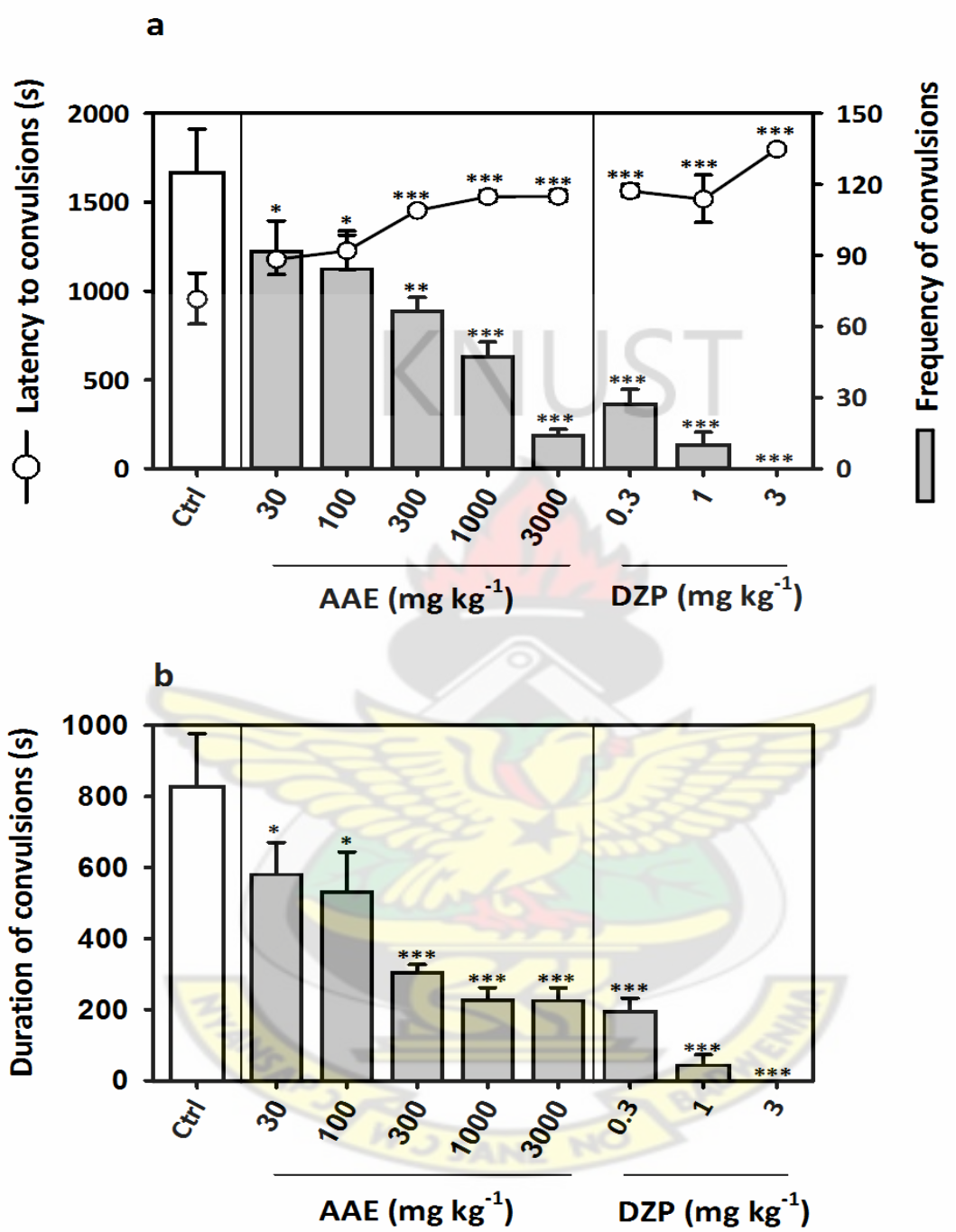

Figure 3.4 Effects of AAE (30-3000 mg kg^{-1}, *p.o.)* and diazepam (0.3, 1.0 and 3.0 mg kg^{-1}, i.p.) on the latency and frequency of seizures (a) and duration of convulsions (b) in PTZ-induced seizures. Data are presented as mean±SEM. **P*<0.05, ***P*<0.01, ****P*<0.001 compared to vehicle-treated group (One-way analysis of variance followed by Newman-Keuls' *post hoc* test).

3.4 DISCUSSION

Investigations carried out on the aqueous extract of *Antiaris toxicaria* showed that it possesses CNS depressant activity and analgesia at high doses without muscle relaxant properties. It is also metabolized by hepatic enzymes and inhibits PTZ-induced convulsions.

In the Irwin test the extract exhibited potential analgesic properties to tail pinch responses as well as a Straub tail effect. The Irwin test involves systematic observational methods for assessing effects of drugs on the behaviour and physiology of rodents. It is a component of the basic protocols satisfying ICH (International Conference on Harmonization) recommendations for safety pharmacology studies. It was first described by Irwin (1968). It helps one detect potential adverse effects of drugs on the central nervous system (CNS) prior to clinical testing and may also be helpful in revealing novel therapeutic effects (Irwin, 1968; Porsolt *et al.*, 2002; Williams *et al.*, 2007). The Straub effect is often measured in response to opioids and has been shown to be mediated by the μ_2-receptor (Nath *et al.*, 1994; Houshyar *et al.*, 2000). Other agonists have also been shown to be able to produce the said response by other mechanisms such as nicotinic and serotoninergic receptor activation (Evangelista de Duffard *et al.*, 1995; Zarrindast *et al.*, 2001; Fonck *et al.*, 2003; Diaz and Maroteaux, 2011). This suggests possible opioidergic, serotoninergic or even nicotinic mechanisms of action. Since no deaths were recorded, the LD_{50} may be estimated to be above 3000 mg kg^{-1}.

Pentobarbitone is a hypnotic at appropriate doses. Its sedative or hypnotic effect is by potentiation of GABA-mediated postsynaptic inhibition at GABA receptors (fFrench-Mullen *et al.*, 1993; Brust, 2004). Potentiation of pentobarbitone-induced hypnosis is an indication of central depressant activity (Fujimori, 1965). Such substances either decrease the time for onset of sleep and/or prolong the duration of sleep. Diazepam was used as the positive control. It is a hypnotic belonging to the benzodiazepine group. The extract most likely possesses depressant action on the CNS similar to that of diazepam. Pretreatment with phenobarbitone for two days prior to testing induced liver metabolising enzymes (Ioannides and Parke, 1975; Whysner *et al.*, 1996; Kushikata *et al.*, 2003). Results indicate that the duration of sleep produced by the

extract is shortened in the presence of metabolising enzymes. It is a strong indication that the extract might be metabolized by cytochrome-P450 enzymes. This brings to the fore the possibility of drug interactions with other drugs that may be metabolised in the same manner since 60% of drugs are known to be metabolised by cytochrome-P450 enzymes (Zhou *et al.*, 2005; Sweeney and Bromilow, 2006).

Testing for drug effect on motor coordination is a vital step in CNS drug evaluation. The beam traversal test helps to measure skilled walking, fine motor balance as well as coordination skills (Carter *et al.*, 1999; Meredith and Kang, 2006). Thus, increased errors in experiments indicate impaired motor coordination (Meredith and Kang, 2006). The extract showed no significant effect on motor coordination as shown by the results. Benzodiazepines at high doses have muscle relaxant effects (Woods and Winger, 1995; Charney *et al.*, 2001) hence, the effect of diazepam on motor coordination.

AAE however reduced spontaneous locomotor activity in mice in the activity meter test at 100 mg kg^{-1}. Motor impairment by drugs can result in decreased locomotor activity (Porsolt *et al.*, 2002). Since motor impairment was absent in the beam traversal it can be ruled out as the cause of the reduction in locomotor activity observed. Another possible cause of reduced locomotion may be sedation which was not observed at any of the doses in the Irwin test. On the other hand, since the extract exhibited CNS depressant activity in the pentobarbitone interaction test, there may be potential sedative effects (Brown, 1961; Carpenedo *et al.*, 1994). Locomotion tests estimate whether a substance is psychostimulant or sedative. Substances with marked psychostimulant properties are expected to increase locomotor activity (Riviere *et al.*, 1999; Kafkafi *et al.*, 2001; Gentry *et al.*, 2004). Such substances can, however, cause a decrease due to animals rotating rapidly in a small space or showing stereotyped behaviours (Morita *et al.*, 2000; Quinn *et al.*, 2003). The increase in locomotor activity at higher doses may be due to some increased exploratory behaviour that may be evident with anxiolytic compounds such as these benzodiazepines (Turski *et al.*, 1982). Nevertheless, this is in contrast to benzodiazepines that produce increased spontaneous activities at low doses and sedation at much higher doses. Diazepam, a CNS depressant, reduced spontaneous activity (Savic *et al.*, 2003) and impaired motor

coordination at the dose used while caffeine, a CNS stimulant, increased the locomotor activity (Kafkafi *et al.*, 2001; Gentry *et al.*, 2004).

The extract also had a significant effect in the tail withdrawal test. This test is considered to be a model of somatosensory pain (Bjorkman, 1995; Shannon *et al.*, 1997; Kalra *et al.*, 2001) and known to be more sensitive to centrally acting analgesics (Prado *et al.*, 1990; Gupta *et al.*, 2005; Santos *et al.*, 2005). Such agents act to elevate pain threshold of animals towards heat and pressure. Central activity involves spinal and supra-spinal mechanisms which can be conferred on the extract since it exhibited significant activity in this model (Jain *et al.*, 2001; Muhammad *et al.*, 2012).

Finally, the extract produced significant inhibition of PTZ-induced seizures which helps to confirm its traditional use in epilepsy management. Pentylenetetrazole exerts its action by acting as an antagonist at the $GABA_A$ receptor complex (Ramanjaneyulu and Ticku, 1984; Katzung, 2004). GABA is a major inhibitory neurotransmitter in the mammalian central nervous system (Katzung, 2004). Inhibition of pentylenetetrazole-induced seizures is an indication that the effects of *Antiaris toxicaria* may be associated with modulation of GABA activity in the central nervous system.

3.5 CONCLUSION

The overall results from this study show that AAE possesses CNS depressant, analgesic and anticonvulsant activity. It also increases spontaneous activity without motor impairment and its LD_{50} may be above 3000 mg kg^{-1}.

Chapter 4

ANTICONVULSANT ACTIVITY OF THE EXTRACT

4.1 INTRODUCTION

In spite of considerable advancements in the treatment of neurological disorders, epilepsy remains a significant therapeutic challenge (Chindo *et al.*, 2009). Currently available antiepileptic drugs (AEDs) have debilitating adverse effects on cognition and behaviour (Duncan, 2002). This problem is further compounded by polypharmacy which characterizes treatment of epilepsy. These problems are known to prevail more in developing countries due to lack of facilities for proper diagnosis and treatment along with monitoring of AED serum levels.

AEDs in use currently are known to suppress seizures without necessarily affecting the underlying disorder or natural history (Loscher, 2002a). Although not well established, a handful of AEDS have been shown to be antiepileptogenic. Examples of such drugs are pentobarbitone and sodium valproate (Duncan, 2002). There is therefore the need for further research into the possible discovery of drugs which are not only anticonvulsant but also antiepileptogenic. These can either prevent epilepsy or alter its natural course.

Natural products and plants already used in traditional medicine can be a good place to start. Numerous plants used for the treatment of epilepsy traditionally have been shown to be potent in models of epilepsy and several such plants remain to be scientifically validated (Raza *et al.*, 2001). Preliminary screening of the aqueous plant extract (Chapter 3) revealed significant anticonvulsant effect in pentylenetetrazole-induced seizure test (Mante *et al.*, 2012). Hence, this study seeks to further explore the extract's potential as an anticonvulsant.

4.2 MATERIALS AND METHODS

4.2.1 Animals

Male ICR mice and Sprague-Dawley rats weighing between 20-25 g and 120-145 g respectively were obtained from Noguchi Memorial Institute for Medical Research, Accra, Ghana and kept in the Departmental Animal House. Animals were maintained under laboratory conditions of temperature, humidity and light, housed in stainless steel

cages (34 x 47 x 18 cm^3) and allowed free access to water and food *ad libitum.* The animals were assigned to treatment groups of eight to ten animals randomly. All animals were handled in accordance with the Guide for the Care and Use of Laboratory Animals (NRC *et al.*, 1996) and experiments were approved by the Faculty Ethics Committee.

4.2.2 Drugs and chemicals

Diazepam (DZP), pentylenetetrazole (PTZ), picrotoxin (PCT), 4-aminopyridine (4-AP), strychnine (STR)), kainic acid (KA) and pilocarpine (PILO) were purchased from Sigma-Aldrich Inc., St. Louis, MO, USA. Flumazenil was obtained from APP Pharmaceuticals, LLC, Schaumburg, Illinois, USA. Carbamazepine (Tegretol®) was from Novartis, Basel, Switzerland and sodium valproate (Epilim®) from Sanofi-Synthelabo, Paris, France. Carbamazepine (Tegretol®) was from Novartis, Basel, Switzerland and nifedipine (Nifecard XL®) from Lek, Ljubljana, Slovenia.

4.2.3 Acute models

4.2.3.1 *Pentylenetetrazole - induced seizures*

Investigation was carried out using the model as described by Vellucci and Webster in 1984. The plant extract was administered at doses of 200, 400 and 800 mg kg^{-1} body weight orally. Intraperitoneal (i.p.) injection of diazepam (0.1, 0.3 and 1 mg kg^{-1}) was used as reference anticonvulsant drug. Animals were pretreated with the plant extract thirty minutes and diazepam fifteen minutes before administration of pentylenetetrazole (PTZ) 85 mg kg^{-1} subcutaneously. Control animals were pretreated with distilled water (10 ml kg^{-1}, *p.o.*). Anticonvulsant activity was scored based on the ability of AAE to delay the onset of clonic seizures, decrease the total duration as well as frequency of clonic seizures.

4.2.3.2 *Picrotoxin –induced seizures*

Animals received AAE orally at doses of 200, 400 and 800 mg kg^{-1} body weight. Picrotoxin (PCT) was injected intraperitoneally at a dose of 3 mg kg^{-1} fifteen minutes after pretreatment with diazepam and thirty minutes after AAE. Diazepam (0.1, 0.3 and 1 mg kg^{-1}) served as positive control. Control animals received distilled water (10 ml kg^{-1}, *p.o.*). Anticonvulsant activity was scored similar to that in the PTZ test (Vellucci and Webster, 1984).

4.2.3.3 *Maximal Electroshock Test (MEST)*

Tonic convulsions of hind limb extremities of mice were induced using electrical current (50 mA, 60 Hz, 0.2 seconds) via ear clip electrodes. Control group animals received distilled water (10 ml kg^{-1}, *p.o.*). Carbamazepine at doses of 3, 10 and 30 mg kg^{-1} orally served as reference anticonvulsant. AAE was tested at doses of 200, 400 and 800 mg kg^{-1} orally. Convulsions were induced thirty minutes after pretreatment. Latency to and total duration of hind limb tonic extension were recorded (Loscher *et al.*, 1991).

4.2.3.4 *Strychnine-induced convulsions*

This test was carried out as described by Bogdanov *et al.*in 1997. AAE was administered orally at doses of 200, 400 and 800 mg kg^{-1} body weight. Strychnine was injected subcutaneously at a dose of 2 mg kg^{-1} thirty minutes after AAE administration. Diazepam (0.1, 0.3 and 1 mg kg^{-1}, i.p.) served as standard anticonvulsant. Animals were observed via video recording. The ability of AAE to delay the onset of, decrease the total duration plus frequency of tonic convulsions was taken as a measure of anticonvulsant activity.

4.2.3.5 *4–aminopyridine induced convulsions (Involvement of potassium ion channels)*

Animals received AAE orally at doses of 200, 400 and 800 mg kg^{-1} body weight. 4-Aminopyridine was dissolved in normal saline and injected subcutaneously at a dose of 10 mg kg^{-1} body weight thirty minutes after drug treatments. Control animals were pretreated with normal saline (10 ml kg^{-1}) and sodium valproate at 100, 200 and 400 mg kg^{-1} served as positive control. Anticonvulsant activity was scored based on the ability of AAE/drug to delay the onset of hind limb tonic extensions. Protection against lethality within the one hour observation period was also assessed. Method was based on work by Morales-Villagran and Tapia, 1996 with slight modifications. This is a mechanistic model indicative of action on potassium ion channels.

4.2.3.6 *Effect of AAE on $GABA_A$*

In order to investigate the mechanism of action of AAE as an anticonvulsant, mice were treated with flumazenil, a benzodiazepine antagonist (1 mg kg^{-1}, i.p) fifteen minutes before the administration of AAE (400 mg kg^{-1}, *p.o*). Thirty (30) minutes later, convulsions were induced with pentylenetetrazole at 85 mg kg^{-1}, subcutaneously. Other groups of animals received either AAE or flumazenil or diazepam (0.3 mg kg^{-1}, i.p) alone. Animals

were observed for thirty minutes after treatment via video recording for latency and duration of convulsions.

4.2.4 Chronic models

4.2.4.1 *Kindling induction*

PTZ kindling was initiated using a subconvulsive dose of PTZ 40 mg kg^{-1} body weight injected into the soft skin fold of the neck on every second day (i.e. Day 1, Day 3, Day 5. . .). The PTZ injections were stopped when the control animals showed adequate kindling, i.e. Racine score of 5. After each PTZ injection, the convulsive behaviour was observed for 30 min. The resultant seizures were scored as follows: Stage 0 (no response); Stage 1 (hyperactivity, restlessness and vibrissae twitching); Stage 2 (head nodding, head clonus and myoclonic jerks); Stage 3 (unilateral or bilateral limb clonus); Stage 4 (forelimb clonic seizures); Stage 5 (generalized clonic seizures with loss of postural control) (Malhotra and Gupta,1997). AAE was tested at doses of 200, 400 and 800 mg kg^{-1} body weight orally and diazepam (0.1, 0.3 and 1 mg kg^{-1}, i.p). PTZ was injected thirty minutes after administration of test drugs. Control animals received 3 ml kg^{-1} of distilled water.

4.2.4.2 *Pilocarpine-induced Status epilepticus*

Seizures were induced by an i.p. injection of PILO (300 mg kg^{-1}, i.p.) into drug or vehicle-treated male rats. Rats were pretreated with AAE (100-1000 mg kg^{-1}, *p.o.*) or diazepam (0.3-3.0 mg kg^{-1}, i.p.) for 30 or 15 minutes, respectively, before PILO injection. To reduce peripheral autonomic effects produced by PILO, the animals were pretreated with hyoscine *n-butyl*-bromide (1 mg kg^{-1}, i.p.) 30 minutes before PILO administration. Animals were placed in observation cages and observed via video recordings. Latency to and duration of seizures were scored (Cavalheiro *et al.*, 1991; Loscher, 2002a).

4.2.4.3 *Rat Kainate Model*

Animals were pretreated with the plant extract thirty minutes as above before administration of kainic acid (10 mg kg^{-1}, i.p.). Other animals were treated with carbamazepine (30 mg kg^{-1}, *p.o*) and nifedipine (30 mg kg^{-1}, *p.o*) thirty minutes before induction of convulsions. Animals were observed for wet dog shakes over a one hour period (Cilio *et al.*, 2001). Brains were harvested for histopathological examination after an hour. Tissues were fixed in 10% buffered formalin (pH 7.2). Dehydration was done

with a series of ethanolic solutions, embedded in paraffin wax and processed for histological analysis. Coronal sections (2 µm thick) were cut and stained with haematoxylin-eosin for examination. The stained tissues were observed through an Olympus microscope (BX-51) and photographed by a chare-couple device (CCD) camera.

4.2.4.4 *Data analysis*

Data were presented as mean±S.E.M and significant differences between means determined by one-way analysis of variance (ANOVA) followed by Newman-Keuls' *post hoc* test. In the 4-Aminopyridine seizure test, the Kaplan-Meier method was used in estimating survival relative to time and survival differences were analyzed with the log-rank (Mantel Cox) test. Statistical analyses were carried out with Graph Pad Prism® Version 5.0 (GraphPad Software, San Diego, CA, USA) and SigmaPlot® Version 11.0 (Systat Software, Inc.). $P < 0.05$ was considered significant in all cases.

4.3 RESULTS

4.3.1 Acute models

4.3.1.1 *Effects in pentylenetetrazole-induced seizures*

Pentylenetetrazole (85 mg kg^{-1}, s.c) produced myoclonic jerks in all mice pretreated with the distilled water. AAE (200-800 mg kg^{-1}) produced a significant (*P=0.0021;* $F_{3, 16}$*=7.671*; Figure 4.1 a) dose-dependent increase in time taken to the onset of clonic seizures. In the extract-treated animals, frequency of seizures was also decreased non-dose dependently and only the 800 mg kg^{-1} dose was significant (*P=0.0511;* $F_{3, 16}$*=3.213*; Figure 4.1 a). AAE produced significant (*P=0.0018;* $F_{3, 16}$*=8.005*; Figure 4.1 c) dose-dependent decrease in the total duration of convulsions in all animals pretreated with the various doses of the extract. The reference anticonvulsant diazepam (0.1-1.0 mg kg^{-1}, i.p) also profoundly delayed the onset of myoclonic jerks, decreased frequency of jerks and significantly (*P<0.0001;* $F_{3, 16}$*=125.8*; Figure 4.1 b and 1 d) antagonized PTZ-induced seizures. GraphPad® prism analysis of dose-response curves (Figure 4.2) revealed that the extract was more efficacious [E_{max}= 100 %] in reducing the duration of convulsions than in affecting the other parameters. It also exhibited higher potency [ED_{50}: 484.20 mg kg^{-1}] in that regard.

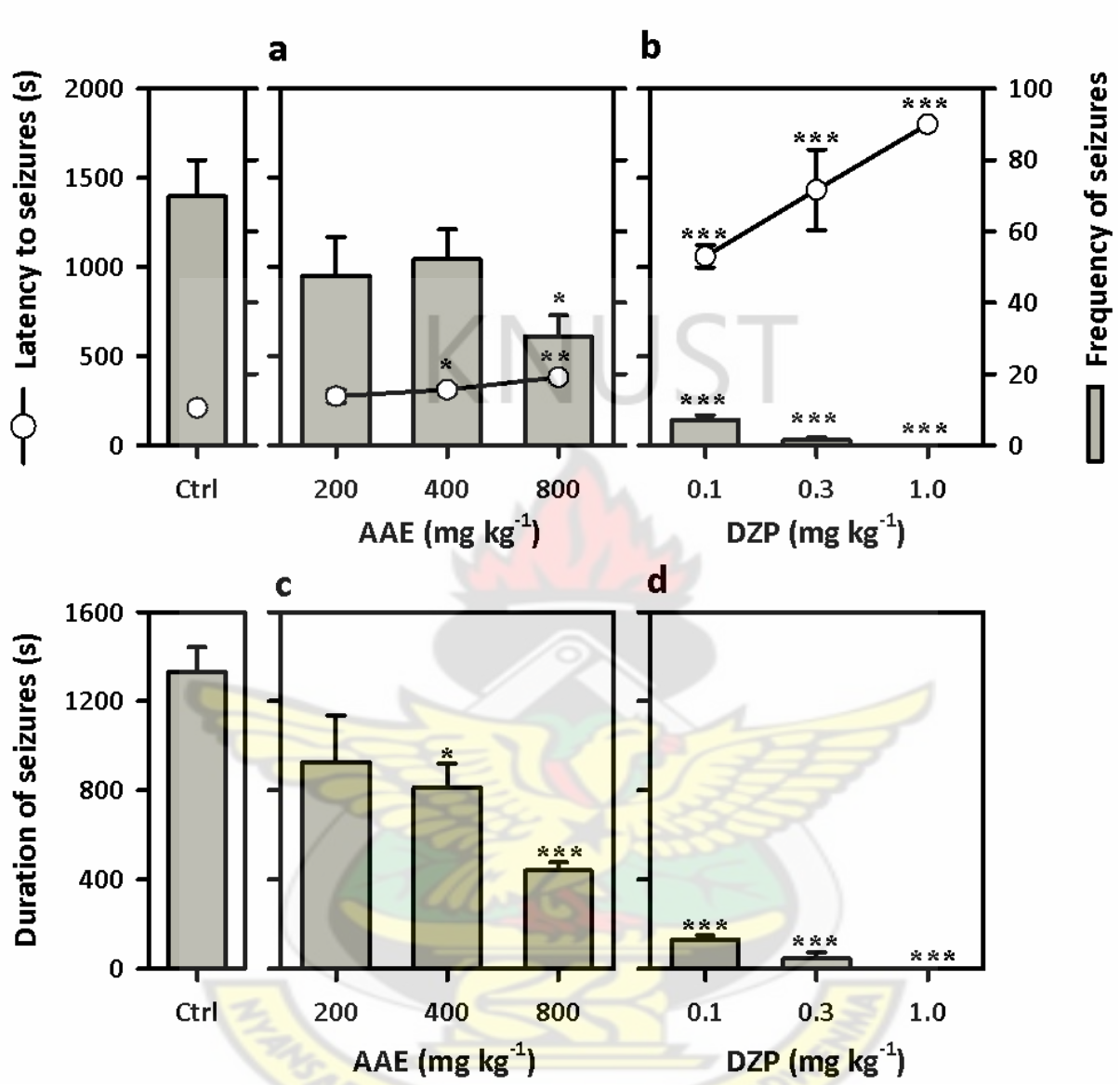

Figure 4.1Effects of AAE (200, 400 and 800 mg kg^{-1}, *p.o.*) and diazepam (0.1, 0.3 and 1.0 mg kg^{-1}, i.p.) on the latency and frequency of seizures (a and b) and duration of convulsions (c and d) in PTZ-induced seizures. Data are presented as mean ±SEM (n=8). *P<0.05, **P<0.01, ***P<0.001 (One-way analysis of variance followed by Newman-Keuls' *post hoc* test).

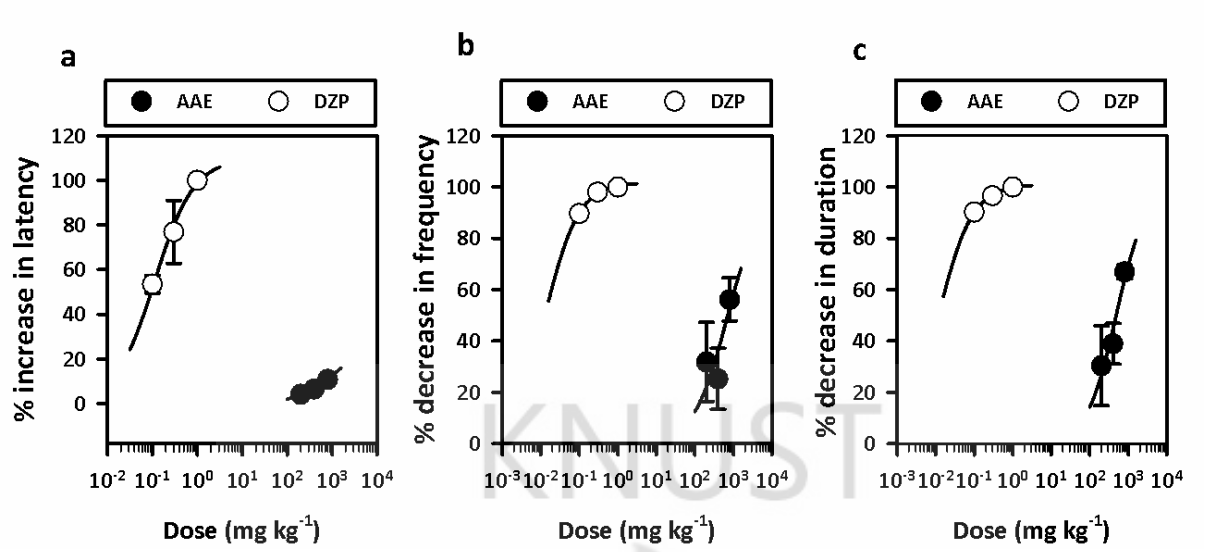

Figure 4.2 Dose-response curves of AAE and diazepam on the % increase in latency to seizures (a), % decrease in frequency (b) and % decrease in duration of seizures (c) in PTZ-induced seizures. Each point represents mean± S.E.M (n=8).

4.3.1.2 *Effects in picrotoxin-induced seizures*

Picrotoxin (3 mg kg^{-1}, i.p) produced clonic seizures in all mice pretreated with the distilled water. AAE (200-800 mg kg^{-1}) produced a dose-dependent increase in latency to seizures which was significant (*P=0.0009; $F_{3, 16}$=9.108*; Figure 4.3 a) at all doses. The frequency of seizures was also significantly (*P=0.0081; $F_{3, 16}$=5.591;* Figure 4.3 a) decreased dose-dependently. AAE produced significant (*P=0.0002; $F_{3, 16}$=12.53*; Figure 4.3 c) dose-dependent decrease in the total duration of seizures in all animals. The reference anticonvulsant diazepam (0.1-1.0 mg kg^{-1}, i.p.) also profoundly delayed the onset of myoclonic jerks, decreased frequency of jerks and significantly antagonised (*P<0.0001; $F_{3, 16}$=17.72*; Figure 4.3 b and 2 d) PCT-induced seizures. Dose-response curve analysis (Figure 4.4) revealed that the extract was more efficacious [E_{max}=100 %] in reducing the duration of convulsions than in other parameters measured. It, however, exhibited higher potency [ED_{50}: 179.20 mg kg^{-1}] in reducing frequency of seizures.

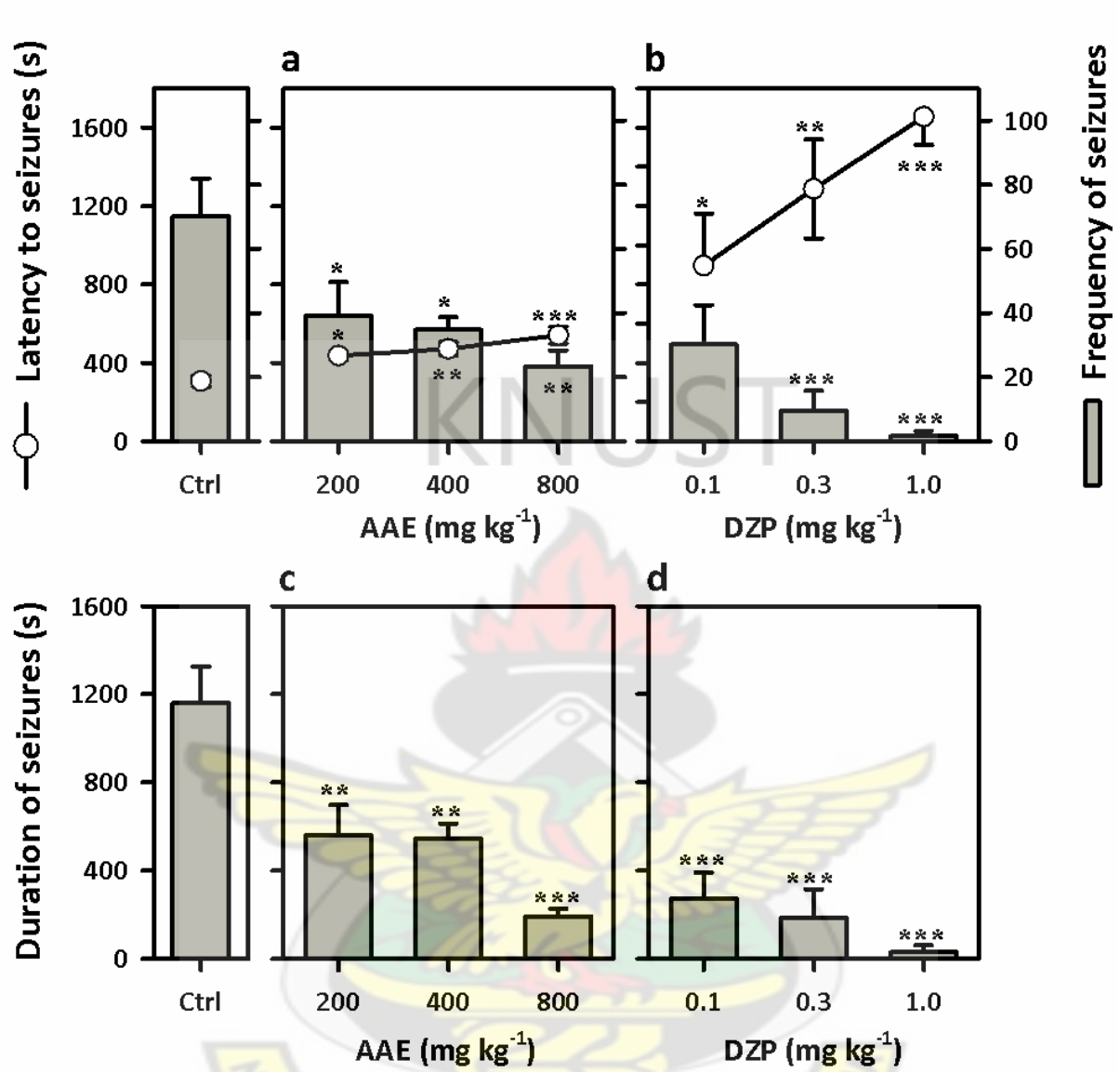

Figure 4.3 Effects of AAE (200, 400 and 800 mg kg^{-1}, *p.o.*) and diazepam (0.1, 0.3 and 1 mg kg^{-1}, i.p.) on the latency and frequency of seizures (a and b) and duration of convulsions (c and d) in picrotoxin-induced seizures. Data are presented as mean±SEM (n=8). **P<0.05, **P<0.01, ***P<0.001* compared to vehicle-treated group (One-way analysis of variance followed by Newman-Keuls' *post hoc* test).

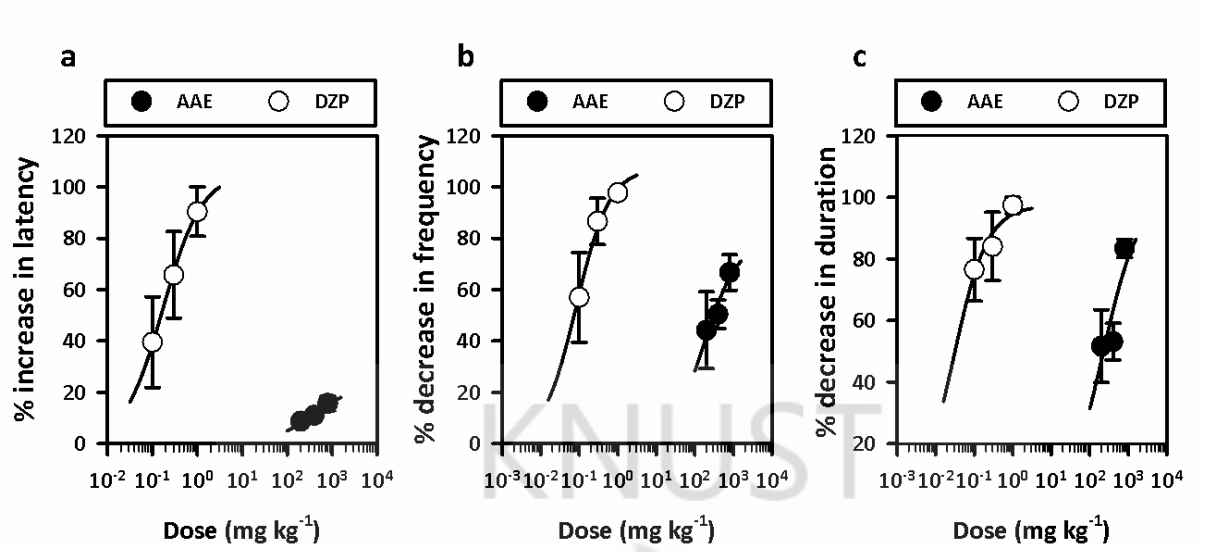

Figure 4.4 Dose-response curves of AAE and diazepam on the % increase in latency to seizures (a), % decrease in frequency (b) and % decrease in duration of seizures (c) in picrotoxin-induced seizures. Each point represents mean± S.E.M (n=8).

4.3.1.3 *Effects in Maximal Electroshock Test*

Electroshock produced hind limb tonic extensions (HLEs) in all mice. The extract did not affect the latency to onset of hind limb tonic extensions and duration of convulsions significantly (Figure 4.5). The reference anticonvulsant carbamazepine (3, 10 and 30 mg kg^{-1}, *p.o.*), however, delayed the onset of HLEs (*P=0.0054;* $F_{3,\ 16}$*=6.193*; Figure 4.5) and significantly (*P<0.0001;* $F_{3,\ 16}$*=15.84*; Figure 4.5) decreased the total duration of electroshock-induced convulsions. From the dose response curves, it can be seen that the extract exhibited less efficacy [E_{max}=53.55 %] as well as lower potency [EC_{50}=238.80 mg kg^{-1}] as compared to carbamazepine [E_{max}=104.60 %; EC_{50}=14.60 mg kg^{-1}]. The non-parallel nature of the curves suggests that the extract and carbamazepine do not act on similar receptors (Figure 4.6).

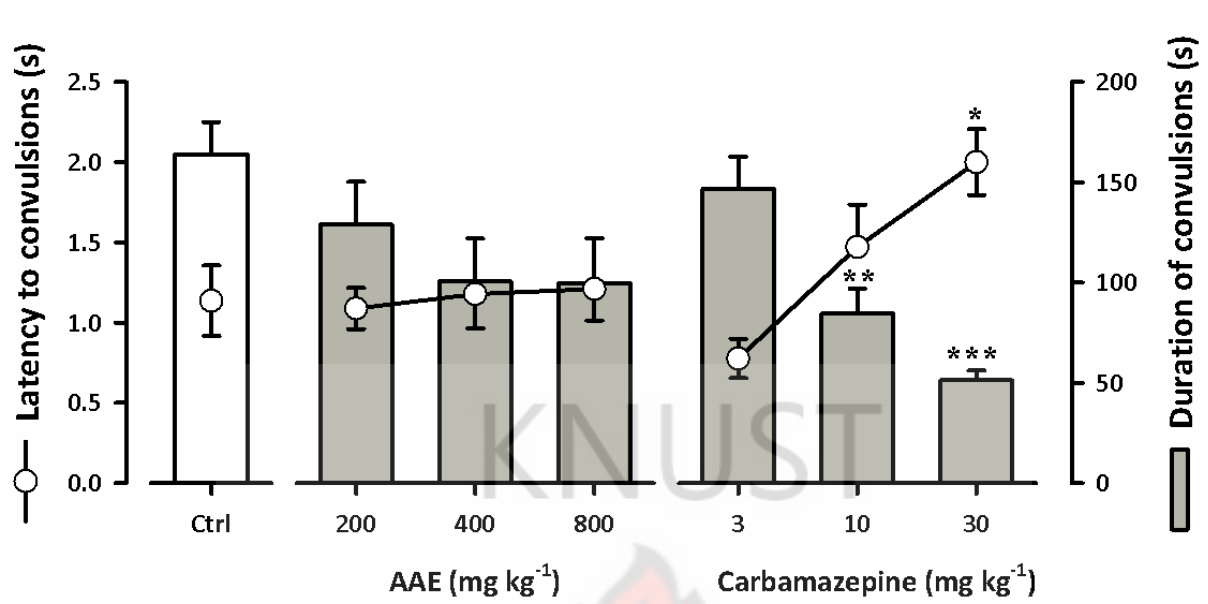

Figure 4.5 Effects of AAE (200, 400 and 800 mg kg^{-1}, *p.o.*) and carbamazepine (3, 10 and 30 mg kg^{-1}, *p.o.*) on the latency to and duration of convulsions in the MEST. Each point and column represents mean ±SEM (n=8). **P<0.05, **P<0.01***P<0.001* compared to vehicle-treated group (One-way analysis of variance followed by Newman-Keuls' *post hoc* test).

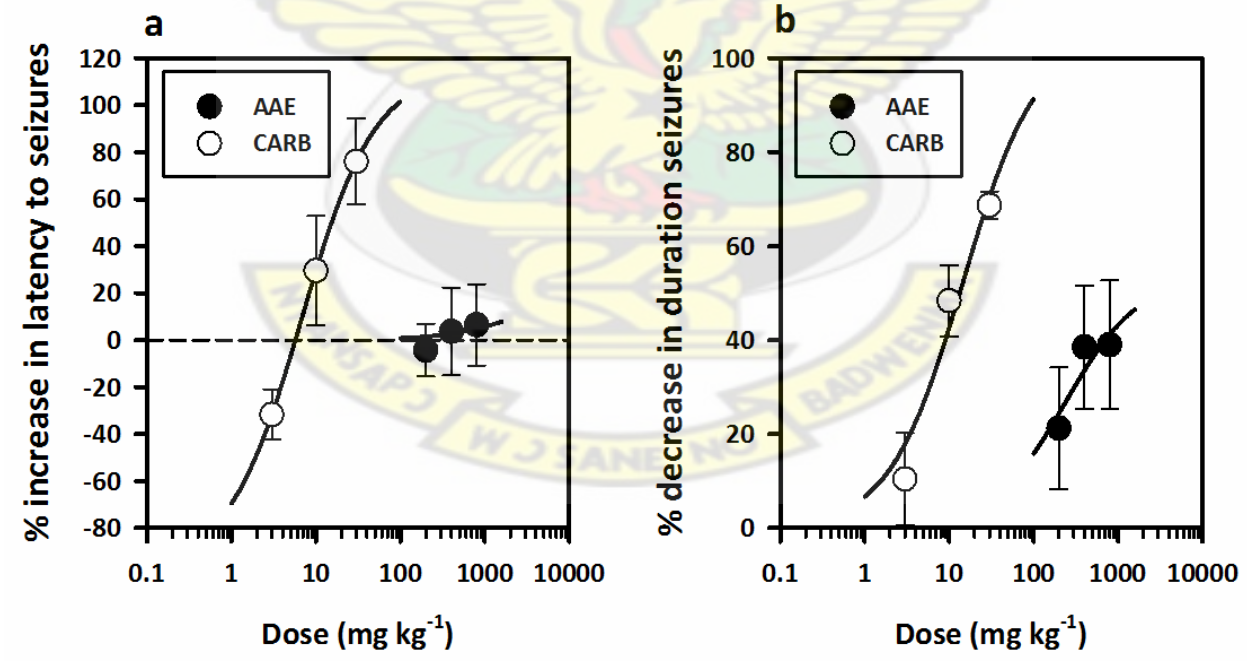

Figure 4.6 Dose-response curves of AAE and carbamazepine on the % increase in latency to seizures (a) and % decrease in duration of seizures (b) in MEST. Each point represents mean± S.E.M (n=8).

4.3.1.4 *Effect in strychnine-induced convulsions*

Strychnine produced hind limb tonic extensions (HLEs) in all mice. AAE did not affect the latency to onset of hind limb tonic extensions and duration of convulsions significantly (Figure 4.7). The reference anticonvulsant diazepam (1 mg kg^{-1}, i.p), on the other hand, delayed the latency to convulsions significantly (*P<0.0001; $F_{4, 20}$=36.60*; Figure 4.7). The duration of convulsions was also significantly (*P<0.0001; $F_{4, 20}$=14.31*; Figure 4.7) reduced. Dose response curves (Figure 4.8) show a lack of efficacy of the extract in this model [E_{max}=7.78 %]. EC_{50} obtained was 137.10 mg kg^{-1}.

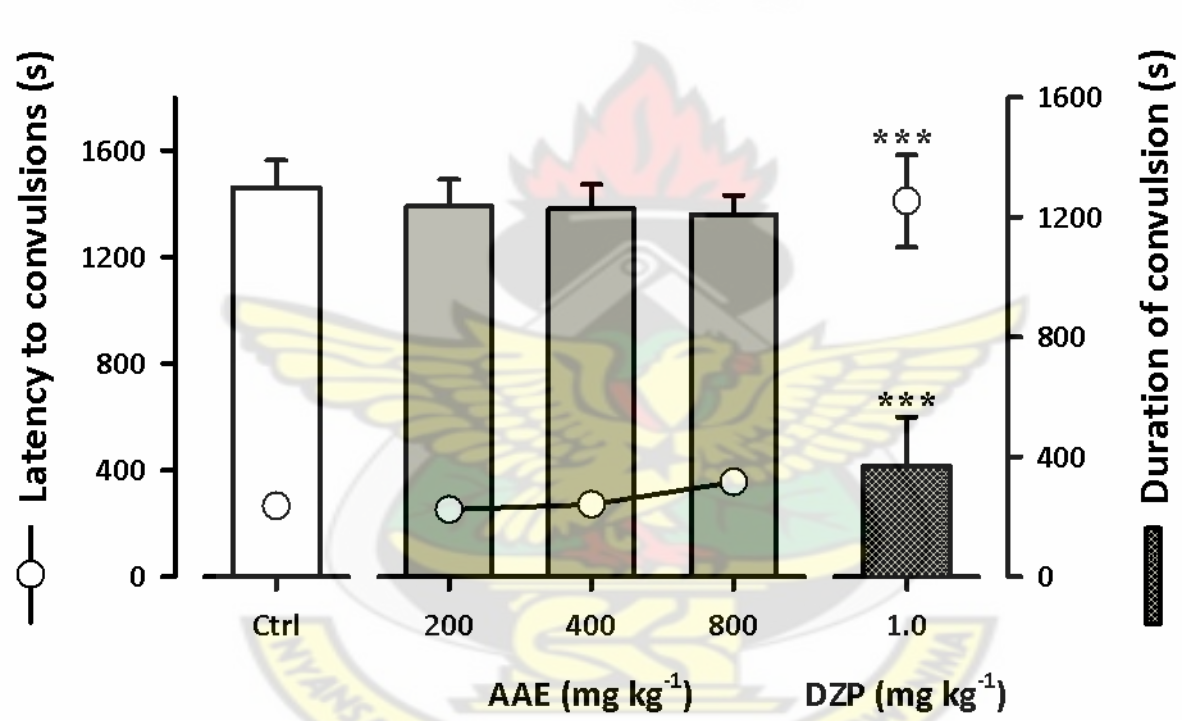

Figure 4.7 Effects of AAE (200, 400 and 800 mg kg^{-1}, *p.o.*) and diazepam (1 mg kg^{-1}, i.p.) on the latency to and duration of convulsions in strychnine-induced seizures. Data are presented as mean±SEM (n=10). ***$P<0.001$ compared to vehicle-treated group *(O*ne-way analysis of variance followed by Newman-Keuls' *post hoc* test).

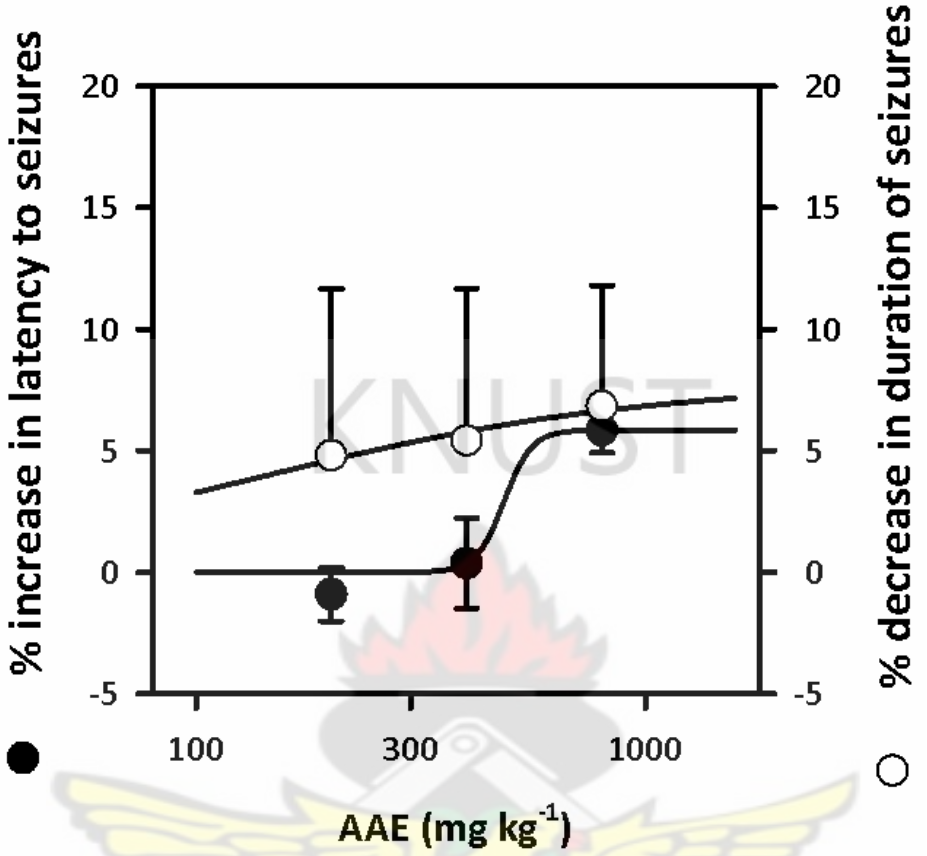

Figure 4.8 Dose-response curves of AAE on the % increase in latency to seizures and % decrease in duration of seizures in strychnine-induced seizures. Each point represents mean± S.E.M (n=10).

4.3.1.5 *Effects in 4- Aminopyridine induced convulsions*

4-aminopyridine (10 mg kg^{-1}, i. p) produced hind limb tonic extensions in all animals. The extract produced a significant (*P<0.0001; $F_{3, 28}$=22.02*; Figure 4.9 a) increase in time taken to the onset of convulsions. Sodium valproate (100-400 mg kg^{-1}) also significantly (*P<0.0001; $F_{3, 28}$=17.61*; Figure 4.9 b) delayed the onset of convulsions. The extract significantly (P=0.0004, χ^2 (*df* =3) = 18.11) improved survival of the animals after induction of convulsions. The test for trend presented significant (P<0.0001, χ^2 (*df* =1) = 15.79) effect of the treatment groups on median survival indicating a linear trend. Sodium valproate also produced similar effects on survival (P<0.0001, χ^2 (*df* =3) = 21.23) and linear trend (P<0.0001, χ^2 (*df*=1) = 19.17). Survival curves show a decrease in probability of survival with time. However, probability of survival increased with increase in dose.

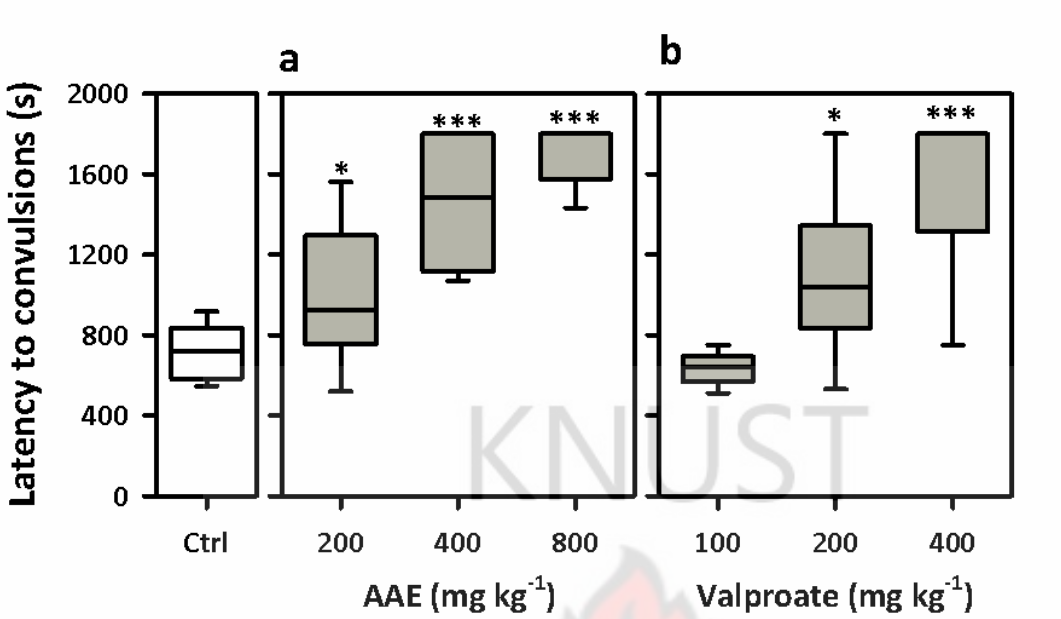

Figure 4.9 Effects of AAE (200, 400 and 800 mgkg^{-1}, *p.o.*) and sodium valproate (100, 200 and 400 mgkg^{-1}, *p.o.*) on the latency to convulsions in 4-aminopyridine induced seizure test. Data are presented as group means±SEM (n=8).The lower and upper margins of the boxes represent the 25th and 75th percentiles, with the extended arms representing the 10th and 90th percentiles respectively. The median is shown as a horizontal line within the box. **P<0.05, ***P<0.001* compared to vehicle-treated group (One-way analysis of variance followed by Newman-Keuls *post hoc* Test).

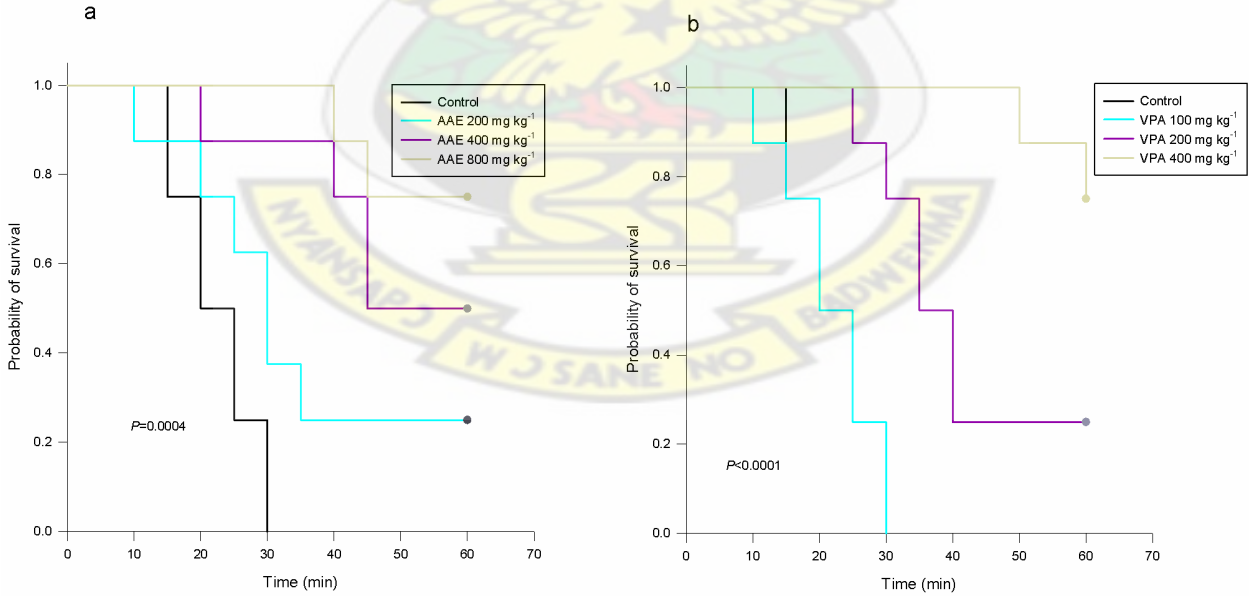

Figure 4.10 Kaplan –Meier estimates of overall survival of animals treated with (a) AAE (200, 400 and 800 mg kg^{-1}) and (b) sodium valproate (100, 200 and 400 mg kg^{-1}) in the 4-aminopyridine seizure test over one hour observation period (n=8).

4.3.1.6 *Effects of flumazenil*

Flumazenil significantly (*P=0.0009; $F_{1, 8}$=26.09*; Figure 4.11 c) reversed the reduction in duration of seizures produced by the extract. A significant reversal (*P=0.0003; $F_{1, 8}$=37.51*; Figure 4.11 a) was also obtained for the latency to seizures. The frequency of seizures as compared to the control showed no significant effect. Effects of diazepam were significantly (*P<0.0001*; Figure 4.11 a, b, c) reversed by flumazenil.

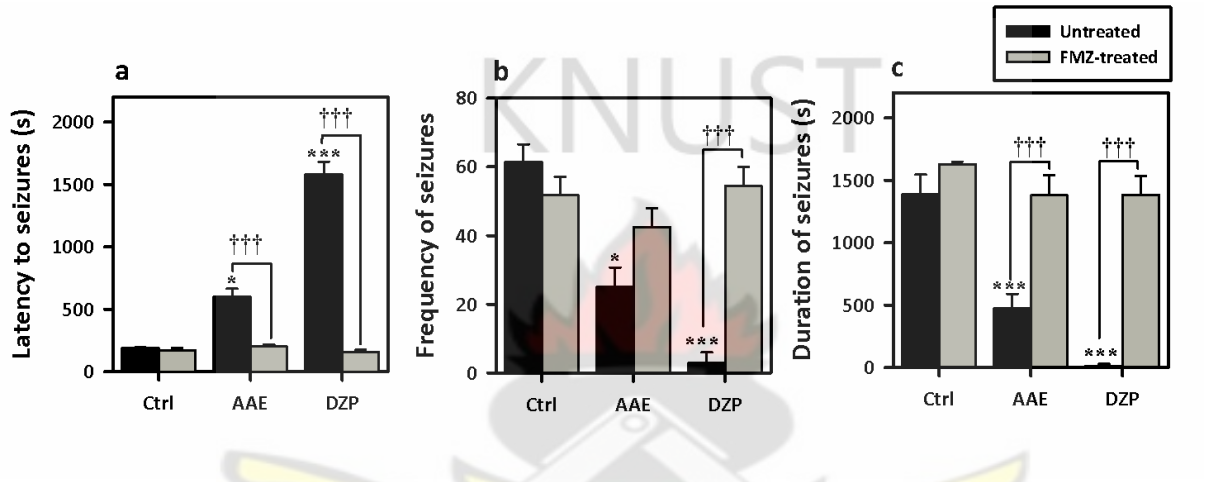

Figure 4.11 Effect of flumazenil on the latency (a), frequency (b) and duration of seizures (c) of AAE (400 mg kg^{-1}, *p.o.*) and diazepam (0.3 mg kg^{-1}, i.p.) (c) in PTZ-induced seizures. Data are presented as mean ±SEM (n=8). **P<0.05, **P<0.01, ***P<0.001* compared to vehicle-treated group (One-way analysis of variance followed by Newman-Keuls' *post hoc* Test). ††† *P<0.001 (*Two-way ANOVA followed by Bonferroni post test).

4.3.2 Chronic Models

4.3.2.1 *Effects in kindling*

In PTZ + vehicle-treated group, repeated administration of subconvulsive dose of PTZ (40 mg kg^{-1}) on every alternate day for 20 days resulted in increasing convulsive activity leading to generalized clonic seizures (Racine score of 5). Administration of AAE in the dose of 200 mg kg^{-1} and 400 mg kg^{-1} did not modify the course of kindling induced by PTZ significantly. However, a higher dose of 800 mg kg^{-1} suppressed the kindled seizure significantly ($P < 0.05$) as the group could not achieve a mean score of 5. The standard anticonvulsant diazepam significantly ($P<0.01$) modified the course of kindling at all three dose levels compared to the control. ED_{50} obtained for the extract was 276.70 mg kg^{-1}

compared to 0.05 mg kg^{-1} for diazepam. The extract was however more efficacious than diazepam achieving an E_{max} of 88.83 % compared to 60.36 % for diazepam.

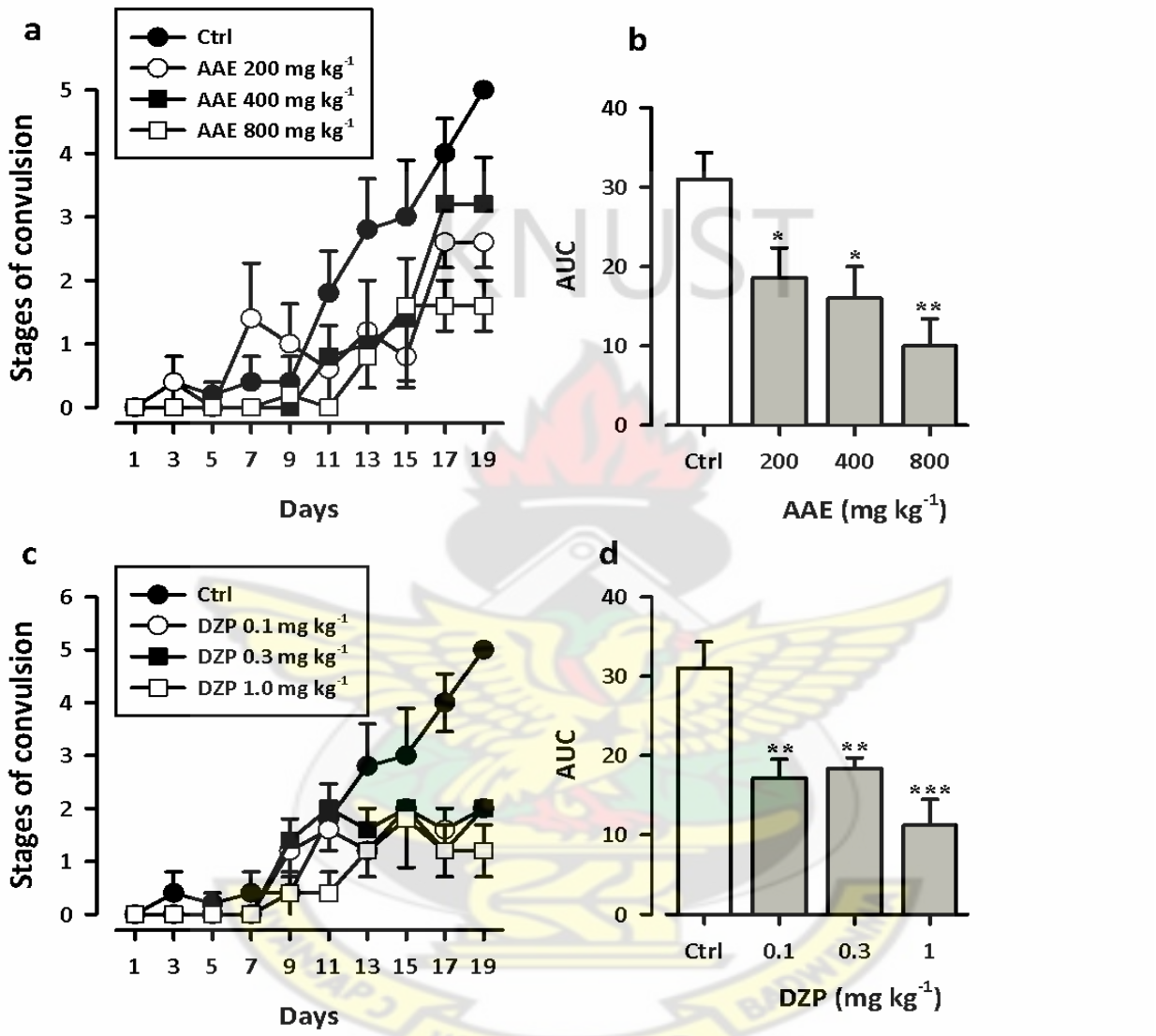

Figure 4.12 Effects of AAE (200, 400 and 800 $mgkg^{-1}$, *p.o.*) and diazepam (0.1, 0.3 and 1 $mgkg^{-1}$, i.p.) on the stages of convulsion attained in PTZ-induced kindling. Data are presented as group mean±SEM (n=8). **P<0.05, ** P<0.01, ***P<0.001* compared with vehicle treated group (One-way analysis of variance followed by Newman-Keuls *post hoc* test).

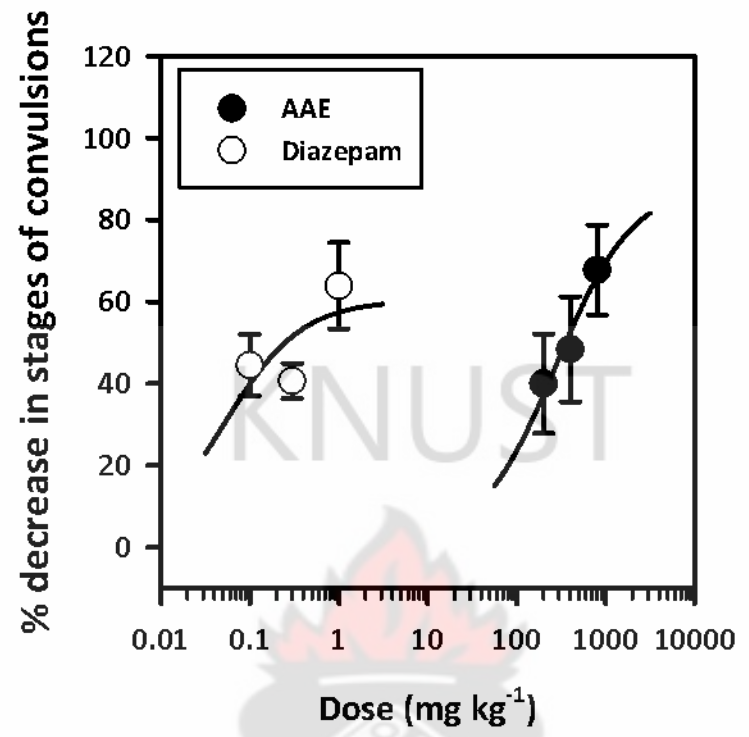

Figure 4.13 Dose-response curves of AAE and diazepam on the % decrease in stages of convulsions in PTZ-induced kindling. Each point represents mean± S.E.M (n=8).

4.3.2.2 *Pilocarpine-induced status epilepticus*

Pilocarpine induced behavioural changes including hypoactivity, tremor and myoclonic movements of the limbs progressing to recurrent myoclonic convulsions with rearing, falling, and status epilepticus. AAE produced significant effect (P<0.01, Figure 4.14 a) on the latency to first myoclonic jerks as compared to control at the highest dose only. It had a similar effect on the total duration of seizures (Figure 4.14 c). Diazepam was used as the reference drug and it also significantly reduced the total duration of seizures (P<o.o1, Figure 4.14 d) and latency ($P<0.001$, Figure 4.14 b) at 1 and 3 mg kg^{-1}. Diazepam was more potent than the extract in increasing the % latency with an ED_{50} of 0.66 mg kg^{-1} as a against 424.50 mg kg^{-1} for the extract. Diazepam was also more efficacious achieving an E_{max} of 108.90 % compared to 100 % for the extract. Likewise, for the % duration AAE produced ED_{50} = 80.06 mg kg^{-1} and E_{max}= 100 % while the standard diazepam achieved ED_{50} = 1.67 mg kg^{-1} and E_{max}= 100%.

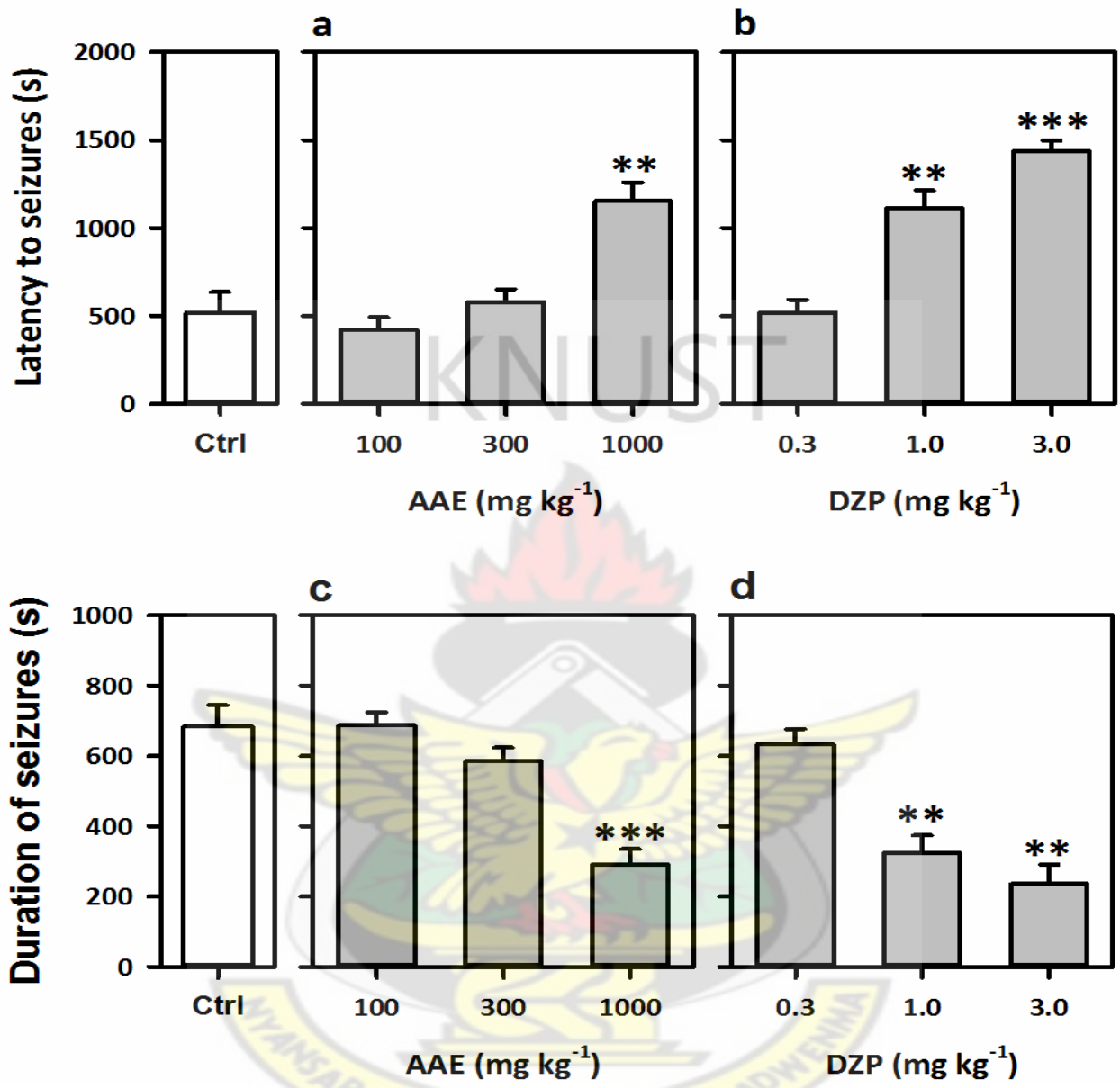

Figure 4.14 Effect of AAE (100-1000 mg kg^{-1}, *p.o.*) and diazepam (0.3-3 mg kg^{-1}, i.p.) on the latency to (a and b) and total duration of seizures (c and d) induced by PILO. Each column represents the mean ± SEM (n=8). $^{**}P < 0.01$, $^{***}P < 0.001$ compared to vehicle-treated group (One-way ANOVA followed by Newman-Keuls *post hoc* test).

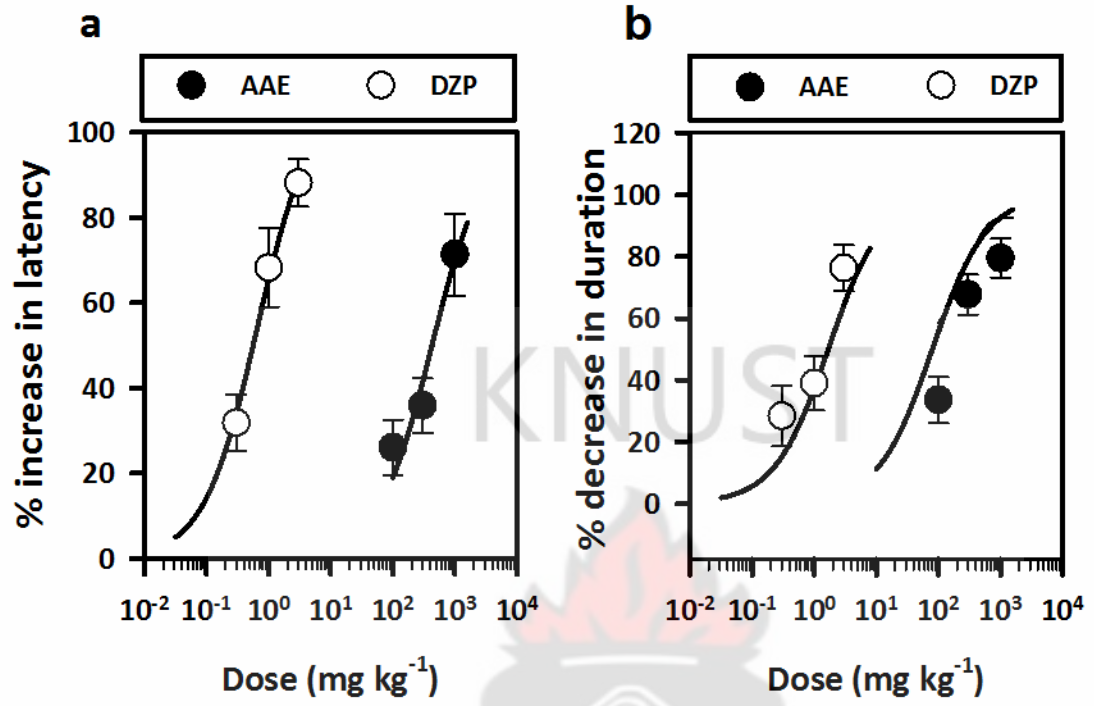

Figure 4.15 Dose-response curves of AAE and diazepam on the % increase in latency and % decrease in durations of status epilepticus induced with pilocarpine. Each point represents mean± S.E.M (n=8).

4.3.2.3 *Effects in Rat Kainate Model*

Kainic acid (10 mg kg^{-1}, i. p) produced wet dog shakes in all animals. AAE (400 mg kg^{-1}) produced a significant (p<0.05) increase in time taken to the onset of wet dog shakes (Figure 4.16). Carbamazepine (30 mg kg^{-1}) and Nifedipine (30 mg kg^{-1}) also delayed the onset. Histopathological examination of the coronal section of the brain showed no protective effect on hippocampal cells by AAE and nifedipine. Carbamazepine offered better preservation of hippocampal cells in the CA1, CA2 and CA3 regions (Figure 4.17). The brain to body ratio decreased significantly (p<0.001) with all three treatments.

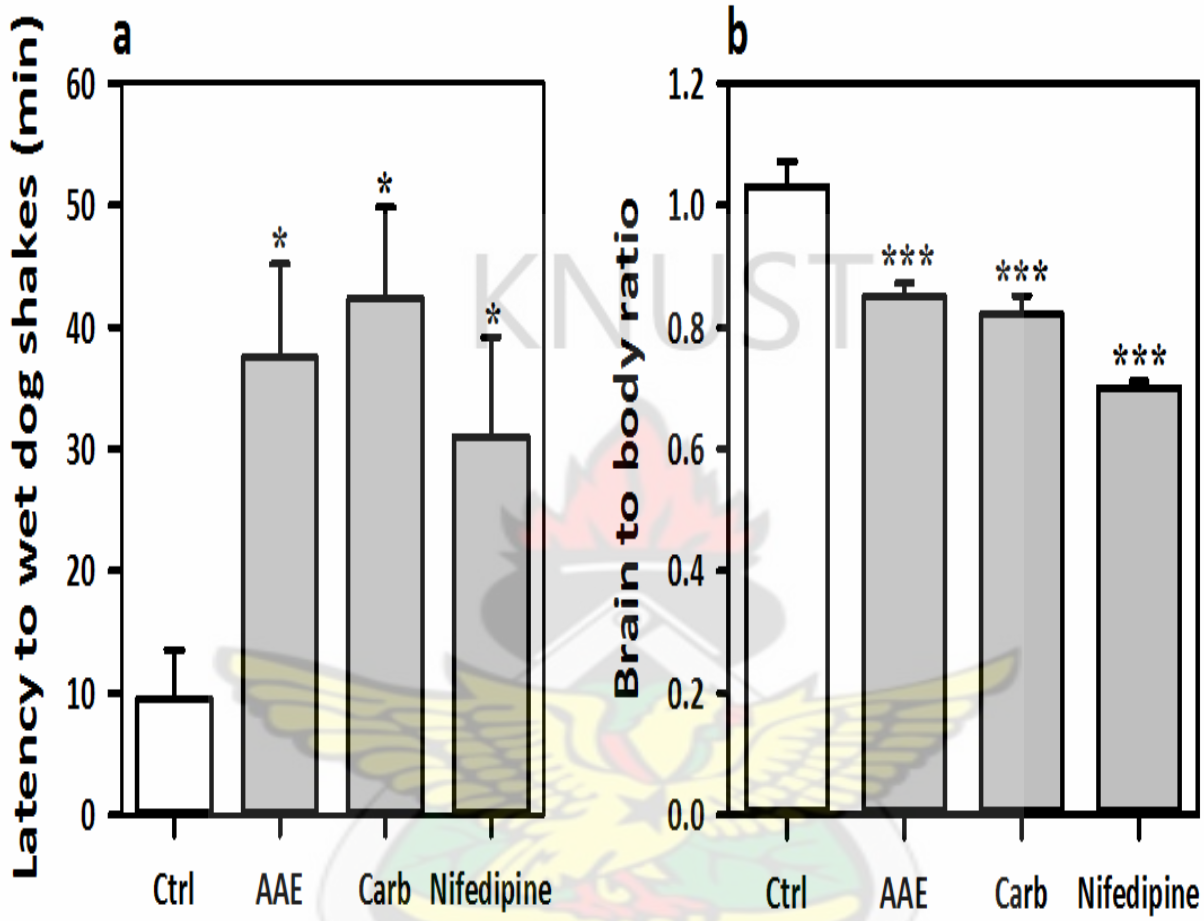

Figure 4.16 Effects of AAE (400 mgkg^{-1}, *p.o.*), carbamazepine (30 mgkg^{-1}, *p.o.*) and nifedipine (30 mgkg^{-1}, *p.o.*) on the latency to wet dog shakes (a) and % brain to body ratio in rat kainate model. Data are presented as group means±SEM (n=8). *$P<0.05$, ***$P<0.001$ compared to vehicle-treated group (One-way analysis of variance followed by Newman-Keuls' *post hoc* Test).

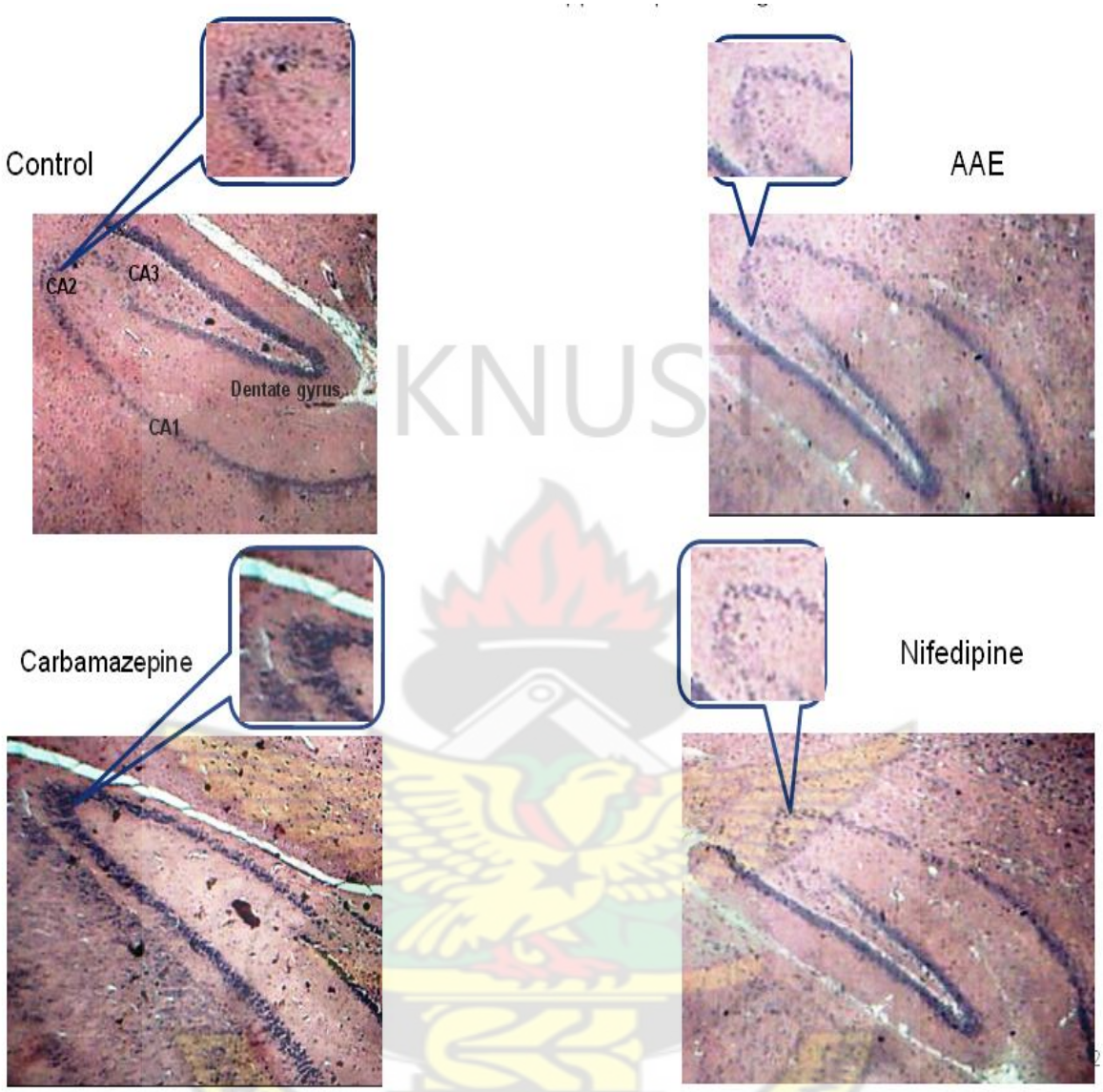

Figure 4.17 Photomicrographs of coronal sections of the brain of rats AAE (400 mg kg^{-1}), carbamazepine (30 mg kg^{-1}) and nifedipine (30 mg kg^{-1}) on kainate– induced hippocampal damage (H & E, x 100).

4.4 DISCUSSION

The outcome of this study provides evidence that the aqueous extract of the stem bark of *Antiaris toxicaria* possesses anticonvulsant activity. The ability of AAE to delay the onset of convulsions and/or shorten the duration of convulsions was considered an indication

of anticonvulsant activity. Judging from the data obtained, the plant extract exhibited anticonvulsant activity in the PTZ test just like diazepam which can be due to action on GABA system (Meldrum, 1975; Gale, 1992; De Sarro *et al.*, 2003). The exact mechanism by which PTZ produces seizures is still unclear. It, however, has been shown to be due to inhibition and/or attenuation of GABAergic neurotransmission (Gale, 1992; Katzung, 2004). It is therefore likely that AAE produces its anticonvulsant effect directly or indirectly by enhancing GABAergic neurotransmission in the brain. The PTZ test models human generalized and absence seizures (Loscher and Schmidt, 1988; Upton, 1994). Hence, the extract may be effective in managing such conditions.

Picrotoxin is $GABA_A$–receptor antagonist (Ticku and Olsen, 1977; Olsen, 1981). GABAergic ionotropic receptors can mediate both pre- and post-synaptic inhibition. Pre-synaptic inhibition mediated by GABA often leads to inhibition of neurotransmitter release from the excitatory arm (MacDermott *et al.*, 1999). PCT-induced seizures which are due to the decreased $GABA_A$-receptor-mediated inhibition in turn promote the excitatory arm of the CNS mainly mediated by glutamate (Gale, 1992). The extract being effective in the PCT-induced seizure test points to a more specific action on GABA-mediated neurotransmission. This has been further confirmed by the use of flumazenil to reverse the anticonvulsant properties of AAE in the PTZ-induced seizure model. Flumazenil is a benzodiazepine antagonist at the $GABA_A$ receptor possessing the ability to reverse anticonvulsant effects and the accompanying alterations in extracellular glutamate concentration (Khan *et al.*, 2000). The specificity of flumazenil to this receptor has been proven as several authors have demonstrated that the antagonist has no effect on diazepam's action on voltage-dependent Na^+ channels (Backus *et al.*, 1991; Ishizawa *et al.*, 1997; Khan *et al.*, 2000).

4-aminopyridine is a potassium ion channel antagonist. AAE significantly and dose dependently increased the latency to convulsions induced with 4-aminopyridine. This may be due to activation of potassium channels or conductance. GABAergic receptor activation can result in enhancement of potassium conductance. AAE may therefore be acting either directly or indirectly to enhance potassium ion conductance. Retigabine is currently the only approved drug for the treatment of epilepsy which functions through activation of potassium currents (Rostock *et al.*, 1996; Rundfeldt, 1997; Hecht, 2012) and has promise in the treatment of pharmacoresistant epilepsy. It is also known that 4-aminopyridine stimulates the release of neurotransmitters, including glutamate, in

numerous preparations such as brain slices (Hu *et al.*, 1991; Tapia *et al.*, 1999) and the neuromuscular junction (Lundh, 1978). Furthermore, it has been recently found by a microdialysis procedure *in vivo* that glutamate is by far the amino acid predominantly released by 4-aminopyridine in the striatum (Morales-Villagran and Tapia, 1996). Therefore, due to the well recognized function of excitatory amino acid receptors in convulsive and excitotoxic mechanisms (Meldrum, 1992), it seems feasible that the excess glutamate released may be involved in the excitatory effects. This indicates that the extract may be acting, in addition to the above, by modulating the release of glutamate. From the pattern of survival curves, it can be inferred that the dose of the extract does affect survival rate offering significant protection against death from 4-aminopyridine-induced convulsions. This was evident from the log-rank analysis which indicated significant differences between survival curves for each dose level. Median survival time ranged from 30 min and death may be prevented totally at the highest dose of AAE. The exact mechanism by which the extract offers this protection may be linked to its ability to reduce excitotoxic effects mediated by glutamate release. Sodium valproate also increased median survival time and was more effective than the extract.

The extract showed no effect in the MEST at all doses. This indicates that it is not effective in partial and generalized tonic seizures (Macdonald and Kelly, 1995) and is unable to prevent seizure spread (Raza *et al.*, 2001). It was also ineffective in strychnine-induced convulsions. Strychnine induces convulsions by antagonising competitively the postsynaptic inhibitory effects of glycine (Bigler, 1977). The fact that AAE produced no protective effects against strychnine-induced convulsions suggests it does not interact with the glycine-mediate inhibitory pathway.

Kindling is a chronic model of epilepsy and epileptogenesis. Repeated administration of a subconvulsive dose of PTZ (a blocker of the $GABA_A$ receptor) results in the progressive intensification of convulsant activity, culminating in a generalized seizure (Corda *et al.*, 1990). The highest dose of AAE (800 mg kg^{-1}) significantly delayed progression of convulsion similarly to diazepam.

Many substances interacting with GABA receptors have been shown to produce potent anticonvulsant effects on seizures in previously kindled animals (Morimoto *et al.*, 1987; Bittencourt *et al.*, 2010). It has been shown that AAE produces anticonvulsant effects by interacting with the $GABA_A$ receptor. The fact that it acts via GABAergic mechanisms

may be a possible explanation for anticonvulsant effects being exhibited in the kindling model.

There is some evidence that free radicals are actively involved in physiological processes during oxidative stress induced by administration of convulsants (Coyle and Puttfarcken, 1993). Of all the free oxygen radicals that occur *in vivo*, the hydroxyl-free radicals (OH^-) are considered to be most hazardous (Halliwell, 1992). Different mechanisms may lead to the increase of the free radicals in PTZ-induced convulsions. It may be assumed that further reason exist for the increased formation of OH^- in kindled animals during PTZ seizure, such as reduced activity of superoxide dismutase (SOD), a major defence system for counteracting the toxic effects of reactive oxygen species such as O^{2-}. However, antioxidant activity of AAE has not been firmly established.

AAE exhibited anticonvulsant effects against pilocarpine-induced seizures. Pilocarpine is a cholinergic agonist, widely used experimentally to induce limbic seizures in structures containing a high concentration of muscarinic receptors such as the cerebrum (Clifford *et al.*, 1987; Turski *et al.*, 1989). *Status Epilepticus* produces significant decreases in M_1, M_2, and GABAergic receptor densities (Freitas *et al.*, 2004) and hence neurotransmission. Freitas *et al.* have also reported in 2004 on increased levels of superoxide dismutase and catalase and reductions in acetylcholinesterase enzymatic activities in the rat frontal cortex and hippocampus. During pilocarpine-induced seizures and SE in adult rats, lipid peroxidation processes are increased (Freitas *et al.*, 2004; Xavier *et al.*, 2007) suggesting free radical involvement in the pilocarpine-induced brain damage. Certain antioxidants, such as ascorbic acid, have therefore been shown to possess anticonvulsant activity against pilocarpine-induced SE (Tejada *et al.*, 2007; Xavier *et al.*, 2007). Muscarinic receptor stimulation is alleged to be responsible for the onset of pilocarpine-induced seizures, while glutamate acting on NMDA receptors sustains seizure activity (Jope *et al.*, 1986; Turski *et al.*, 1989). Analysis of the brain morphology after pilocarpine administration demonstrates that the CA1 hippocampal neurones and the hilus of dentate gyrus are predominantly susceptible to neuronal cell loss (Turski *et al.*,1983;Turski *et al.*,1986). Neuronal cell death during SE occurs largely by excitotoxic injury caused by the activation of glutamatergic pathways (Cavalheiro *et al.*,1994;Costa *et al.*,2004). Thus, the ability of AAE to attenuate seizures induced by pilocarpine could be attributed to cholinergic antagonism at the M_1 or M_2 receptors, increase in GABA, and/or its receptor densities, decrease in glutamate levels or through antioxidant pathways. Activation of

potassium ion conductance can also contribute as it results in inhibition of the release of glutamate (Morales-Villagran and Tapia, 1996). AAE may therefore have potential in the management of *status epilepticus*.

Kainic acid is a neuroexcitotoxic analogue of glutamate used in studies of epilepsy to model experimentally induced limbic seizures (Ben-Ari, 1985; Ben-Ari and Cossart, 2000). Kainate-treated rats may respond differently. Some may produce wet dog shakes (equivalent to a class III seizure on the Racine scale) or more severe seizures (Hellier and Dudek, 2005). Previous studies have shown pattern of neurodegeneration in the hippocampus with high concentration of high affinity KA binding sites (CA3 pyramidal cells of the hippocampus) (Ben-Ari, 1985; Fisher, 1989). The dentate gyrus from kainate-treated rats has shown the presence of mossy fibre sprouting in the inner molecular layer. (Nadler *et al.*, 1980; Buckmaster and Dudek, 1997a, 1997b). Examination of the hippocampus after seizures revealed hippocampal damage, especially in the CA3 and CA2 regions as shown in the photomicrographs. The extract showed no significant protection against such damage even though it significantly delayed the latency to wet dog shakes. This implies that the extract possesses general anticonvulsant properties but offers no protection against morphological changes. The kainate-treated rat model is used to study temporal lobe epilepsy. However, similarity of seizure occurrence to human temporal lobe epilepsy has not been studied comprehensively. But there are several characteristics of the seizures that resemble temporal lobe epilepsy in humans. For instance, some of the animals produce a few observed motor seizures (even with several months of observation) after a latent period, while other animals have seizures at a frequency as high as 1–2 h which is reminiscent of epilepsy in the human population (French *et al.*,1993). The rats often demonstrate confusion (e.g. hyperactive exploration of their cage) after a seizure, resembling the post-ictal confusion in most humans with temporal lobe epilepsy (Fenwick,1995). Rats treated with the extract exhibited fewer seizures as compared to the control implying a possibility that it might be effective in the treatment of temporal lobe epilepsy. The CA1and CA3 regions of the hippocampus are also known to posses one of the highest densities of the dihydropyridine receptors in the rat brain (Cortes *et al.*, 1984; Meyer *et al.*, 1987). The results of many experimental studies have shown that calcium channel blockers are effective against several different types of seizures (van Luijtelaar *et al.*, 2000; Kriz *et al.*, 2003). And hence, nifedipine proved its effect in this model.

4.5 CONCLUSION

Results presented here indicate that the aqueous extract of *Antiaris toxicaria* exhibits anticonvulsant activity, possibly through GABA-mediated inhibition and inhibition of glutamate mediated excitation via activation of potassium ion channels.

Antiaris toxicaria possesses anticonvulsant properties in kindling and status epilepticus murine models and may be a candidate for the management of temporal lobe epilepsy and antiepileptogenesis.

Chapter 5

ANXIOLYTIC ACTIVITY OF THE EXTRACT

5.1 INTRODUCTION

Anxiety disorders are the most common of mental illnesses in the world (Rabbani *et al.*, 2008). There is substantial research on the neurobiology of anxiety. Anxiety can be a normal emotion or associated with a pathological condition. Anxiety disorders do not exist solely as psychological or psychosocial phenomena, but can also occur as a direct result of neurobiological processes (Mensah *et al.*, 2007). For instance, Hughlings Jackson has identified that fear may represent part of a seizure rather than simply a reaction to the impending seizure (Jackson, 1879).

Drug therapy for anxiety is laden with dependence, tolerance and adverse effects (Nutt, 2005). For this reason, the development of newer therapies is essential and plants have always been an invaluable source.

GABA is the major inhibitory neurotransmitter in the mammalian CNS (Sinclair and Nutt, 2007). Useful anticonvulsants, anxiolytics and sedative-hypnotics act via modulation of the $GABA_A$ receptor (Polc, 1988). *Antiaris toxicaria* aqueous extract has exhibited $GABA_A$ receptor activity previously (Chapter 4). It was therefore investigated for potential anxiolytic activity in acute murine models of anxiety-elevated plus maze; light/dark box test; social interaction test.

5.2 MATERIALS AND METHODS

5.2.1 Animals

Male ICR mice (20-25 g) were obtained from Noguchi Memorial Institute for Medical Research, Accra, Ghana and kept in the departmental Animal House. Animals were maintained under laboratory conditions in stainless steel cages with free access to water and food *ad libitum*. All animals were handled in accordance with the Guide for

the Care and Use of Laboratory Animals (NRC *et al.*, 1996) and experiments were approved by the Faculty Ethics Committee.

5.2.2 Drugs and chemicals

Diazepam (DZP) and pentylenetetrazole (PTZ) were purchased from Sigma-Aldrich Inc., St. Louis, MO, USA.

5.2.3 Elevated plus maze (EPM)

Evaluation was done by using the method as described for rats (Pellow *et al.*, 1985) with some modifications. The elevated plus maze consisted of two opposite open arms (15cm×5 cm) without side walls and two enclosed arms (15 cm ×5 cm×5 cm), extending from a common central square platform (5×5 cm). A rim of Plexiglas (0.5 cm in height) surrounded the perimeter of the open arms to provide additional grip and prevent the mice from falling off. The EPM was raised 50 cm above the floor. The animals were divided into ten groups of six animals each, and pretreated with either doses of the extract (200, 400 and 800 mg kg^{-1}) or reference drugs diazepam (0.1, 0.3 and 1mg kg^{-1}) and pentylenetetrazole (3, 10 and 30 mg kg^{-1}). Diazepam served as reference anxiolytic drug and pentylenetetrazole reference anxiogenic drug. Control group animals were pretreated with the vehicle (distilled water, 3 ml kg^{-1}; *p.o*). Animals were placed individually in the central platform of the EPM facing the open arm at the start of the experiment and their behaviour recorded on a videotape with a digital camera placed 75 cm above the maze for five minutes each. The following spatiotemporal behavioural parameters were scored from video recordings as follows: number of closed and open arm entries (absolute value and percentage of the total number); time spent in the open and closed arms of the maze (absolute time and percentage of the total time of testing). Arm entry was counted only when all four limbs of the mouse were within a given arm of the EPM. Ethological parameters were also assessed namely: number of head-dips (absolute value and total number)—projecting head over the edge of either an open (unprotected) or closed (protected) arm and down toward the floor); number of stretch-attend postures (absolute value and percentage of the total number)—mouse stretches forward and retracts to original position from a closed (protected) or an open (unprotected) arm; rearing behaviour—

standing upright on the hind legs. The total distance travelled in the EPM was also measured. Raw data for the total distance travelled by mice in the EPM was obtained from the videos with the software Behavior Collect (http://cas.bellamrmine.edu/tietjen/DownLoads/computer_programs_for_data_colle.htm). Distances between X-Y coordinate pairs were calculated with Microsoft® Office Excel 2007 from the formula:

$$\sqrt{[(X1 - X2)^2 + (Y1 - Y2)^2]}$$

Where X1, X2, Y1 and Y2 represent coordinate pairs.

5.2.4 Light-dark Exploration Test

Evaluation using this apparatus was based on the initial model described by Crawley, 1981 and as modified by other workers (Belzung *et al.*, 1987; Belzung and Le Pape, 1994). It consists of wooden boxes (36 cm long × 30 cm wide × 30 cm deep), which are divided into two equal compartments by a wooden board. An 8 × 8 cm^2 opening located centrally at the floor level of the central divide connects the two compartments. One compartment was painted black and covered with a wooden lid and the other compartment painted white was not covered but lit by a 60-W light bulb set above the box. Mice were grouped and treated with drugs as for the elevated plus maze described above. At the beginning of the experiment, mice were placed individually in the centre of the dark compartment, facing away from the light compartment. Behaviours of the animals were recorded for 5 minutes with a digital camera placed 75cm above the box. Videotapes were scored manually with the aid of a computer program, J-Watcher® Version 1.0 for the frequency of compartment entries and total time spent by mice in each compartment.

5.2.5 Social Interaction

Animals were housed in groups of four animals prior to the test. The apparatus used consisted of plexiglas box with an open top. (40 x40 x 20 cm^3) with 15 x15 cm^2 marked areas on the floor. A 60-W light bulb was used to illuminate the box 17 cm above. Drug treatment was done as for the elevated plus maze test. Two naïve mice from

separate cages were placed into the box and their exploratory and social interaction behaviour such as following, climbing over, frequency of transitions and rearing was observed for ten minutes by video recording (de Angelis and File, 1979).

5.2.6 Data analysis

Data were presented as mean±S.E.M and significant differences between means determined by one-way analysis of variance (ANOVA) followed by Newman-Keuls' *post hoc* test and two-way ANOVA followed by Bonferroni post test. $P < 0.05$ was considered significant. Statistical analyses were carried out with Graph Pad Prism® Version 5.0 (GraphPad Software, San Diego, CA, USA).

5.3 RESULTS

5.3.1 Effects in the elevated plus maze

5.3.1.1 *Spatiotemporal Effects*

Administration of *A. toxicaria* (200-800 mg kg^{-1}) decreased anxiety in mice. AAE caused an increase in the number of open arm entries at the 200 mg kg^{-1} dose though it was not statistically significant. When open arm entries are expressed as a percentage, all doses show a significant increase in open arm entry ($P=0.004$, $F_{3,20}=6.103$, Figure 5.1 d). Diazepam, an anxiolytic (0.1, 0.3 and 1 mg kg^{-1}) also showed no significant increase in open arm entry but significantly ($P=0.005$, $F_{3,20}=5.811$, Figure 5.1 b) decreased the closed arm entry. It showed a non-dose dependent increase in percentage entry into the open arms ($P=0.0035$, $F_{3,20}=6.282$, Figure 5.1 e). Pentylenetetrazole (3, 10 and 30 mg kg^{-1}), increased open arm avoidance by decreasing the percentage open arm entries ($P=0.0079$, $F_{3,20}=5.227$, Figure 5.1 f). Number of arm entries was however not significant for both arms. Two-way analysis of variance (treatment group x arm type i.e. open or closed) revealed no significant arm type effect on the number of arm entries by the extract treated group. Diazepam ($P=0.0412$, $F_{1,30}=5.48$, Figure 5.1 b) and pentylenetetrazole ($P<0.0001$, $F_{1,30}=58.35$, Figure 5.1 c), however, showed significant arm type effect.

The time spent in the open arms was increased significantly ($P=0.0225$, $F_{3,20}=3.977$,Figure 5.2 a) at the 800 mg kg^{-1} dose of AAE and likewise, the % open arm

time showed a similar pattern of increase ($P=0.0225$, $F_{3,20}=3.978$, Figure 5.2 d). Diazepam showed significant ($P<0.0001$, $F_{3,20}=15.39$, Figure 5.2 b) increase in open arm time while showing no significant effect on closed arm time. Pentylenetetrazole decreased open arm time at the highest dose significantly ($P=0.0020$, $F_{3,20}=7.070$, Figure 5.2 c). Time spent in the closed arm was increased but not significantly (Figure 5.2 c).A decrease in the percentage open time ($P=0.0020$, $F_{3,20}=7.070$,Figure 5.2 f) was also obtained with PTZ in contrast to an increase by diazepam significantly($P=0.0035$, $F_{3,20}=6.282$,Figure 5.2 e) at all doses. Two-way analysis of variance (treatment group x arm type i.e. open or closed) revealed no significant arm type effect on time spent in the various arms by the extract treated group. Diazepam ($P=0.0412$, $F_{1,30}=5.48$, Figure 5.2b) and pentylenetetrazole ($P<0.0001$, $F_{1,30}=48.52$, Figure 5.2 c), however, showed significant arm type effect.

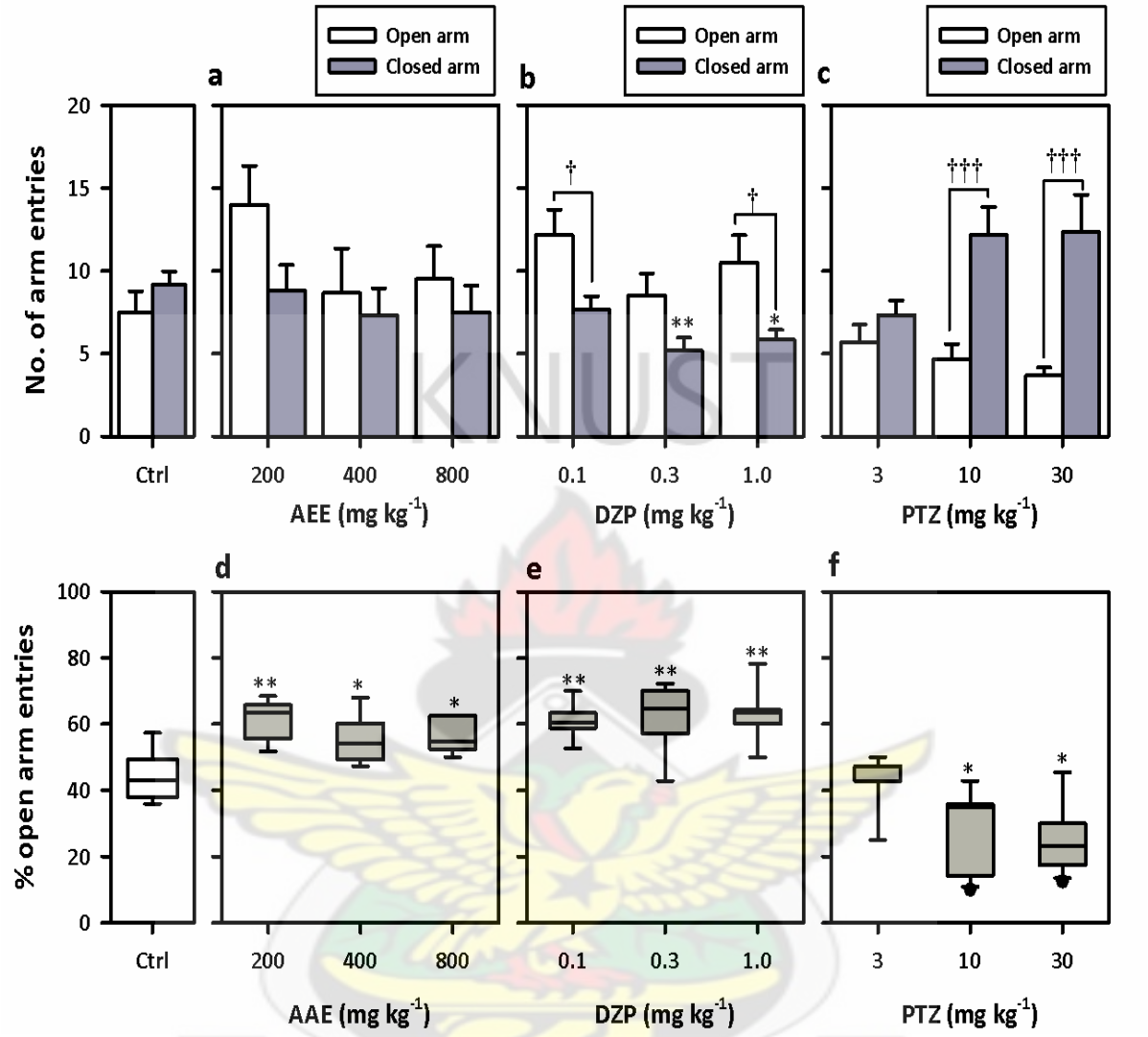

Figure 5.1 Effects of AAE (200, 400 and 800 mg kg^{-1}, *p.o.)* (a, d), diazepam (0.1, 0.3 and 1 mg kg^{-1}, *p.o.*) (b, e) and pentylenetetrazole (3, 10 and 30 mg kg^{-1}, i.p.) (c, f) treatment on frequency of arm entry and % open arm entry respectively in the elevated plus maze test. Data are presented as group means ±SEM (n=6). **P< 0.05, **P< 0.01* indicates significant differences between vehicle-treated group and drug treated groups (One-way ANOVA followed by Newman-Keuls' *post hoc* test). † $P \leq 0.05$, ††† $P \leq$ 0.001 (two-way ANOVA followed by Bonferroni post test).

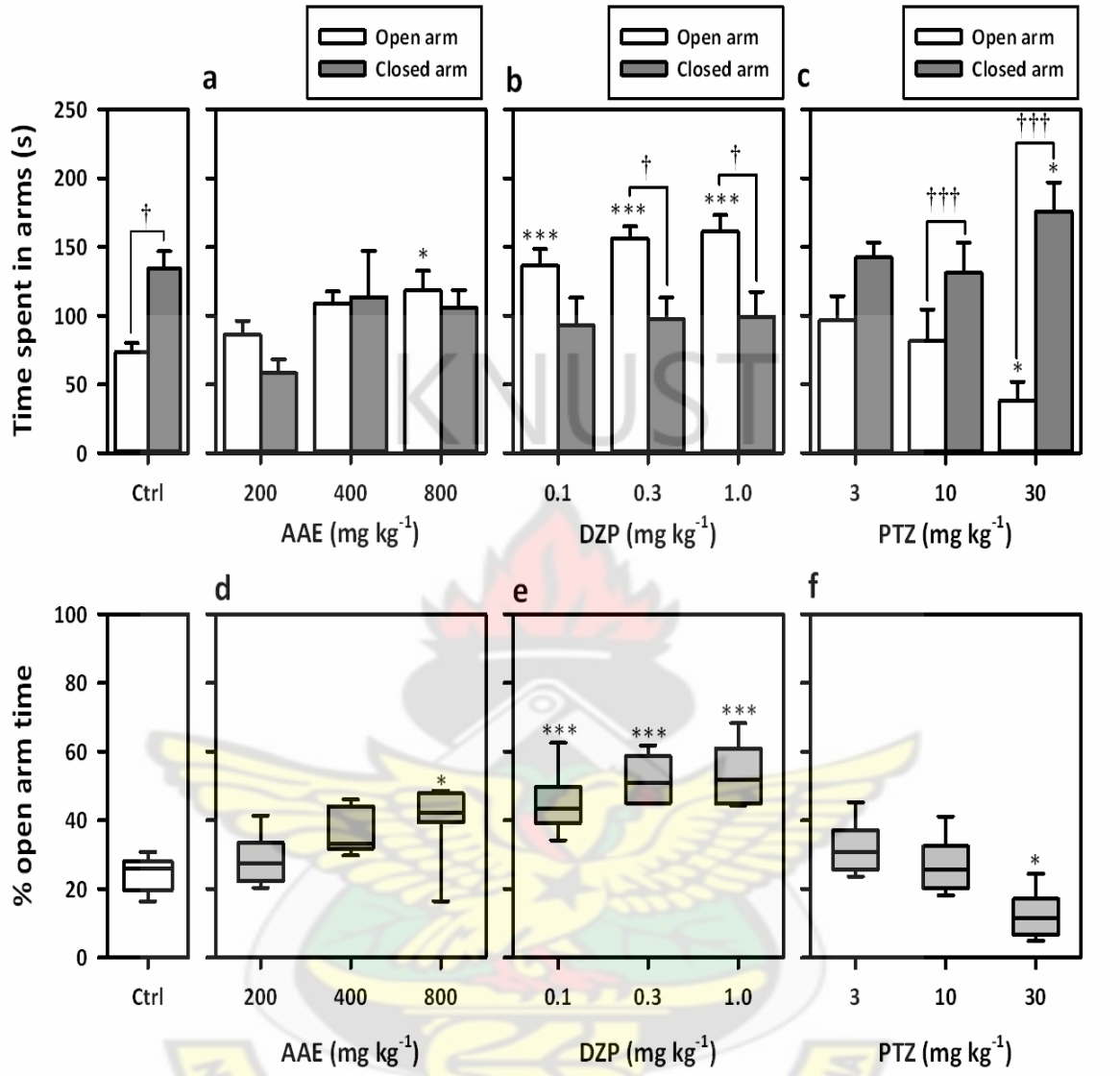

Figure 5.2 Effects of AAE (200, 400 and 800 mg kg^{-1}, *p.o.)* (a, d), diazepam (0.1, 0.3 and 1 mg kg^{-1}, *p.o.*) (b, e) and pentylenetetrazole (3, 10 and 30 mg kg^{-1}, i.p.) (c, f) on treatment on time spent in arms and % open arm time respectively in the elevated plus maze test. Data are presented as group means±SEM (n=6). *$P< 0.05$, **$P< 0.01$ indicates significant differences between vehicle-treated group and drug treated groups (One-way ANOVA followed by Newman-Keuls' *post hoc* test). †$P \leq 0.05$, †††$P \leq 0.001$ by two-way ANOVA followed by Bonferroni post test.

With regard to horizontal exploration, AAE caused significant ($F_{3, 20} = 55.76$, $P<0.0001$; Figure 5.3 a) increase in locomotor activity dose-dependently as reflected on the maze.

Diazepam produced similar increase in explorative activity in a dose dependent manner ($F_{3, 20}$=*20.29, P<0.0001*; Figure 5.3 b). Pentylenetetrazole produced an opposite response, decreasing the horizontal exploration ($F_{3, 20}$=*24.68, P<0.0001;* Figure 5.3 *c).*

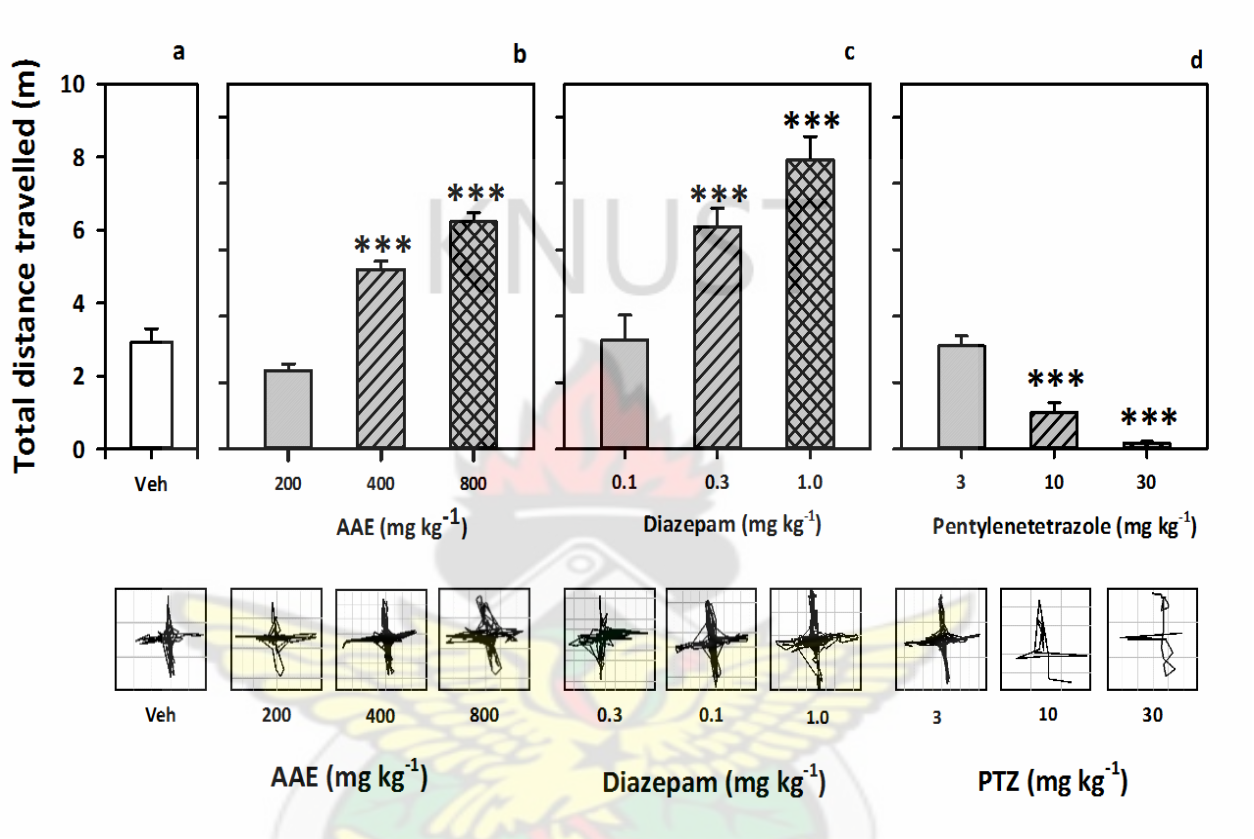

Figure 5.3 Effects of AAE (200, 400 and 800 mg kg^{-1}, *p.o.)* (b), diazepam (0.1, 0.3 and 1 mg kg^{-1}, *p.o.*) (c) and pentylenetetrazole (3, 10 and 30 mg kg^{-1}, i.p.) (d) on total distance travelled in the EPM. Data are presented as group means ±SEM (n=6). ****P<0.001* indicates significant differences between vehicle-treated group and drug treated groups (One-way ANOVA followed by Newman-Keuls' *post hoc* test). Line plots (lower panel) 3D plots were generated from the time and XY data obtained using SigmaPlot Version 12.0 (Systat Software Inc., Point Richmond, CA, USA).

5.3.1.2 ***Ethological Effects***

AAE caused a significant increase in the frequency of unprotected head dips (UHDs) in a dose-dependent manner. However, it was only significant (*P=0.0407*, $F_{3, 20}$=*3.319*, Figure 5.4 a) at the highest dose. There was also a significant (*P=0.0214*, $F_{3, 20}$=*4.037* Figure 5.4 a) decrease in protected head dips (PHDs) performed by extract treated animals by the 400 mg kg^{-1} dose. The total number of head dips (HDs) exhibited an

increase with increasing dose with effect being significant ($P=0.0237$, $F_{3,20}=3.920$, Figure 5.4 d) only at the highest dose. Diazepam caused a significant ($P=0.0100$, $F_{3,20}=4.934$, Figure 5.4 b) decrease in protected head dips at the highest dose but the increase in unprotected head dips was not significant statistically. The total number of head dips increased dose dependently and was significant ($P=0.0046$, $F_{3,20}=5.919$, Figure 5.4 e) at the 1 mg kg^{-1} dose. Pentylenetetrazole however showed no significant effect. Two-way analysis of variance (treatment group x HD type i.e. protected an d unprotected) revealed significant head dip type effect was produced by the extract where the UHDs increased significantly compared to the protected HDs. *Post hoc* analysis showed that all the doses were significant ($P=0.0005$, $F_{1,30}=25.38$, Figure 5.4 a). Diazepam ($P<0.0001$, $F_{1,30}=48.89$, Figure 5.2 b) in addition showed significant effect.

The number of stretch attend postures (SAPs) were not significantly affected by the extract. There was also a significant ($P=0.0023$, $F_{3,20}=6.851$, Figure 5.5 b) increase in unprotected stretch attend postures (USAPs) by diazepam treated animals at all doses. The decrease in protected stretch attend postures (PSAPs) was not significant statistically. The total number of SAPs showed no significance for all treatment groups. % protected SAPs was significantly decreased by both AAE ($P=0.0032$, $F_{3,20}=6.4111$, Figure 5.5 g) and diazepam ($P=0.0002$, $F_{3,20}=10.49$, Figure 5.5 h) at all three dose levels. Two-way analysis of variance (treatment group x SAP type i.e. protected and unprotected) revealed significant SAP type effect was produced by the extract where the USAPs increased significantly compared to the protected SAPs. *Post hoc* analysis showed that all the doses were statistically significant ($P=0.0003$, $F_{1,30}=28.88$, Figure 5.5 a). Diazepam in addition showed significant effect ($P<0.0001$, $F_{1,30}=51.92$, Figure 5.5 b). Pentylenetetrazole however showed no significant effect.

Rearing behaviour was increased by the extract. Both the number ($P=0.0471$, $F_{3,20}=3.162$, Figure 5.6 a) and duration ($P=0.0042$, $F_{3,20}=6.040$, Figure 5.6 a) of rears were significantly affected by the highest dose. Diazepam showed a similar pattern of increase producing significant effects on number ($P=0.0030$, $F_{3,20}=6.487$, Figure 5.6 b) and duration ($P=0.0002$, $F_{3,20}=10.57$, Figure 5.6 b) of rears at the highest dose only. PTZ decreased the number ($P=0.0072$, $F_{3,20}=5.350$, Figure 5.6 c) and duration ($P=0.002$, $F_{3,20}=7.097$, Figure 5.6 c) of rears also at the highest dose only.

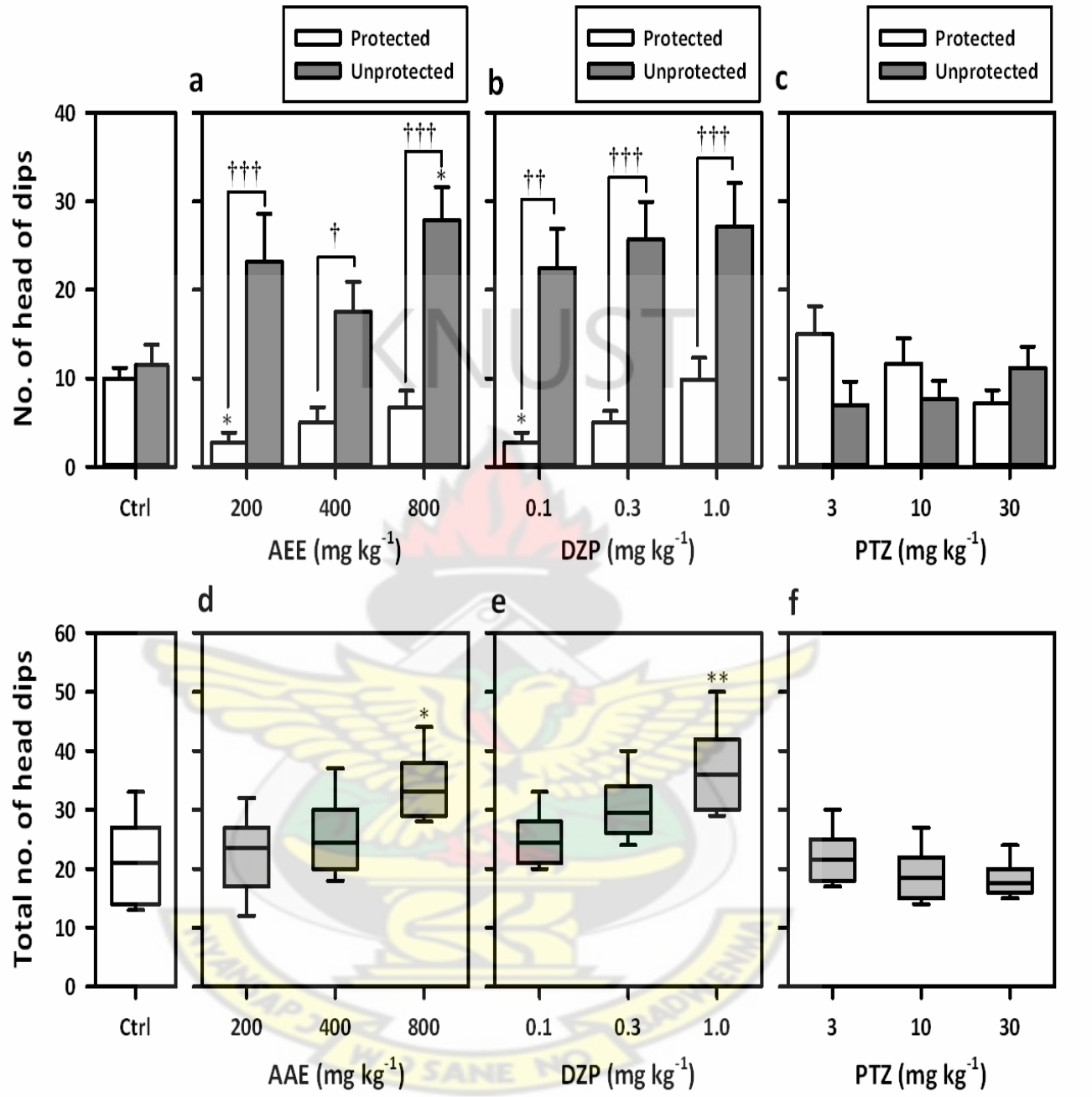

Figure 5.4 Effects of AAE (200, 400 and 800 mg kg^{-1}, *p.o.)* (a, d), diazepam (0.1, 0.3 and 1 mg kg^{-1}, *p.o.*) (b, e) and pentylenetetrazole (3, 10 and 30 mg kg^{-1}, i.p.) (c, f) on head dips in the EPM. Data is presented as mean±S.E.M (n=6). *$P<0.05$, ** $P< 0.01$ compared to vehicle-treated group (one-way ANOVA followed by Newman-Keuls' *post hoc* test) and †$P \leq 0.05$, ††$P \leq 0.01$, †††$P \leq 0.001$ (two-way ANOVA followed by Bonferroni post test).

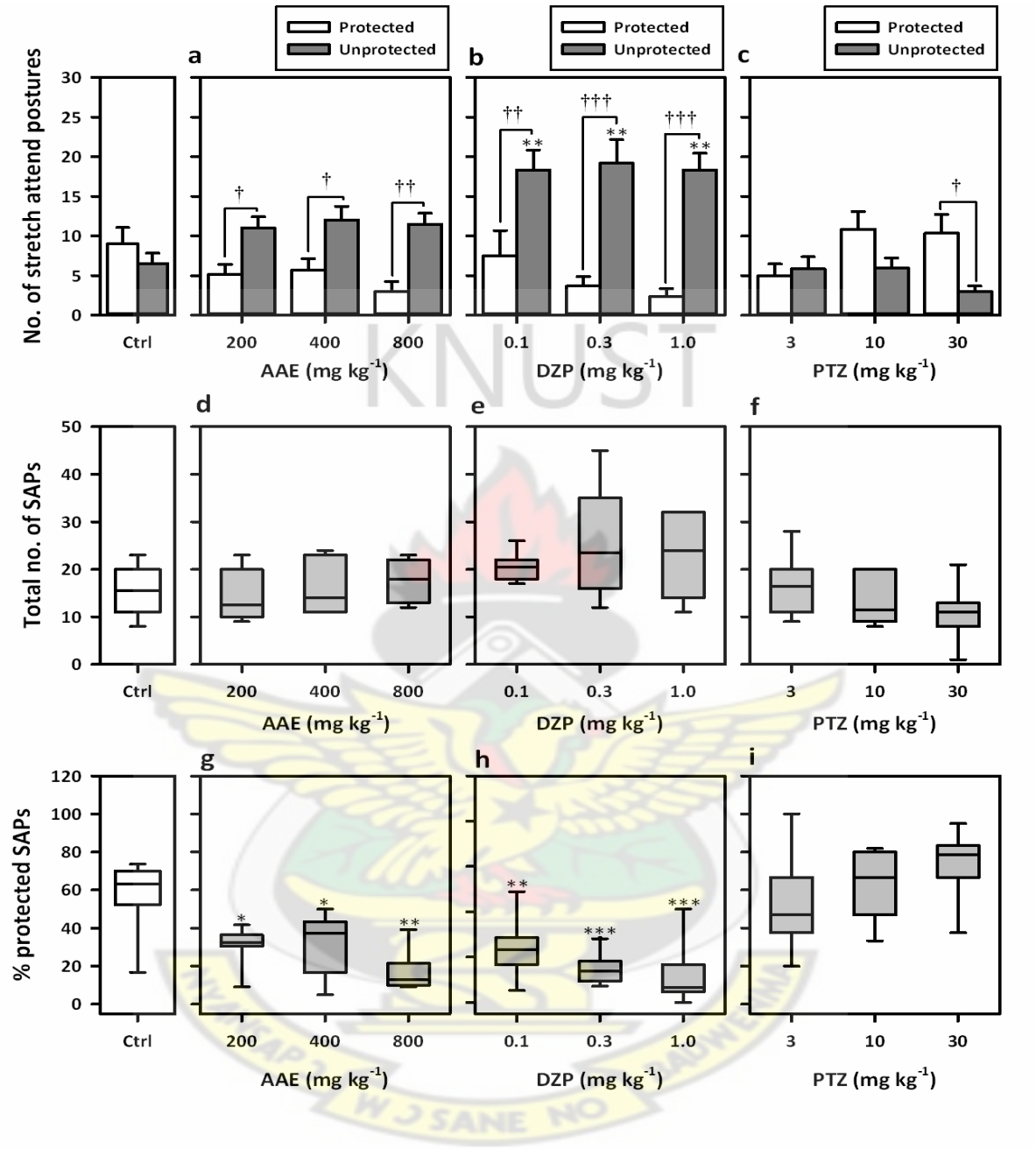

Figure 5.5 Effects of AAE (200, 400 and 800 mg kg^{-1}, *p.o.)* (a, d), diazepam (0.1, 0.3 and 1 mg kg^{-1}, *p.o.*) (b, e) and pentylenetetrazole (3, 10 and 30 mg kg^{-1}, i.p.) (c, f) on stretch attend postures in the EPM. Data is presented as mean±S.E.M (n=6) *$P<0.05$, ** $P<0.01$, *** $P<0.001$ compared to vehicle-treated group (one-way ANOVA followed by Newman-Keuls' *post hoc* test) and †$P \leq 0.05$, ††$P \leq 0.01$, †††$P \leq 0.001$ (two-way ANOVA followed by Bonferroni post test).

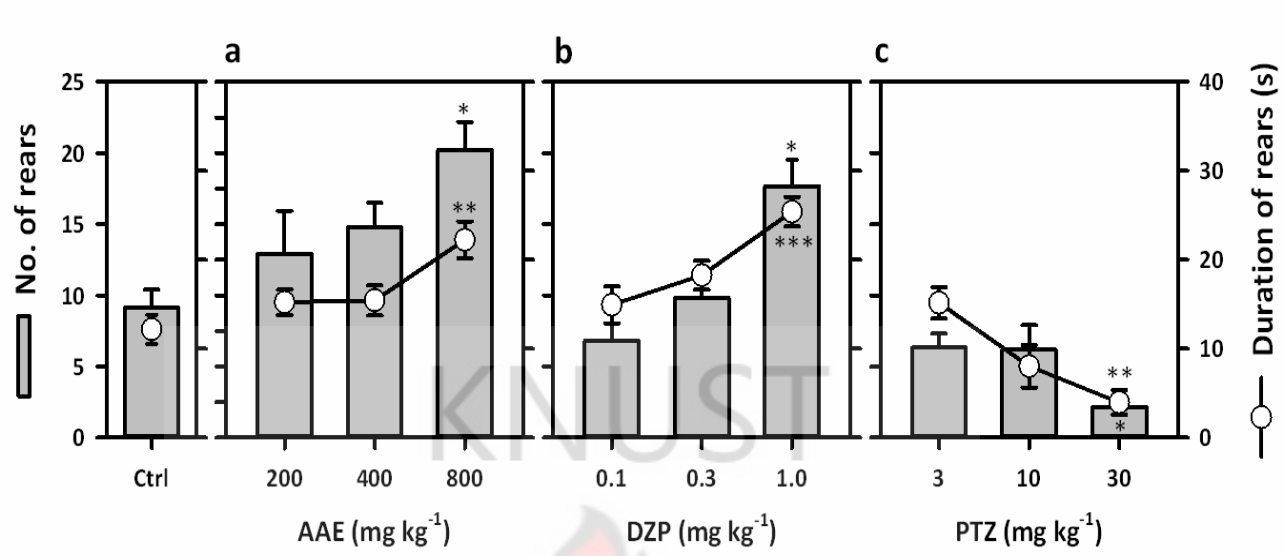

Figure 5.6 Effects of (a) AAE (200, 400 and 800 mg kg^{-1}, *p.o.*), (b) diazepam (0.1, 0.3 and 1 mg kg^{-1}, *p.o.*) and (c) pentylenetetrazole (3, 10 and 30 mg kg^{-1}, i.p.) on rearing behaviour in the EPM. Data is presented as mean±S.E.M (n=6). *$P<0.05$, ** $P<0.01$, *** $P<0.001$ compared with vehicle-treated group (one-way ANOVA followed by Newman-Keuls' *post hoc* test).

5.3.2 **Effects of light/ dark exploration**

In the light/dark test, AAE (200, 400 and 800 mg kg^{-1}, *p.o*) induced anxiolytic-like effects. Time spent in the lit box was increased significantly ($P=0.0276$; $F_{3,20}=3.747$, Figure 5.7 d) by the middle dose compared to the vehicle treated group. The time spent in the dark compartment was reduced by the extract but not significantly. Diazepam increased the time spent in the lit box significantly ($P=0.0101$; $F_{3,20}= 4.931$, Figure 5.7 e) also and with only the low dose significantly ($P=0.0262$; $F_{3,20}= 3.808$, Figure 5.7 e) decreasing time spent in the dark compartment. PTZ exhibited increase in time spent in the dark compartment and was significant ($P<0.0001$; $F_{3,20}= 11.64$, Figure 5.7 f) at the 30 mg kg^{-1} dose. The latency to emergence from the dark into the light compartment decreased significantly ($P=0.0003$; $F_{3,20}= 10.26$, Figure 5.7 a) for AAE in a linear fashion. Diazepam produced similar but less effective decreases ($P=0.0167$; $F_{3,20}= 4.321$, Figure 5.7 b). In contrast, pentylenetetrazole increased the latency to emergence but showed no statistical significance (Figure 5.7 c). Frequency

of transitions was not significantly affected by all treatments except diazepam at the 1 mg kg^{-1} dose ($P=0.0170$; $F_{3,16}= 4.576$, Figure 5.7 b).

Two-way analysis of variance assessed the effect of compartments (light and dark) on the treatment groups. Significant effect was produced by the extract where the time spent in the light compartments increased significantly compared to that of the dark compartment. *Post hoc* analysis showed statistical significance ($P=0.0374$, $F_{1,10}=5.76$, Figure 5.7 d) for the middle dose of the extract. The highest dose of diazepam ($P=0.0018$, $F_{3,30}=6.36$, Figure 5.7 e) in addition showed significant effect as well as all three dose of PTZ ($P<0.0001$, $F_{1,10}=74.49$, Figure 5.7 f).

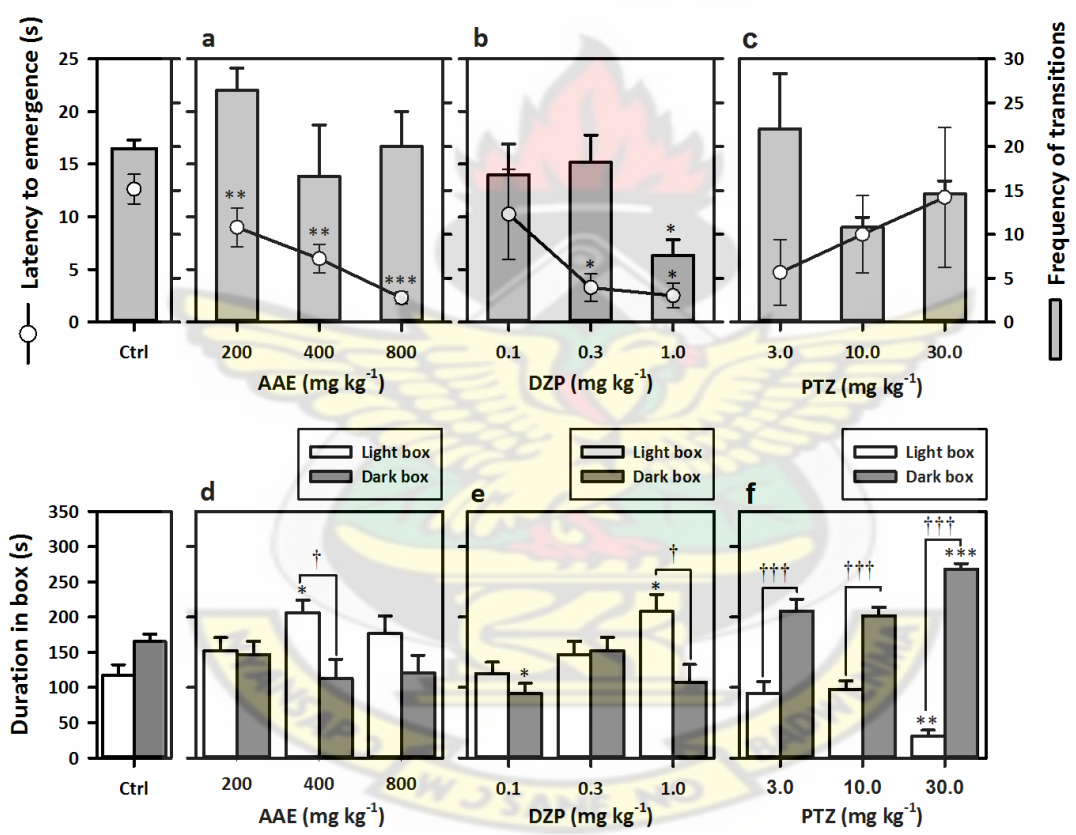

Figure 5.7 Effects of AAE (200, 400 and 800 mg kg^{-1}, *p.o.*) (a, d), diazepam (0.1, 0.3 and 1 mg kg^{-1}, *p.o.*) (b, e) and pentylenetetrazole (3, 10 and 30 mg kg^{-1}, i.p.) (c, f) on treatment on the frequency of compartmental transitions, latency to emergence and time spent in compartments. Data are presented as mean±SEM (n=6). *$P<0.05$, ** $P<0.01$, ***$P<0.001$ compared with vehicle-treated group (one-way ANOVA followed by Newman-Keuls' *post hoc* test) and †$P \leq 0.05$, †††$P \leq 0.001$ (two-way ANOVA followed by Bonferroni post test).

5.3.3 **Effects in social interaction test**

Rearing behaviour was increased by the extract. Both the frequency (*P=0.0022*, $F_{3, 28}$*=6.237*, Figure 5.8 a) and duration (*P=0.0037*, $F_{3, 28}$*=5.667*, Figure 5.8 a) of rears were significantly affected. Diazepam showed a higher increases producing significant effects on number (*P<0.0001*, $F_{3, 28}$*=15.83*, Figure 5.8 b) and duration (*P<0.0001*, $F_{3, 28}$*=28.88*, Figure 5.8 b). PTZ exhibited a paradoxical significant (*P=0.0150*, $F_{3, 28}$*=4.147*, Figure 5.8 c) increase in duration of rears at the middle dose.

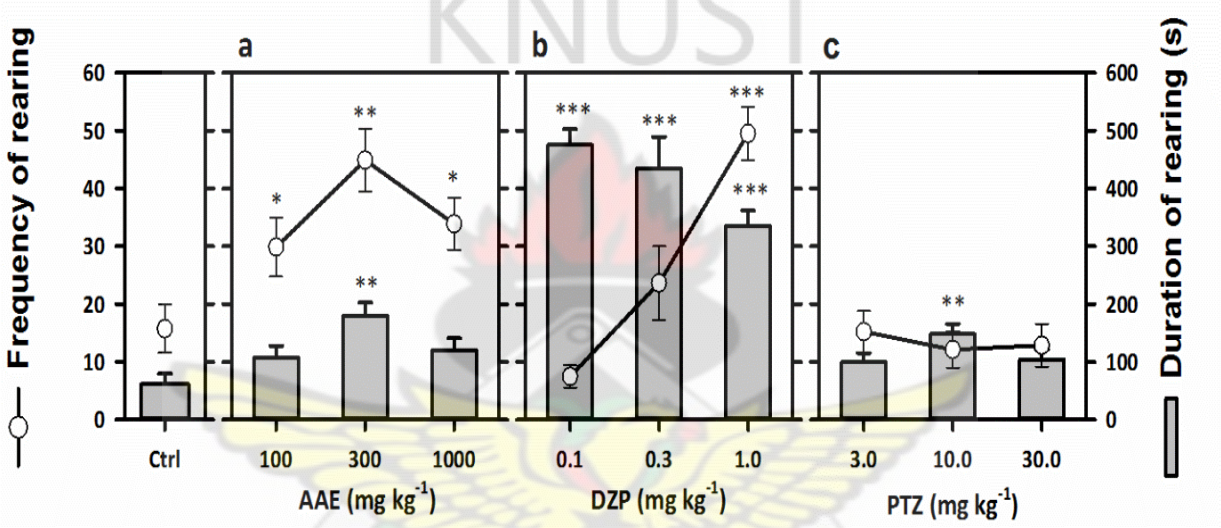

Figure 5.8 Effects of (a) AAE (100-1000 mg kg^{-1}, *p.o.*), (b) diazepam (0.1, 0.3 and 1 mg kg^{-1}, *p.o.*) and (c) pentylenetetrazole (3, 10 and 30 mg kg^{-1}, i.p.) on rearing behaviour in the social interaction test. Data is presented as mean±S.E.M (n=12). **P<0.05*, *** P<0.01*, **** P<0.001* compared with vehicle-treated group (one-way ANOVA followed by Newman-Keuls' *post hoc* test).

Frequency of transitions across the marked areas was significantly (*P=0.0050*, $F_{3, 28}$*=5.307*, Figure 5.9 a) increased by all the doses of AAE. Diazepam produced significant effects on frequency (*P=0.0023*, $F_{3, 28}$*=6.217*, Figure 5.9 b) only at the highest dose. Duration was significantly (*P=0.0003*, $F_{3, 28}$*=8.864*, Figure 5.9 b) increased also.

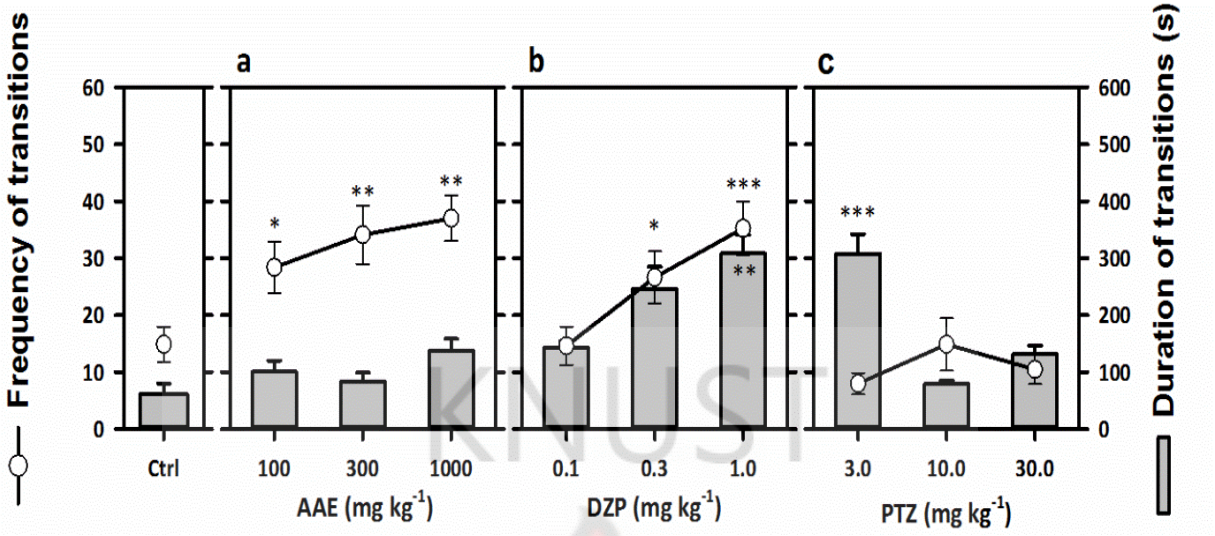

Figure 5.9 Effects of (a) AAE (100-1000 mg kg^{-1}, *p.o.*), (b) diazepam (0.1, 0.3 and 1 mg kg^{-1}, *p.o.*) and (c) pentylenetetrazole (3, 10 and 30 mg kg^{-1}, i.p.) on transitions in the social interaction test. Data is presented as mean±S.E.M (n=12). *$P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ compared with vehicle-treated group (one-way ANOVA followed by Newman-Keuls' *post hoc* test).

AAE showed no significant effects on climbing and following behaviours (Figure 5.10 a and 5.11 a). Diazepam however showed significant increases ($P<0.0001$, Figure 5.10 b and 5.11 b) in both behaviours in accordance with literature.

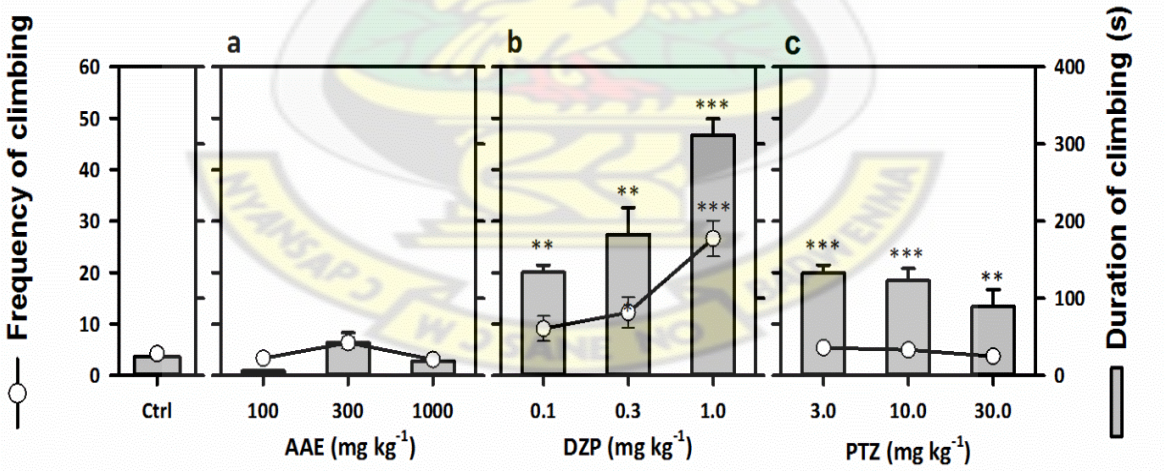

Figure 5.10 Effects of (a) AAE (100-1000 mg kg^{-1}, *p.o.*), (b) diazepam (0.1, 0.3 and 1 mg kg^{-1}, *p.o.*) and (c) pentylenetetrazole (3, 10 and 30 mg kg^{-1}, i.p.) on climbing behaviour in the social interaction test. Data is presented as mean±S.E.M (n=12) *$P<0.05$, ** $P< 0.01$, *** $P<0.001$ compared with vehicle-treated group (one-way ANOVA followed by Newman-Keuls' *post hoc* test).

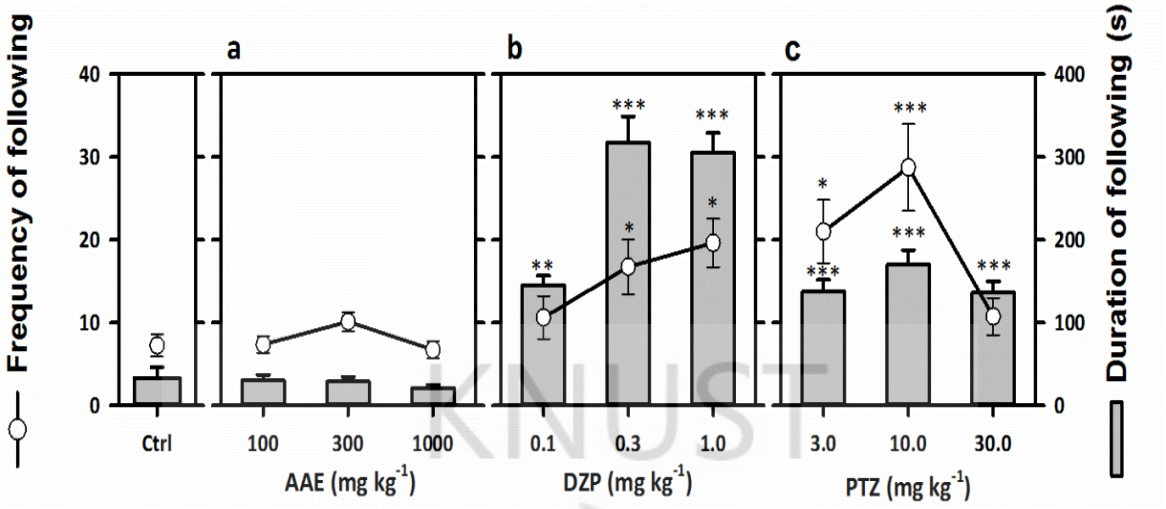

Figure 5.11 Effects of (a) AAE (100-1000 mg kg^{-1}, *p.o.*), (b) diazepam (0.1, 0.3 and 1 mg kg^{-1}, *p.o.*) and (c) pentylenetetrazole (3, 10 and 30 mg kg^{-1}, i.p.) on following behaviour in the social interaction test. Data is presented as mean±S.E.M (n=12) *$P<0.05$, ** $P<0.01$, *** $P< 0.001$ compared to vehicle-treated group (one-way ANOVA followed by Newman-Keuls' *post hoc* test).

5.4 DISCUSSION

The present study has shown potential anxiolytic activity in the aqueous stem bark extract of *Antiaris toxicaria*, as assessed by the light/dark box, elevated plus maze and social interaction tests. Behavioural models used in the study are based on unconditioned responses to stimuli which are thought to be indicative of human generalized anxiety symptoms (Crawley, 1999; Ohl, 2005).

The light/dark test is an approach-avoidance conflict test and it is sensitive to drugs that affect anxiety (Costall *et al.*, 1989; Chaouloff *et al.*, 1997; Crawley *et al.*, 1997; Ohl, 2005). AAE treated–mice, just like diazepam, spent more time in the lit chamber of the box more than control animals. The time spent in the illuminated compartment according to Young and Johnson, 1991, is the most consistent parameter for evaluating anxiolytic activity as compared to the frequency of transitions. The frequency of transitions however reflects both anxiety and exploration. Time spent in the light area being a stronger indication in the study of anxiety and the most robust indicator is also affirmed by Lepicard *et al.*, 2000.

The elevated plus maze uses natural stimuli which is fear of a novel, brightly-lit open space and a raised platform. Animals thus prefer to remain in the closed arms rather than venture into the open. It has been suggested that this preference is derived from the possibility of thigmotaxis, so the avoidance of the open arms occurs primarily because of the absence of walls and not due to distance from the ground (Treit *et al.*, 1993). This test has been shown to be bidirectionally sensitive to drugs that are designed to affect anxiety (Handley and Mithani, 1984; Pellow *et al.*, 1985; Pellow and File, 1986; Lister, 1987).

Anxiolytic agents, such as diazepam, are expected to increase the time spent in the open arms of the EPM as well the frequency of transitions (Moser, 1989; Helton *et al.*, 1998). Oral administration AAE induced an anxiolytic-like effect in mice, since it increased the percentage number of open arm entries and the time spent in the open arms of the EPM.

Analysis of exploratory behaviour in the EPM has been extended to include ethological factors. These factors have a biological function of monitoring behavioural patterns of rodents in potentially dangerous situations (Blanchard *et al.*, 1993). These behaviours have been shown by factor analysis to be strongly indicative of anxiety (Rodgers and Johnson, 1995; Rodgers *et al.*, 1997). The ethological assessment revealed an increase in unprotected head dips with a corresponding decrease in protected head dips by both the extract and diazepam. They also produced an increase in total head dips. This is a clear indication of reduced anxiety as well as an increase in explorative tendencies (Rodgers *et al.*, 1997). Likewise, unprotected stretch attend postures were increased with a reduction in % protected SAPs. Total number of SAPs is an indication of risk assessment behaviour. Unlike head-dips, SAPs are generally more defensive behaviour rather than approach (Lepicard *et al.*, 2000). AAE, however, had no significant effect on total SAPs. Rodents display risk assessment behaviours even after ceasing to avoid unprotected areas, suggesting the defensive pattern may be even more sensitive to anxiety modulating drugs than avoidance-related measures (Rodgers *et al.*, 1997; Albrechet-Souza and Brandão, 2010). Both the duration and number of rears were increased by the highest dose indicating that the extract promotes vertical activity in rodents.

In an unfamiliar, brightly lit environment, social interaction of rodents is suppressed. The social interaction test, therefore measures equal or less exploration of a novel social target over a familiar one (Crawley, 2004; Moy *et al.*, 2007). Social interaction is assessed by timing behaviours such as following, climbing over and sniffing of partner. AAE did not significantly affect these parameters assessed indicating that it has little effect on social interaction in rodents. It however showed an increase in transitions or crossings across the marked areas. This is a measure of exploratory motion. An increase in rearing behaviour further confirmed its anxiolytic properties. Anxiolytics have been proposed to increase the social interaction time in the social interaction test (File, 1985) and this was confirmed by diazepam. In the present study, AAE treated mice exhibited a lack of social interaction in spite of reduced anxiety related behaviour.

5.5 CONCLUSION

Results presented here indicate that the aqueous stem bark extract of *Antiaris toxicaria* produces anxiolytic effects devoid of an increase in social interaction in rodents unlike diazepam.

Chapter 6

ANTIDEPRESSANT ACTIVITY OF THE EXTRACT

6.1 INTRODUCTION

Depression is a common comorbid condition with epilepsy and epileptic patients have been reported to be more susceptible to suicide ideation (Christensen *et al.*, 2007). An imbalance in levels of monoamines such as serotonin and noradrenaline is considered as the most important cause of clinical depression. Hence, a number of antidepressants in use currently interact with monoaminergic systems (Berton and Nestler, 2006; Yacoubi *et al.*, 2011). These medications, however, are plagued with a myriad of adverse effects (Poleszak *et al.*, 2011) and are slow in onset of action (Gourion, 2008). Furthermore, between 30 to 40% of patients are believed to have conditions refractory to current therapy (Belmaker and Agam, 2008).

Consequently, there is an unmet need for newer therapeutic agents possessing fewer adverse effects and faster onset of action as well as wider patient spectrum (Cryan *et al.*, 2002; Nestler *et al.*, 2002). Besides, a growing number of anticonvulsants are receiving attention as antidepressants and mood stabilizers (Bourin *et al.*, 2009). This attention is based chiefly on the assumption that the antimanic efficacy of anticonvulsants makes them appropriate as mood stabilizers due to their mechanisms of action (Ernst and Goldberg, 2003). This makes *Antiaris toxicaria* a potential for antidepressant effect. Although traditional approaches to health are not always easily translated into biomedical categories, numerous plant species used traditionally have shown antidepressant activity in animal models (Piato *et al.*, 2009).

Thus, this study investigated the effect of the aqueous stem bark extract of *Antiaris toxicaria* in acute antidepressant models—the forced swim (FST) and tail suspension tests (TST).

6.2 MATERIALS AND METHODS

6.2.1 Animals

Male ICR mice (20-25 g) were obtained from the Noguchi Memorial Institute for Medical Research. Animals were cared for in the Departmental Animal House for use in this study. Prior to testing, animals were allowed to acclimatize to laboratory conditions of temperature, humidity and light. They were also allowed free access to water and food and housed in standard cages. Groups of eight to ten animals were used. Animals were treated according to the Guide for the Care and Use of Laboratory Animals (NRC, 1996) and experiments were approved by the Faculty Ethics Committee.

6.2.2 Drugs and chemicals

Fluoxetine hydrochloride (Prozac®) (FLX), imipramine hydrochloride (IMI), α-methyldopa (α-MD), reserpine, ρ-chlorophenylalanine (PCPA), α-methyl-ρ-tyrosine (AMPT), D-tubocurarine (d-TC), diazepam (DZP), D-serine, D-cycloserine (D-CS), desipramine (DES), 5-hydroxytryptophan (5-HTP), adrenaline, carbachol and propranolol (PROP) were purchased from Sigma-Aldrich Inc., St. Louis, MO, USA.

6.2.3 Forced swimming test (FST)

The test was performed as previously described by (Porsolt *et al.*, 1977). Mice were placed in vertical cylindrical plastic containers (25 cm high, 10 cm internal radius) filled with water (25°C to 28°C) up to a level of 20 cm, for 7 minutes each. Five identical cylinders were used separated by opaque screens. Each session was recorded by a video camera suspended approximately 75 cm above the cylinders. Five animals were tested simultaneously. After each session, animals were removed from the cylinders, dried with absorbent towels and then returned to their home cages. Water was changed for each mouse. Scored behaviours were defined as mobility (swimming and struggling) and immobility (floating with only minimal movements needed to keep head above water). Mice were divided into seven groups (n=8) and received either the extract (200, 400 or 800 mg kg^{-1}, *p.o.*), the vehicle (10 ml kg^{-1}, *p.o.*) or the standard reference drug imipramine and fluoxetine (3, 10 or 30 mg kg^{-1}, *p.o.*).

6.2.4 Tail suspension test (TST)

The test was carried out as previously described by Steru *et al.*, 1985. Mice were divided into ten groups of five (n=10) and received either extract (200, 400 or 800 mg kg^{-1}, *p.o.*), the vehicle (10 ml kg^{-1}, *p.o.*) or the standard reference drug imipramine and fluoxetine (3, 10 or 30 mg kg^{-1}, *p.o.*). Mice were individually suspended by the tail from a horizontal bar raised 30 cm above the bench top using adhesive tape (distance from tip of tail = 1 cm). Scored behaviour was defined as mobility (struggling) and immobility (lack of movement).

6.2.5 Effect of monoamine depletion on the anti-depressant actions of AAE

The possible role of noradrenergic and serotoninergic systems in the actions of AAE was investigated by carrying out serotonin (5-HT) and catecholamine depletion. Doses of *para*-chlorophenylalanine (PCPA), *α*-methyldopa (*α*-MD) and reserpine were chosen based on previous work by O'Leary *et al.*, 2007 and Woode *et al.*, 2010. To selectively deplete 5-HT, mice were pretreated with PCPA, a tryptophan hydroxylase inhibitor, at a dose of 300 mg kg^{-1}; i.p twice daily for three consecutive days. Animals were tested on the fourth day 20 hours after the last dose. Mice were treated with a single dose of α-MD (200 mg kg^{-1}, i.p.) three and a half hours before behavioural testing, in order to deplete newly synthesized pools of noradrenaline (NA). To deplete vesicular pools of NA and 5-HT, mice received a single dose of reserpine (1 mg kg^{-1}, s.c.) twenty-four hours before behavioural testing. Both the vesicular and cytoplasmic pools of NA and 5-HT were depleted with a combination of reserpine (1 mg kg^{-1}, s.c.) 24 h and α-MD (200 mg kg^{-1}, i.p.) 3.5 h, before behavioural testing, respectively. All control animals received normal saline. The tail suspension test was then carried out following monoamine depletion.

6.2.6 Involvement of glycine/NMDA receptors

Investigation into the possible involvement of glycine/NMDA receptors in the mechanism of action was carried out. The procedure as described by Poleszak *et al.*, 2011 was used with slight modifications. D-serine (320 mg kg^{-1}; i.p), a full agonist at the glycine/NMDA receptor was used to antagonise the antidepressant effects of the extract and standard antidepressants. D-cycloserine is a partial agonist at the

glycine/NMDA site that acts as an antidepressant. Monoamine depletion was carried out to investigate the effects of monoamines on NMDA receptor activity. 5-HT was depleted using PCPA at a dose of 300 mg kg^{-1}; i.p twice daily for three consecutive days. Animals were tested on the fourth day 20 hours after the last dose. α-methyl-ρ-tyrosine (400 mg kg^{-1}, i.p.) was administered three and a half hours before behavioural testing, in order to deplete newly synthesized pools of noradrenaline (NA).

6.2.7 Potentiation of 5-Hydroxytryptophan-induced head twitches

Groups of 10 mice (20-30 g) were used. They were treated with either AAE (400 mg kg^{-1}; *p.o*), Fluoxetine (20 mg kg^{-1}; *p.o*) or distilled water. Thirty minutes later, the mice received 200 mg kg^{-1} of 5-Hydroxytryptophan via intraperitoneal route. The number head twitches exhibited by the mice were recorded for the next thirty minutes and presented as the head twitch score (Koe *et al.*, 1983).

6.2.8 Involvement of opioidergic mechanisms in the tail suspension test

This evaluation was done as described by Berrocoso *et al.*, 2011. Swinging behaviour was defined as when the animal moved alternately from side to side with the body straight. Pedalling behaviour was when the animal moved its paws continuously without moving its body. When the animal raised its head towards its hind paws it was defined as curling. Behaviours were assessed in the tail suspension test as carried out above.

6.2.9 β_2-Adrenoceptor activity

This was tested using the isolated rat uterus preparation. The rat uterus muscle was obtained from a freshly killed Sprague-Dawley rat (200 g). The muscle was suspended under a resting tension of 0.5 g in a 10 ml organ bath containing De Jalon's solution. The solution was maintained at 32 ºC and aerated with oxygen (95 %) and carbon dioxide (5 %). Isotonic contractions were recorded on a single channel pen recorder. Dose-response curves to carbachol were produced and mean (n=3) EC_{80} (8.76×10^{-7} M) was chosen. Increasing concentrations of the extract were administered in the presence of the EC_{80} of carbachol and responses compared to the carbachol control response. Propranolol (10^{-7} to 10^{-5} M) was then added to the physiological saline

solution for the remainder of the experiment and the above procedure repeated. Responses to adrenaline were used as standard following the same protocol. The % inhibition of carbachol-induced contractions was calculated.

6.2.10 **Motor co-ordination - Rotarod test**

Effect of the extract on motor coordination was assessed using the rotarod apparatus. The rotarod apparatus (model 7600, Ugo Basile, Cormerio, Italy) rotated at a speed of 18 rpm. This apparatus consists of a base platform and a rotating rod of 3 cm diameter with a non-skid surface. The rod, 50 cm in length, is divided into five equal sections by six disks. Before the start of the experiment, animals were trained to stay on the rotarod for 300 s. Mice that did not achieve 300 s endurance were excluded from the study. Mice were taken through five training runs. On the test day, five mice were tested simultaneously. The length of time each mouse remained on the rod (maximal score 300 s) was measured after administration of the test compounds or vehicle. Integrity of motor coordination was assessed by the time spent on the rotating rod. Animals received either distilled water (10 ml kg^{-1}; *p.o*), extract (200, 400 and 800 mg kg^{-1}; *p.o*), diazepam (0.1, 0.3 and 1.0 mg kg^{-1}, i.p.) or *d*-tubocurarine (3, 10 and 30 *u*g kg^{-1}, i.p.) (Dunham and Miya, 1957).

6.2.11 **Data analysis**

Values were presented as mean±S.E.M. One-way analysis of variance (ANOVA) followed by Newman-Keuls' *post hoc* test was used to determine significant differences between means. Two-way ANOVA followed by Bonferroni test was used in the forced swimming and tail suspension tests. Statistical analyses were carried out with Graph Pad Prism® Version 5.0 (GraphPad Software, San Diego, CA, USA). $P < 0.05$ was considered significant.

6.3 RESULTS

6.3.1 Effect in forced swimming test

In the FST, mobility time was increased significantly (*P= 0.0338; $F_{3, 16}$= 3.703;* Figure 6.1 a) only by the 800 mg kg^{-1} dose of the extract. Fluoxetine showed a similar increase while imipramine showed higher increases which were significant (*P= 0.0031; $F_{3, 16}$= 7.059;* Figure 6.1 c) at the 10 and 30 mg kg^{-1} doses. Immobility time was significantly (*P=0.0423; $F_{3, 16}$ = 3.434*; Figure 6.1 a) decreased by the middle dose of AAE. A significant (*P=0.0142; $F_{3, 16}$ = 4.809*; Figure 6.1 b) decrease in immobility period occurred at all doses for fluoxetine. Imipramine showed significant (*P=0.0035; $F_{3, 16}$ = 6.841*; Figure 6.1 b) decrease in immobility periods in a non-dose-dependent manner. Dose-response curves (Figure 6.2) showed slight changes in the percentage decrease in immobility with the middle dose showing the most significant change. Slopes of the AAE mobility curves were similar to that of fluoxetine indicating possible action at similar receptor sites. AAE was less potent in increasing % mobility as compared to the standards by about 41.85 times for fluoxetine and 77.69 times for imipramine. Efficacies were however comparable as all three attained an E_{max} of 100%.

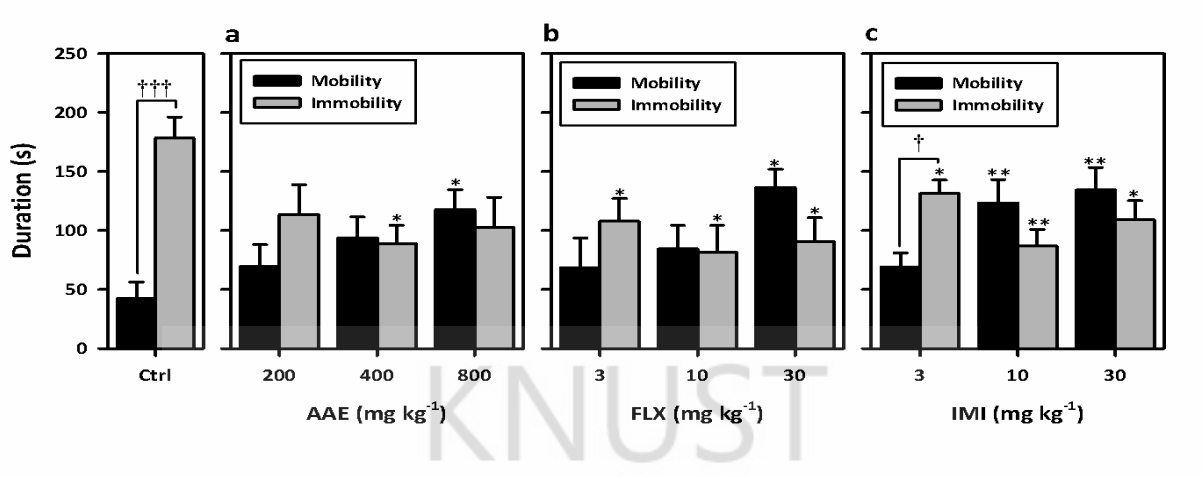

Figure 6.1Effects of acute AAE (200, 400 and 800 mgkg^{-1}, *p.o.*), fluoxetine (3, 10 and 30 mgkg^{-1}, *p.o.*) and imipramine (3, 10 and 30 mgkg^{-1}, *p.o.*) treatment on mobility and immobility times in FST. Data are presented as group means±SEM (n=8). Significantly different from control: **P*<0.05, ***P*<0.01(one-way ANOVA followed by Newman-Keuls' *post hoc* test) and †*P* <0.05, †††*P* < 0.001 by two-way ANOVA followed by Bonferroni post test.

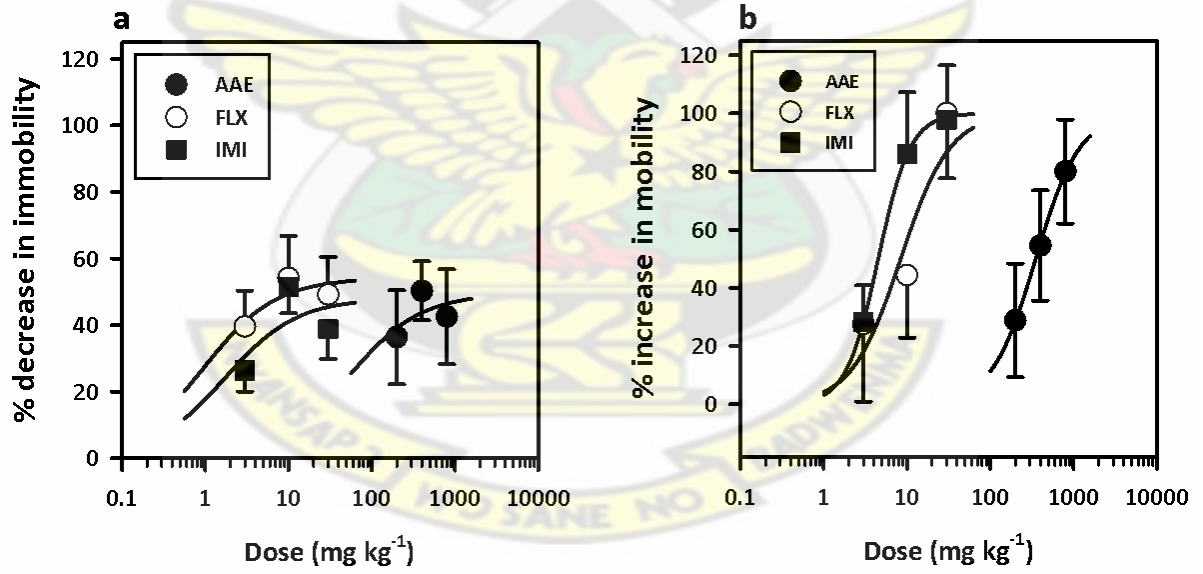

Figure 6.2 Dose-response curves of AAE, fluoxetine and imipramine showing % decrease in immobility (a) and % increase in mobility (b) in the forced swim test. Each point represents mean ± S.E.M (n=8).

6.3.2 **Effect in the tail suspension test**

All doses showed a significant decrease in immobility periods for extract treated groups (*P=0.0019; $F_{3, 16}$ = 7.826*; Figure 6.3 a) with only the highest dose showing a significant (*P=0.0206; $F_{3, 16}$ = 4.321;* Figure 6.3 a) increase in mobility periods. Imipramine produced a significant (*P=0.0003; $F_{3, 16}$ = 11.77*; Figure 6.3 c) decrease in immobility time which was dose dependent. The increase in mobility period was also significant (*P=0.0023*; *$F_{3, 16}$ = 7.574;* Figure 6.3 c) at all doses. Fluoxetine produced a decrease in immobility periods as well (*P<0.0011*; *$F_{3, 16}$= 8.910*, Figure 6.3 b) in addition to a significant increase in mobility time (*P<0.0008*; *$F_{3, 16}$= 9.531;* Figure 6.3 b). Slopes of the AAE curves (Figure 6.4), however, were not as similar to that of fluoxetine. AAE was less potent in increasing % mobility as compared to the standards by about 102.62 times for fluoxetine and 164.05 times for imipramine. It proved to be almost equipotent in comparison with the FST. Efficacies were however comparable as all three treatments attained an E_{max} of 100%.

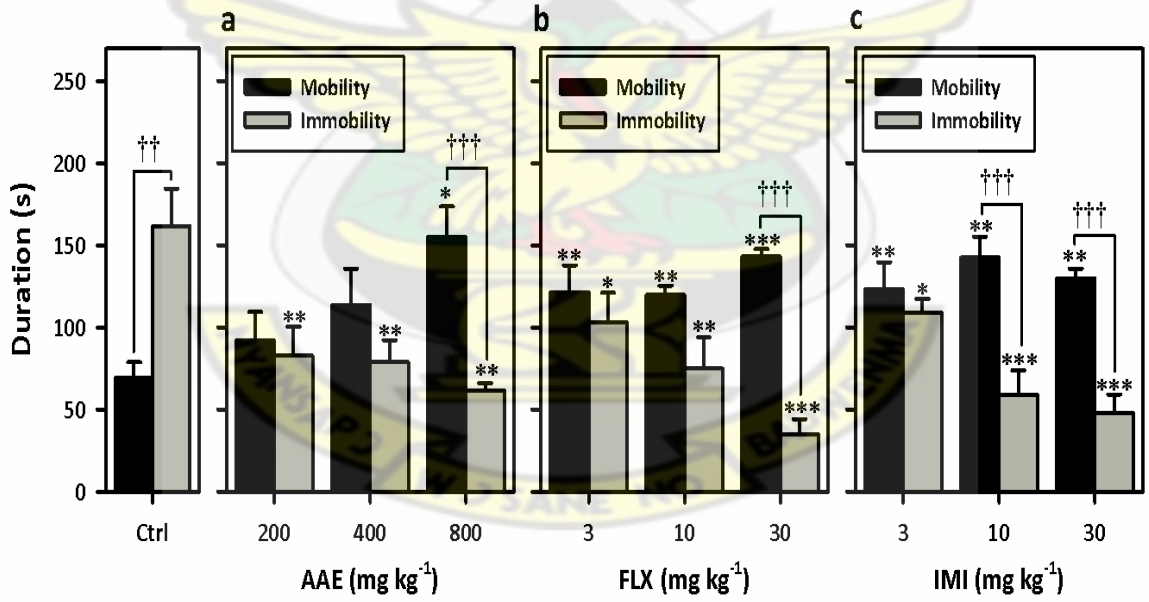

Figure 6.3 Effects of acute AAE (200, 400 and 800 mgkg^{-1}, *p.o.*), fluoxetine (3, 10 and 30 mgkg^{-1}, *p.o.*) and imipramine (3, 10 and 30 mgkg^{-1}, *p.o.*) treatment on mobility and immobility times in TST. Data are presented as group means±SEM (n=10). Significantly different from control: **P*<0.05, ***P*<0.01, ****P*<0.001(one–way ANOVA followed by Newman-Keuls' *post hoc* test). ††*P* < 0.01, †††*P* <0.001 by two-way ANOVA followed by Bonferroni post test.

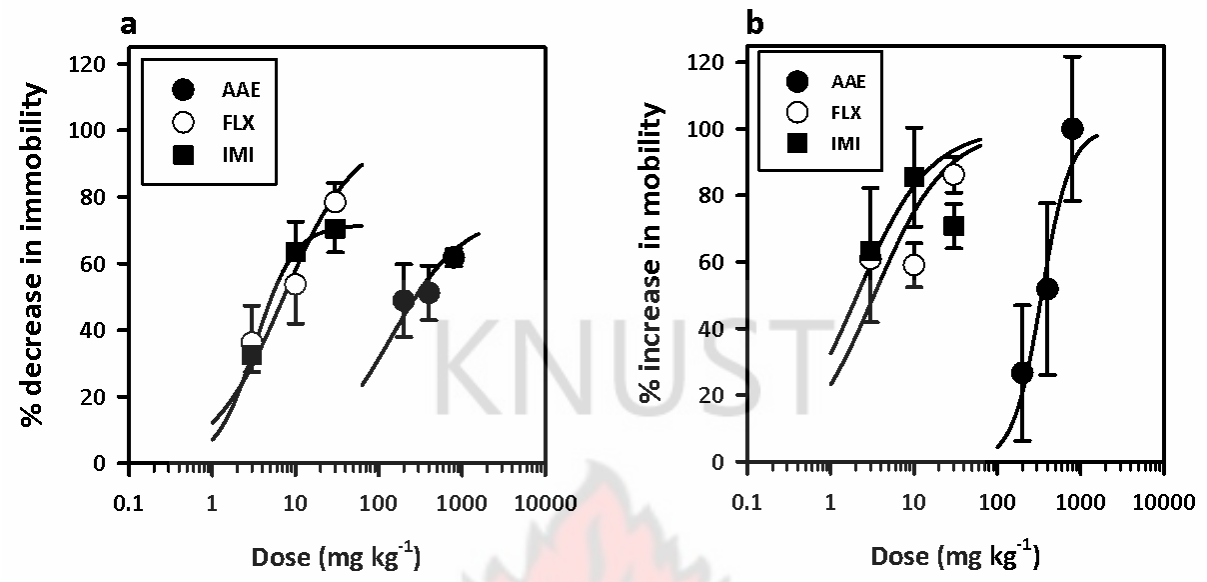

Figure 6.4 Dose- response curves of AAE, fluoxetine and imipramine showing % decrease in immobility (a) and % increase in immobility (b) in the tail suspension test. Each point represents mean ± S.E.M (n=10).

6.3.3 **Effects in evaluation of opioidergic mechanisms**

Swinging frequency was not affected by the extract. The duration, however, showed significant (P<0.05; Figure 6.5a) increase at the highest dose. A significant decrease in both pedalling frequency and duration (P<0.01; Figure 6.5b) in a non-dose-dependent manner was obtained. AAE showed no significant increase in curling frequency but a significant (P<0.01; Figure 6.5 c) increase in the duration of curling.

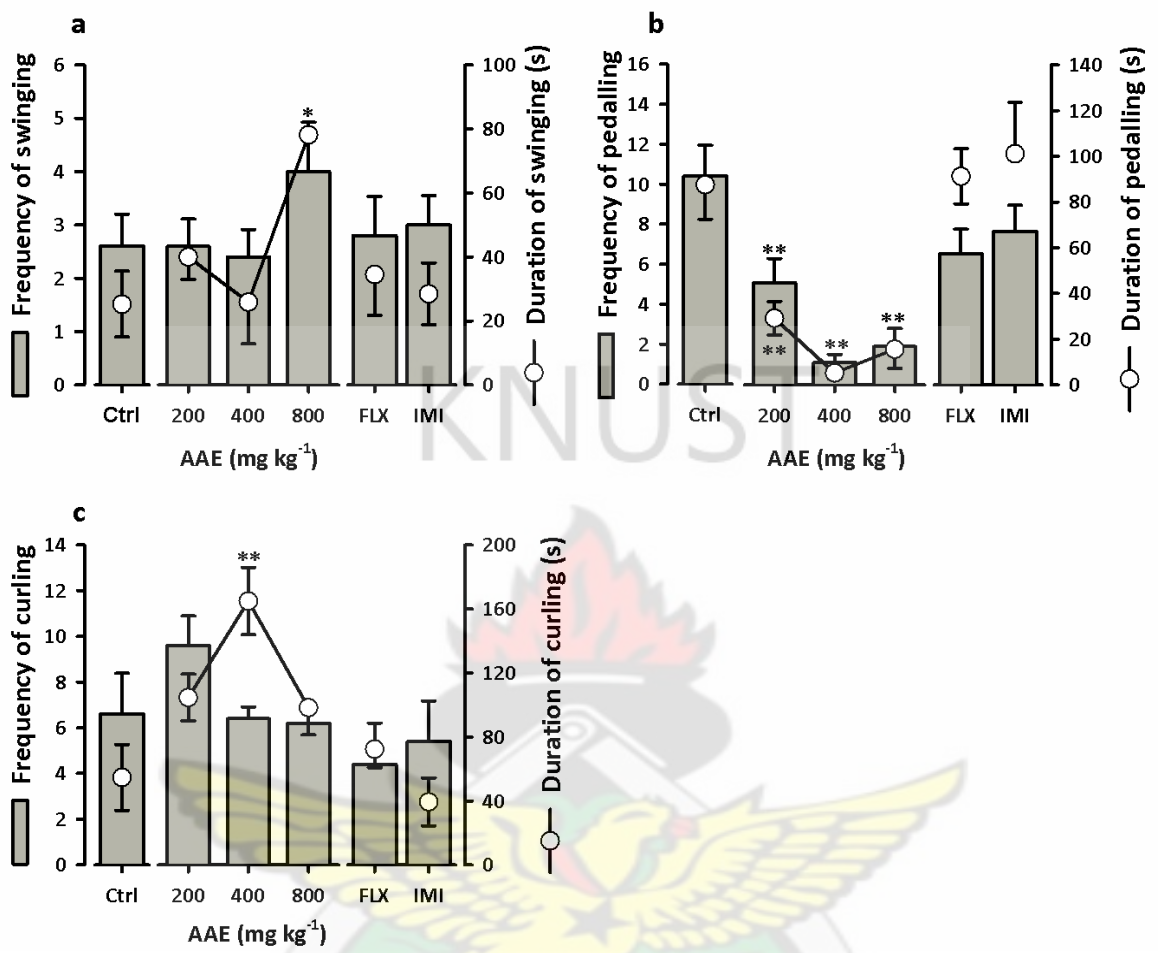

Figure 6.5 Effects of acute AAE (200, 400 and 800 mg kg^{-1}), imipramine (30 mg kg^{-1}) and fluoxetine (30 mg kg^{-1}) treatment on swinging (a), pedalling (b) and curling behaviour (c) in TST. Data are presented as group mean±SEM (n=10). **P<0.05, **P<0.01* compared to vehicle-treated group (one-way ANOVA followed by Newman-Keuls' *post hoc* test).

6.3.4 **Effect of monoamine depletion on the effect of AAE**

AAE, imipramine (TCA) and fluoxetine (SSRI) were not able to significantly attenuate or reverse the immobility induced by α-MD. Two-way ANOVA shows significant (*P<0.0001*; $F_{1, 18} = 25.70$; Figure 6.6 a) increase in immobility periods by α-MD. The extract was also not able to significantly reverse the immobility periods induced by pretreatment with PCPA just as fluoxetine. Imipramine, however, significantly *(P<0.001*; Figure 6.6 b) reversed the increase in immobility time compared to control. PCPA caused significant (*P<0.05*; $F_{1, 18} = 5.64$, Figure 6.6 b) depletion of serotonin hence

the inability of fluoxetine to act. From the effects of pretreatment with reserpine, fluoxetine, AAE and imipramine were not able to cause any significant change in reserpine-induced immobility.

Pretreatment with a combination of reserpine (1 mg kg^{-1}, s.c.) 24 h before the TST and α-MD (200 mg kg^{-1}, i.p.) 3.5 h before the TST depleted both the newly formed stores of catecholamines as well as vesicular storage respectively. The behavioural effects of all the tested antidepressant drugs were completely blocked by this combination.

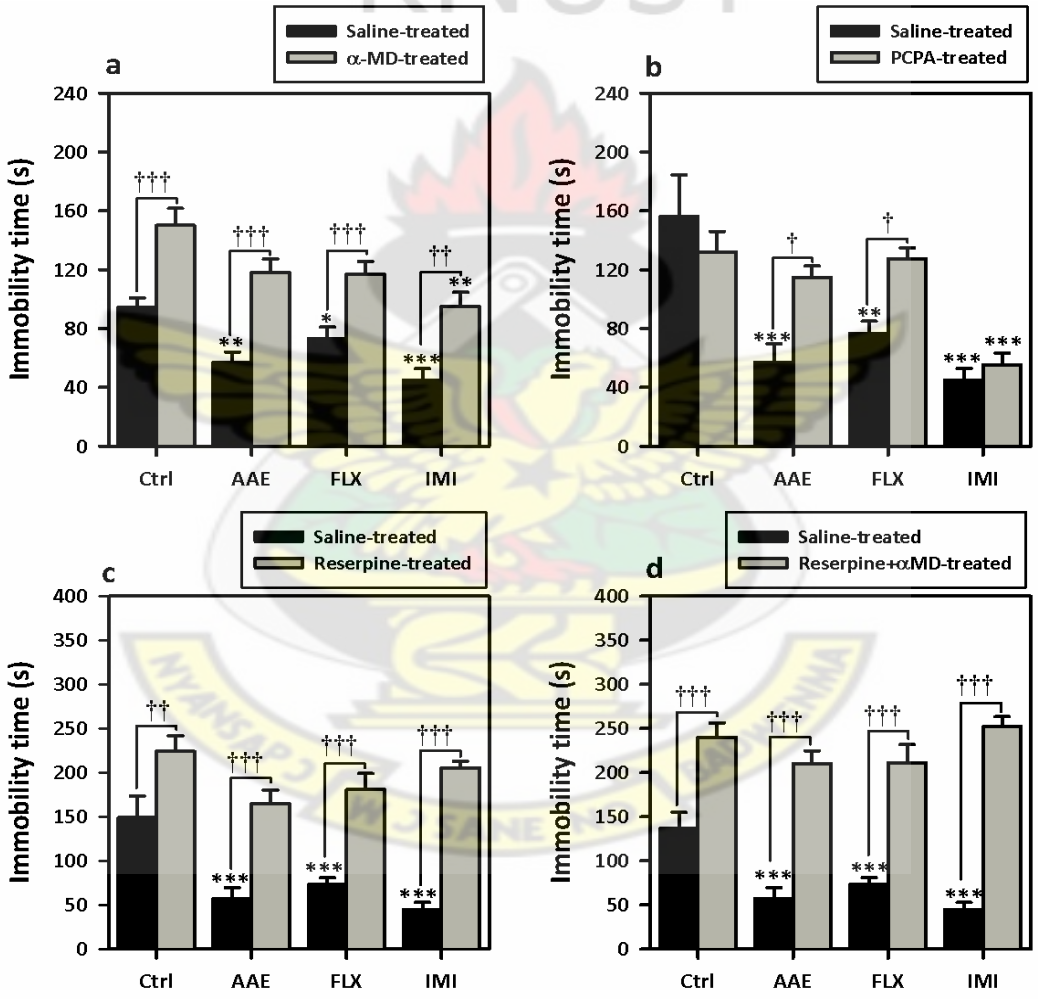

Figure 6.6 Effects of *α*-MD (200 mgkg^{-1}, i.p.) (a), PCPA (300 mg kg^{-1} *bd*, i.p.) (b), Reserpine (1 mg kg^{-1}, s.c.) (c) and Reserpine + *α*-MD (d) pretreatment on the behavioural response of AAE (400 mg kg^{-1}, *p.o.*), fluoxetine (10 mg kg^{-1}, *p.o.*) and imipramine (10 mg kg^{-1}, *p.o.*) in the tail- suspension test. Data are presented as group mean±SEM (n=8). *$P<0.05$, **$P<0.01$, ***$P<0.001$ compared to vehicle treated

group (one-way analysis of variance followed by Newman-Keuls' *post hoc* Test). †P <0.05, ††P < 0.01, †††P < 0.001(two-way ANOVA followed by Bonferroni post test).

6.3.5 Effect of 5-Hydroxytryptophan potentiation

The extract was able to significantly (P<0.05; Figure 6.7) increase the number of head twitches produced by 5-hydroxytryptophan in a similar manner to fluoxetine.

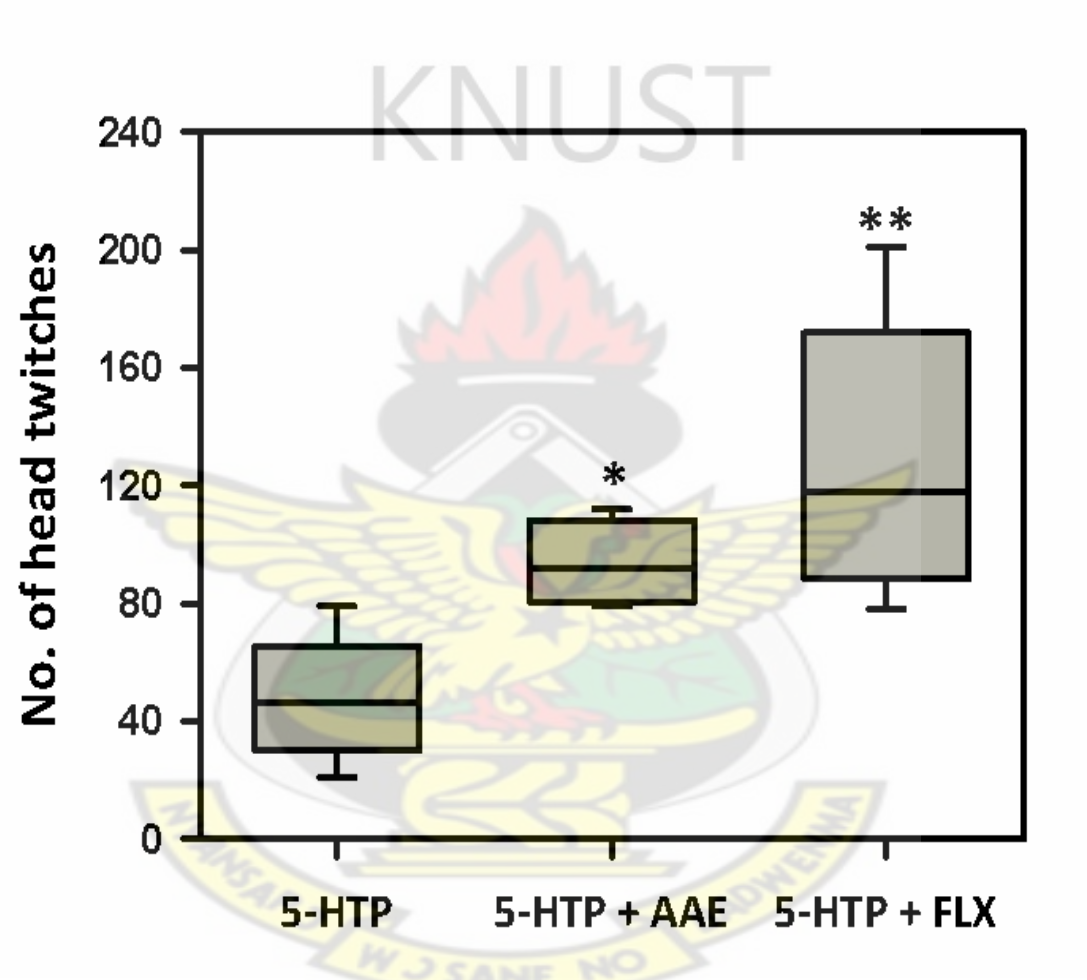

Figure 6.7 Effects of AAE (400 mg kg$^{-1,}$ *p.o.)* and fluoxetine (15 mg kg^{-1}, *p.o.)* on head twitches in 5-hydroxytryptophan potentiation test. Data are presented as group mean±SEM (n=5).The lower and upper margins of the boxes represent the 25th and 75th percentiles, with the extended arms representing the 10th and 90th percentiles respectively. The median is shown as a horizontal line within the box. *P<0.05, **P<0.001 compared to control (one-way analysis of variance followed by Newman-Keuls' *post hoc* test).

6.3.6 Glycine/ NMDA receptor involvement in antidepressant mechanism of AAE

The extract alone produced significant (P<0.001; Figure 6.8 a) decrease in immobility time compared to vehicle-treated animals, as did fluoxetine, desipramine and D-cycloserine (P< 0.001; Figure 6.8 b-d). Treatment with D-serine produced no significant increase in the immobility time produced by the extract (Figure 6.8 a). Fluoxetine (P=*0.0021*; $F_{1, 18}$ = *12.85;* Figure 6.8b) and D-cycloserine (P=*0.0015*; $F_{1, 18}$ = *14.05;* Figure 6.8 d) showed significant increases in immobility time after D-serine treatment at the doses studied.

PCPA pretreatment significantly (P=*0.0235*; $F_{1, 18}$ = *6.12;* Figure 6.8 e) increased the immobility period at the highest dose only of the extract just as fluoxetine (P=*0.0014*; $F_{1, 18}$ = *14.34;* Figure 6.8 f). Desipramine was not affected significantly. The immobility time for the extract was significantly (P<*0.0001*; $F_{1, 54}$ = *204.18;* Figure 6.8 i) reversed by α-methyl-ρ-tyrosine but fluoxetine was unaffected. Desipramine was affected (P<*0.0001*; $F_{1, 54}$ = *48.27;* Figure 6.8 k) at all doses except the highest dose. D-cycloserine was also not affected.

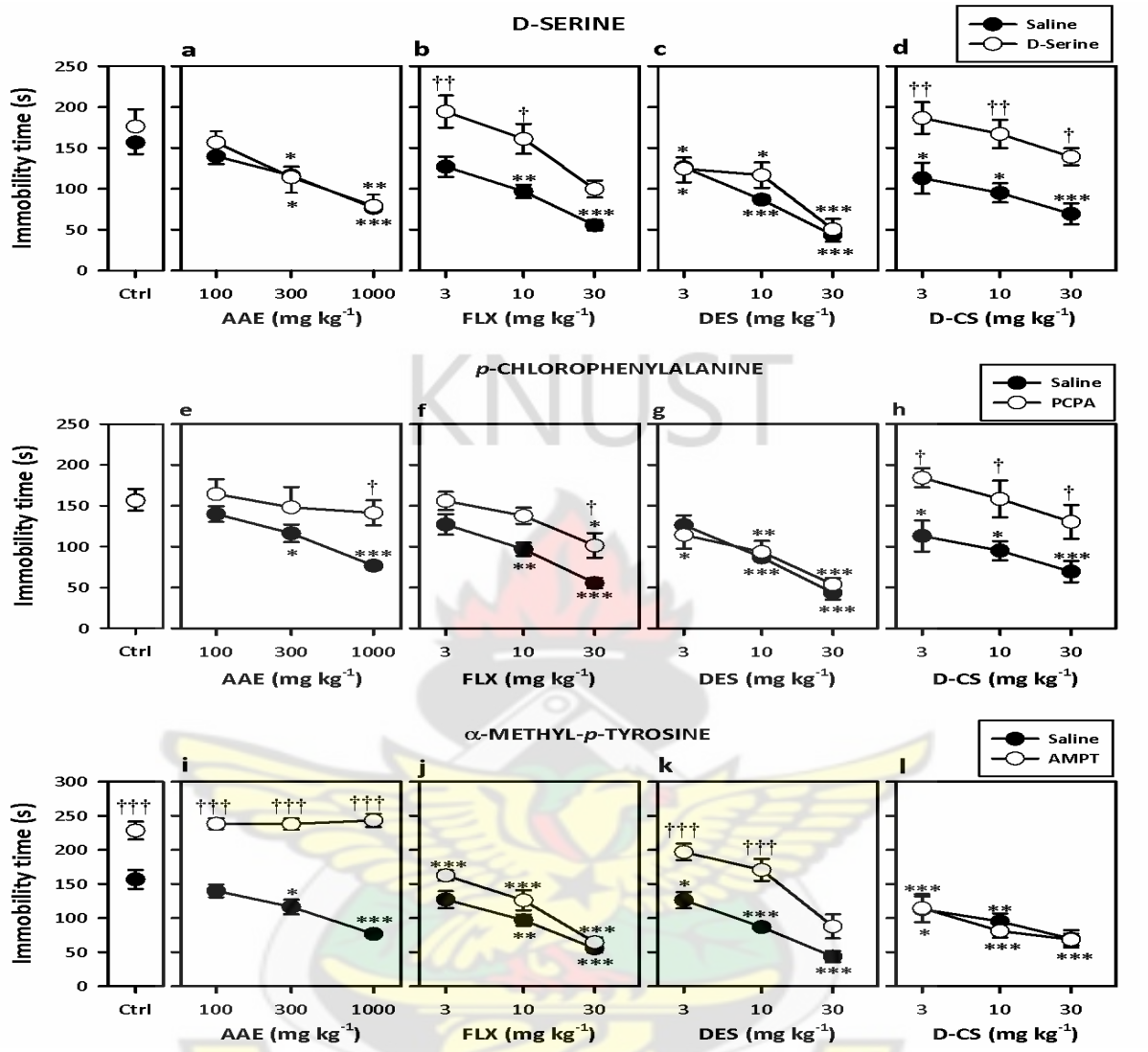

Figure 6.8 Effects D-serine (320 mg kg^{-1}; i.p) on behavioural response of AAE (100-1000 mg kg^{-1}) (a), fluoxetine (3-30 mg kg^{-1}) (b), Desipramine (3-30 mg kg^{-1})(c) and D- cycloserine (3-30 mg kg^{-1}) (d); PCPA (200 mg kg^{-1}) pretreatment on the behavioural response of AAE (100-1000 mg kg^{-1}) (e), fluoxetine (3-30 mg kg^{-1}) (f), Desipramine (3-30 mg kg^{-1})(g) and D- cycloserine (3-30 mg kg^{-1}) (h); α-methyl-ρ-tyrosine pretreatment on the behavioural response AAE (100-1000 mg kg^{-1}) (i), fluoxetine (3-30 mg kg^{-1})(j), Desipramine (3-30 mg kg^{-1})(k) and D- cycloserine (3-30 mg kg^{-1}) (l) in the forced swimming test. Data are presented as group mean±SEM (n=8). *$P<0.05$, **$P<0.01$, ***$P<0.001$ compared to vehicle treated group (one-way analysis of variance followed by Newman-Keuls' *post hoc* Test). †$P < 0.05$, ††$P < 0.01$, †††$P < 0.001$(two-way ANOVA followed by Bonferroni post test).

6.3.7 β_2-Adrenoceptor Activity

The extract produced inhibition of carbachol-induced contractions in a dose-dependent fashion with a maximum inhibition of 100 %. Propranolol (10^{-7}-10^{-5} M) produced a non-parallel rightward shift of increasing concentrations of AAE (Figure 6.9 a). Adrenaline, however, produced a parallel rightward shift in the presence of propranolol (Figure 6.9 b).

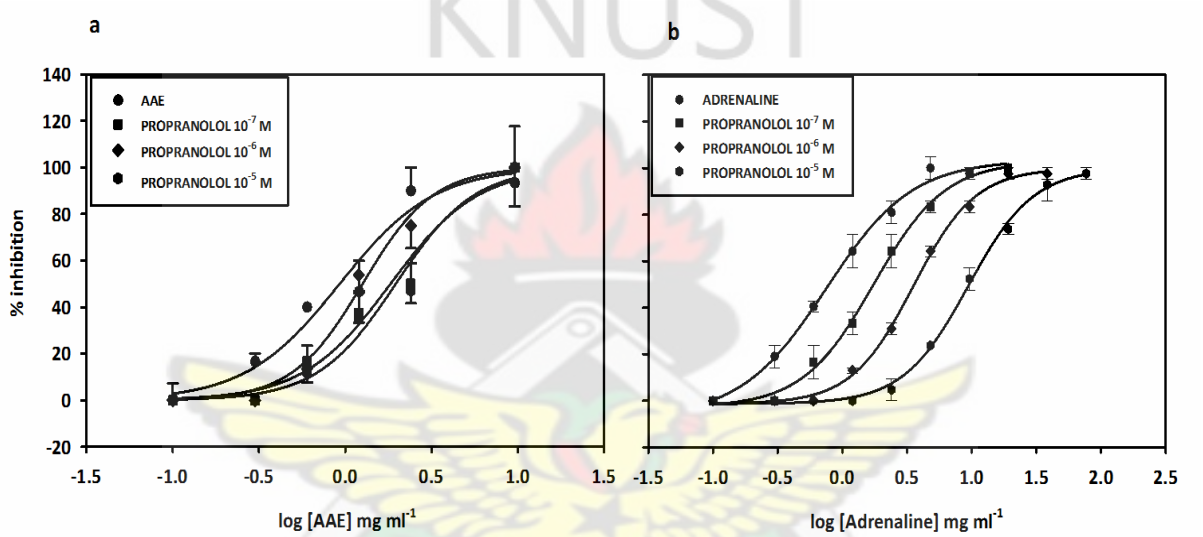

Figure 6.9 Mean concentration-response curves obtained for AAE (a) and adrenaline (b) in the absence and presence of propranolol on the response of isolated rat uterine preparation to the EC_{80} (8.76×10^{-7} M) of carbachol. Each point represents mean ± S.E.M. % maximum response were calculated as % inhibition of carbachol responses.

6.3.8 Locomotor activity

Results show that AAE at the doses used has no significant effect on motor coordination. D-tubocurarine and diazepam however caused significant (*P<0.01*) dose-dependent decrease in time spent on the rod.

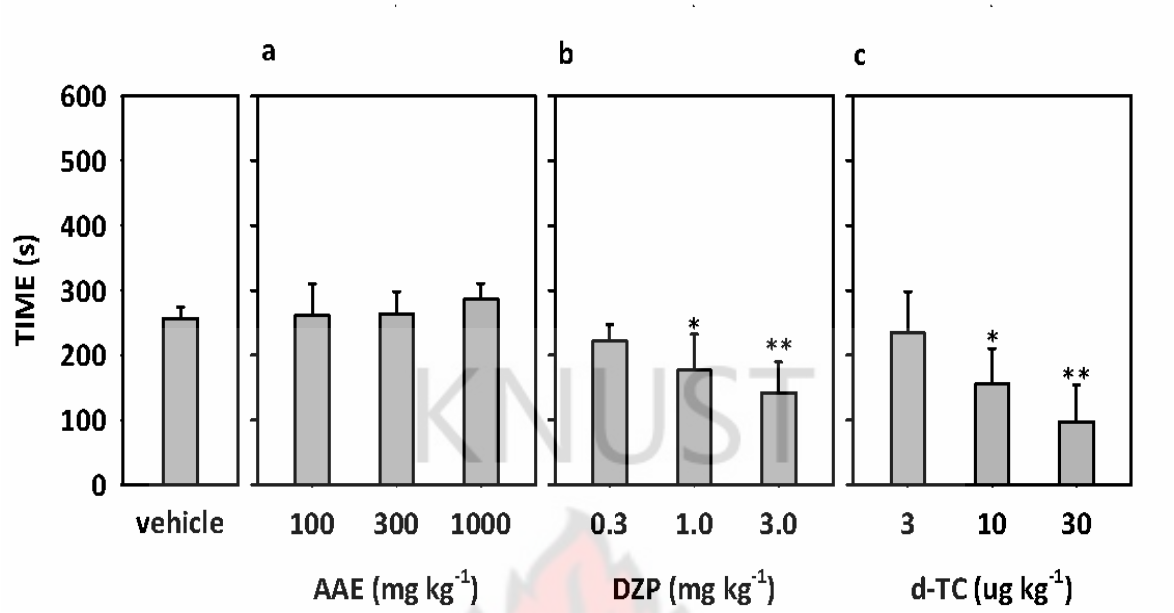

Figure 6.10 Effects of acute AAE (100, 300 and 1000 mg kg^{-1}), diazepam (0.3-3.0 mg kg^{-1}) and D-tubocurarine (3-30 mg kg^{-1}) on motor coordination in the rotarod test. Data are presented as group mean±SEM (n=8). *$P<0.05$, **$P<0.01$ compared to vehicle-treated group (one-way ANOVA followed by Newman-Keuls' *post hoc* test).

6.4 DISCUSSION

Results of the present study indicate that *Antiaris toxicaria* possesses antidepressant-like effect in the models of depression used.

The forced swimming test evaluates 'behavioural despair'; a measure of failure to seek escape from an aversive stimulus (Crawley *et al.*, 1997). FST has high predictive validity and has shown sensitivity to major classes of antidepressants such as tricyclic antidepressants (TCAs) and selective serotonin reuptake inhibitors (SSRIs) (Borsini and Meli, 1988; Dalvi and Lucki, 1999). In the tail suspension test, mice attempt to "escape" by agitation. In both tests, immobility is reduced by a broad range of pharmacological treatments. AAE showed reduced immobility in both tests. It was however more effective in the TST showing it is more sensitive in that test.

Berrocoso *et al.*, 2011 have shown that opioids produce stereotyped behavioural patterns by decreasing pedalling behaviour and increasing the curling behaviour in the tail suspension test. This may help differentiate between standard antidepressants and other compounds possessing antidepressant-like properties but with different

mechanisms of action. The extract showed a significant increase in duration of curling and decreased pedalling behaviour implicating opioidergic properties. Some opioids such as pethidine and methadone have shown a tendency to increase swinging behaviour. As AAE showed some significant increase in duration of swinging, it is likely that its opioidergic properties are more similar to that of pethidine and methadone than to that of morphine.

An attempt was made to investigate the mechanism of the antidepressant action of AAE. Pretreatment of mice with PCPA, α-methyldopa and reserpine are mechanistic models known to alter the monoamine levels in the frontal cortex. These monoamines are crucial in mood, cognition and motor behaviour functions that are compromised in depression (Millan *et al.*, 2000). Acute administration of many antidepressants is accompanied by an increase in extracellular monoamine levels since they block transporters that control neurotransmission (Millan *et al.*, 2000). This implies that elevated levels of monoamines is crucial in the management of depression (Gobert *et al.*, 1997; Brunello *et al.*, 2002; Stone *et al.*, 2003).

α-Methyldopa is an L-aromatic amino acid decarboxylase inhibitor which hampers synthesis of catecholamines and 5-hydroxytryptamine (Oates *et al.*, 1960; Hess *et al.*, 1961; Carlsson and Lindqvist, 1962). α-Methyldopa gives rise to 'false transmitters'; α-methyldopamine and α-methylnoradrenaline (Carlsson and Lindqvist, 1962; Sjoerdsma, 1963). Presynaptic feedback inhibition of noradrenaline occurs since these false transmitters are powerful α_2-adrenoceptor agonists (Hey *et al.*, 1988).This implies that pretreatment with α-methyldopa is expected to have a more sustained effect on catecholamine depletion rather than on the serotoninergic pathway. Results did not confirm this as the effect of fluoxetine was largely blocked though not completely. This, however, confirms the findings of Carlsson and Lindqvist, 1962. Pretreatment with α-methyldopa, similar to imipramine, abolished the antidepressant effects of the extract pointing to the possible involvement of catecholamines in the antidepressant-like properties of *Antiaris toxicaria*. Imipramine is a non-selective inhibitor of both monoamine transporters NET and SERT (Iversen, 2006). Hence, α-methyldopa was not expected to block its action completely—and this was rightly so.

Pretreatment with PCPA did not alter baseline immobility significantly. This agrees with previous reports which demonstrate the depletion of 5-HT with PCPA does not

alter baseline behaviour in many antidepressant animal models (Lucki and O'Leary, 2004; O'Leary *et al.*, 2007). It reversed the antidepressant effect of fluoxetine since 5-HT had been depleted. The effect of the extract was reversed similarly, again implicating serotoninergic involvement in its mechanism of action. Imipramine, however, showed no significant reversal of activity consistent with earlier findings (O'Leary *et al.*, 2007). PCPA is an irreversible tryptophan hydroxylase inhibitor.

Pretreatment with reserpine increased immobility periods reversing the antidepressant effects of imipramine and AAE in the TST. Effect of fluoxetine was also reversed, though not completely. Reserpine irreversibly inhibits vesicular monoamine transporter 2 (VMAT-2) which is responsible for transporting monoamines from the cytoplasm into secretory vesicles mainly in the CNS (Freis, 1954; Metzger *et al.*, 2002). Vesicular monoamine stores of both serotonin and noradrenaline are depleted by reserpine (Fukui *et al.*, 2007). This helps to further implicate both serotonin and noradrenaline in the antidepressant effects of AAE. Pretreatment with a combination of reserpine and α-MD completely inhibited the effects of all three treatments.

Involvement of serotonin in the effects of AAE was confirmed using the 5-hydroxytryptophan potentiation test. 5-HTP is a precursor for serotonin and is known to produce head twitches while increasing serotonergic transmission (Ortmann *et al.*, 1981; Pandey *et al.*, 2008). 5-HTP head twitch response was significantly potentiated by AAE further confirming its antidepressant properties are modulated by serotonin. This test is considered as further evidence for antidepressant activity based on synaptic serotonin uptake inhibition (Pandey *et al.*,2008).

Some antidepressants are known to increase synaptic concentrations of noradrenaline while others act directly on adrenoceptors (Elhwuegi, 2004). β_2-Adrenoceptor agonists have been shown to possess antidepressant-like activity in animals and man, but possess peripheral side-effects that prevent their therapeutic use (Simiand *et al.*, 1992; Consoli *et al.*, 2007). The extract showed β_2-adrenoceptor agonist activity by producing reduced contractions induced by carbachol on the isolated rat uterus (Mattsson *et al.*, 1982; Liu *et al.*, 1998; Tanaka *et al.*, 2005). Various concentrations of propranolol blocked the relaxation effect of the extract non-competitively.

The role of the excitatory neurotransmitter glutamate in the pathophysiology of depression is evident. Disturbances of glutamate levels in depressed patients have been found in clinical studies (Levine *et al.*, 2000). The antidepressant property shown by the extract was not antagonized by pretreatment with D-serine. D-serine is a full agonist at the glycine/NMDA receptor. AAE's antidepressant effects were still apparent in the presence of D-serine indicating that its actions are independent of the NMDA-glycine receptor B. Glycine/NMDA receptor antagonists and partial agonists such as D-cycloserine are known to enhance antidepressant-like effects of serotonin-based, but not noradrenaline-based antidepressants (Poleszak *et al.*, 2011). This was confirmed by monoamine depletion with PCPA and α-methyl-ρ-tyrosine.

Results from the rotarod test show that AAE at the doses employed does not compromise motor activity and coordination.

6.5 CONCLUSION

The aqueous stem bark extract of *Antiaris toxicaria* possesses antidepressant activity and acts via serotoninergic, β_2-adrenoceptor activity and opioidergic mechanisms.

Chapter 7

ANALGESIC ACTIVITY OF THE EXTRACT

7.1 INTRODUCTION

Tissue injury and/or inflammation may result in pathophysiological nociceptive pain. This may manifest as spontaneous pain, such as hyperalgesia and is effectively managed with non-steroidal anti-inflammatory drugs ($NSAID_S$) and opiates (Schaible and Richter, 2004). On the other hand, pain caused by neuronal injury (i.e. neuropathic pain) in the peripheral or central nervous system is poorly managed by these drugs; anticonvulsants or tricyclic antidepressants are often resorted to (Schaible and Richter, 2004).

Anticonvulsants are believed to be effective in alleviating certain forms of neuropathic pain and even acute pain (Lopes *et al.*, 2009). They act by reducing membrane excitability and suppressing abnormal discharges in pathologically altered neurons (Berry *et al.*, 2001).

Many different therapeutic options for pain relief have been obtained from plants. A popular example is *Papaver somniferum* from which morphine was isolated (Almeida *et al.*, 2001). Medicinal plants with anticonvulsant activity may lead to the development of potential analgesics devoid of side effects related to opioids or NSAIDs such as addiction and gastrointestinal erosions. Therefore, the development of more effective analgesic agents of natural origin is desirable.

Antiaris toxicaria aqueous extract was in this study evaluated for antinociceptive activity in the formalin test and acetic acid-induced writhing test.

7.2 MATERIALS AND METHODS

7.2.1 Animals

Male ICR mice (20-25 g) and Sprague-Dawley rats (120-200 g) were obtained from the Noguchi Memorial Institute for Medical Research and were cared for in the Departmental Animal House. Animals were allowed to adapt to laboratory conditions

of temperature, humidity and light. They were also allowed free access to water and food and housed in standard cages. Groups of eight to ten animals were used. Animals were treated according to the Guide for the Care and Use of Laboratory Animals (NRC, 1996) and experiments were approved by the Faculty Ethics Committee.

7.2.2 Drugs and chemicals

Formalin and glacial acetic acid were purchased from Sigma-Aldrich Inc., St. Louis, MO, USA. Diclofenac sodium was purchased from Troge, Hamburg, Germany while morphine hydrochloride was from Phyto-Riker, Accra, Ghana.

7.2.3 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 and Yaksh, 1995. Rats were randomly divided into eleven groups (n=8) and allowed to acclimatize to testing environment before injection of formalin.

Animals were pretreated with either AAE (200, 400 and 800 mg kg^{-1}, *p.o*) or morphine (1, 3 and 10 mg kg^{-1}, i.p.). Control animals received distilled water at 3 ml kg^{-1}, *p.o.* After the above treatments, each rat was injected with 0.1ml of 2 % formalin into the right hind paw intraplantar. Each animal was placed in an observation chamber made of plexiglas. Animals were observed via video recording for an hour. From the videos pain response was scored starting immediately after injection of formalin. The first phase of the formalin test was defined as 0-10 minutes and the second as 11-60 minutes post formalin injection (Wilson *et al.*, 2002). Nociceptive behaviour was quantified based on the incidents of licking or biting of the injected paw (Hayashida *et al.*, 2003) using J-Watcher® (Macquarie University, Australia and UCLA, USA). Behaviour Tracking Software version 1.0. Nociceptive score was determined for each five- minute time block. The product of the frequency and duration of licking was used as the nociceptive score.

7.2.4 Acetic acid-induced writhing assay

The test was carried out according to the method described by Amresh *et al.*, 2007 with slight modifications. 10 ml kg^{-1} of 0.6% glacial acetic acid was injected intraperitoneally. The total number of writhings recorded over a period of 20 min, starting 10 min after the acetic acid injection. Writhing was defined as contraction of the abdominal muscle, together with a stretching of the hind limbs. Animals received AAE (100-1000 mg kg^{-1}, *p.o.*), diclofenac (10-100 mg kg^{-1}, i.p.). Control animals received distilled water (3 ml kg^{-1}, *p.o.*).

7.2.5 Data analysis

Data were presented as mean±S.E.M and significant differences between means determined by one-way analysis of variance (ANOVA) followed by Newman-Keuls' *post hoc* test and two-way ANOVA followed by Bonferroni post tests using GraphPad Prism® (GraphPad Software, San Diego, CA, USA). In all cases, $P < 0.05$ was considered significant.

7.3 RESULTS

7.3.1 Effects in the formalin test

Formalin administration produced a typical pattern of biting and licking behaviour. Treatment of rats with AAE (200-800 mg kg^{-1}, *p.o.*) produced a marked non-dose dependent inhibition of the first phase of formalin-induced nociception *($F_{3, 16} = 7.373$, $P=0.0025$*; Figure 7.1b) with the middle and highest doses. Phase 2 was also inhibited significantly *($F_{3, 16} = 6.549$, $P=0.0043$*; Figure 7.1b). Similarly, morphine (1-10 mg kg^{-1}, *i.p.*) produced marked inhibition of both phase 1(*$F_{3, 16} = 51.20$, $P<0.0001$*; Figure 7.1d) and phase 2 (*$F_{3, 16} = 22.64$, $P<0.0001$*; Figure 7.1d) at all doses. Comparison of ED_{50}s obtained by GraphPad® prism analysis revealed that the extract was more potent in the first [ED_{50}: 418.10 mg kg^{-1}] than the second phase [ED_{50}: 648.90 mg kg^{-1}]. Likewise, morphine was 2.7 times more potent in the first phase [ED_{50}: 0.39 mg kg^{-1}] compared to the second phase [ED_{50}: 1.06 mg kg^{-1}].

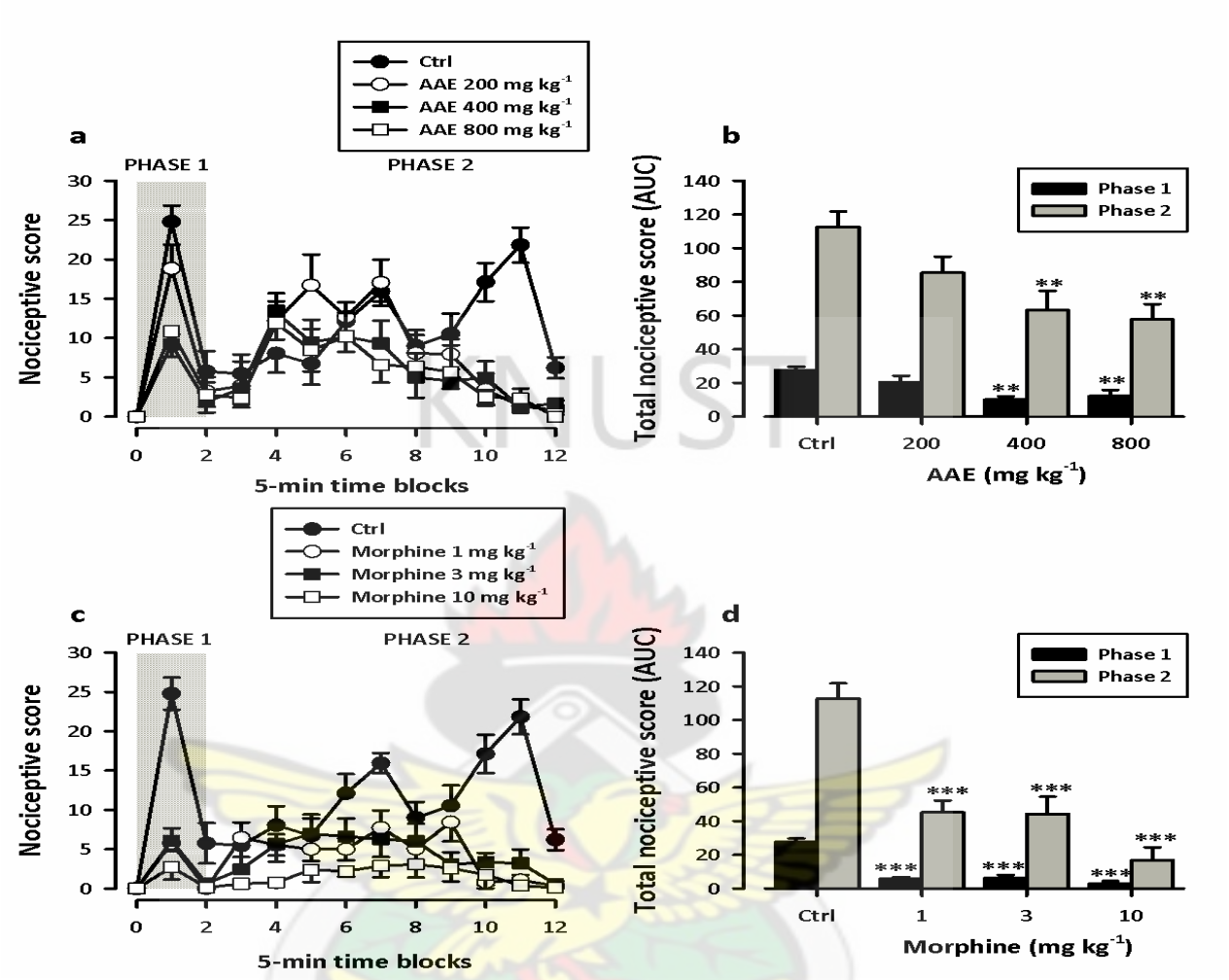

Figure 7.1Effect of AAE and morphine on phase 1 and 2 of formalin induced nociception. (a) Time course effects of AAE (200-800 mg kg^{-1}, *p.o.*) and its total nociceptive score in (b). (c) Time course effects of morphine (1-10 mg kg^{-1}, i.p.) and its total nociceptive score in (d). Nociceptive scores are shown in 5- minute time blocks up to 60 minutes for the time course curves. Data are presented as mean ± SEM (n=8). ***P*<0.01, ****P*<0.001 compared to vehicle-treated group (one-way analysis of variance followed by Newman-Keuls' *post hoc* test).

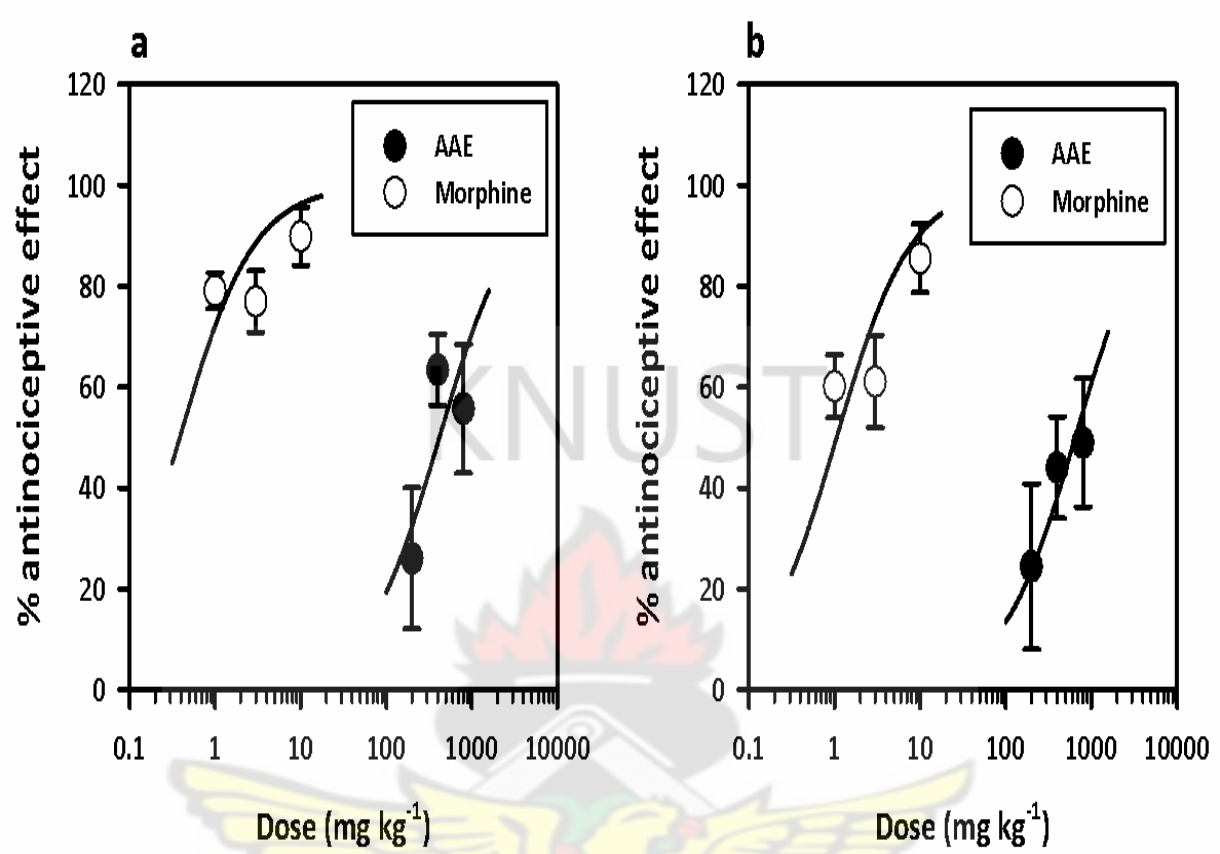

Figure 7.2 Dose response curves of AAE and morphine on the total nociceptive score for the first phase (a) and the second phase (b) of the formalin test in rats. Each point represents mean ± S.E.M (n=8).

7.3.2 **Effect in Acetic acid-induced Writhing Assay**

AAE (100-1000 mg kg^{-1}, *p.o.*) significantly (P=0.0334, $F_{3,28}$= 3.87,Figure 7.3 b) reduced the number of writhes induced by acetic acid in mice with the highest dose used reducing the total writhes by 56.89 %. Diclofenac (10-100 mg kg^{-1}, i.p) in the same vein reduced the number of acetic acid- induced writhings significantly (P=0.001, $F_{3,\ 28}$= 7.136, Figure 7.3 d) by 67.88%.

Comparison of ED_{50}s obtained by non-linear regression (Figure 7.4) revealed that the extract [ED_{50}=1757 mg kg^{-1}] was 70× less potent than diclofenac [ED_{50}=25.07 mg kg^{-1}]. The extract [E_{max}= 100%].was however more efficacious than diclofenac [E_{max}= 90 %].

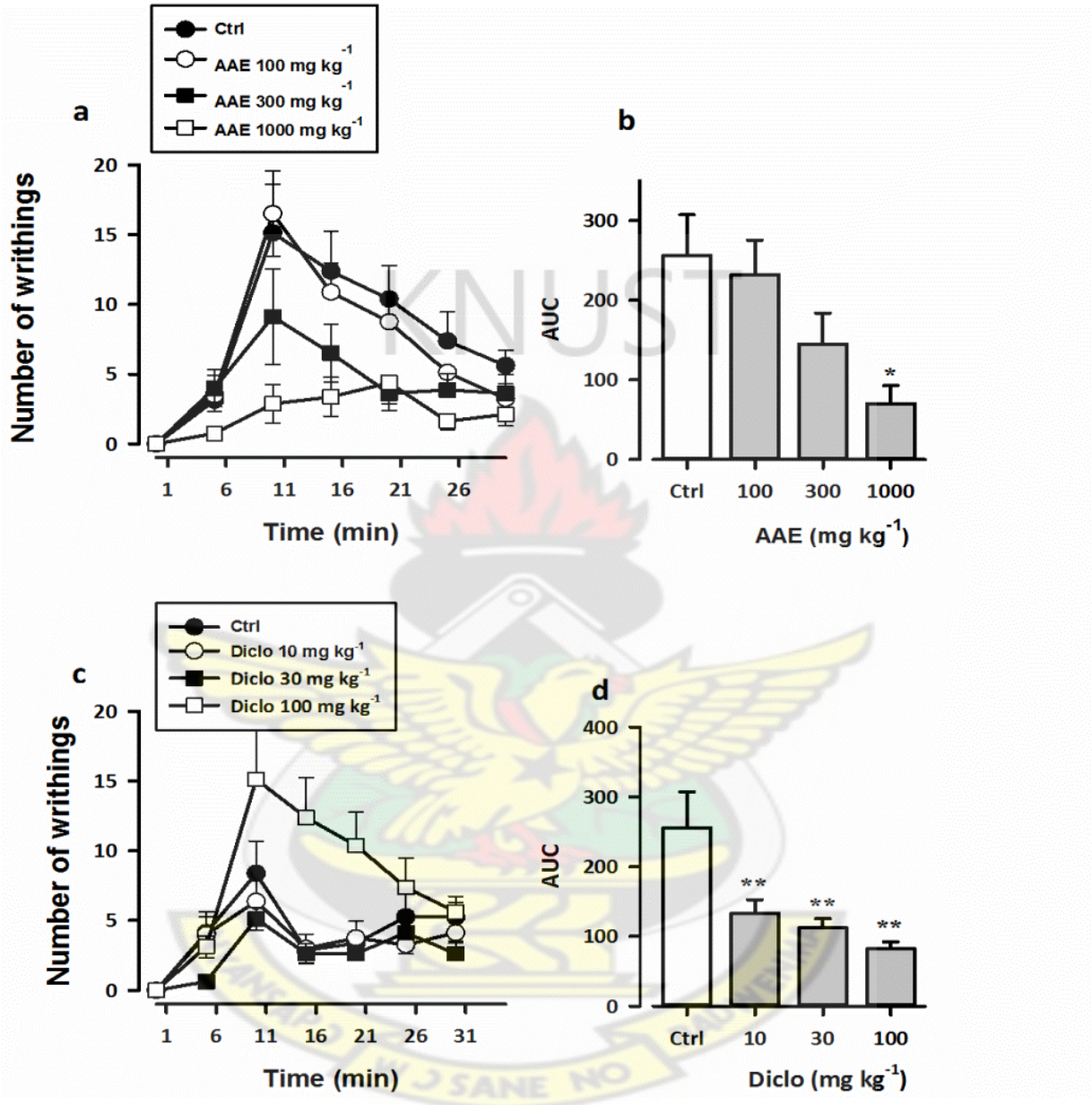

Figure 7.3 Effect of AAE (100-1000 mg kg^{-1}, *p.o.*) and diclofenac (10-100 mg kg^{-1}, i.p.) on acetic acid induced writhing. (a) Time course effects of AAE and its total nociceptive score in (b). (c) Time course effects of diclofenac and its total nociceptive score in (d). Nociceptive scores are shown in 5-minute time blocks up to 30 minutes for the time course curves. Data are presented as mean±S.E.M (n=8). *P<0.05, **P<0.01 compared to vehicle-treated group (one-way analysis of variance followed by Newman-Keuls' *post hoc* test).

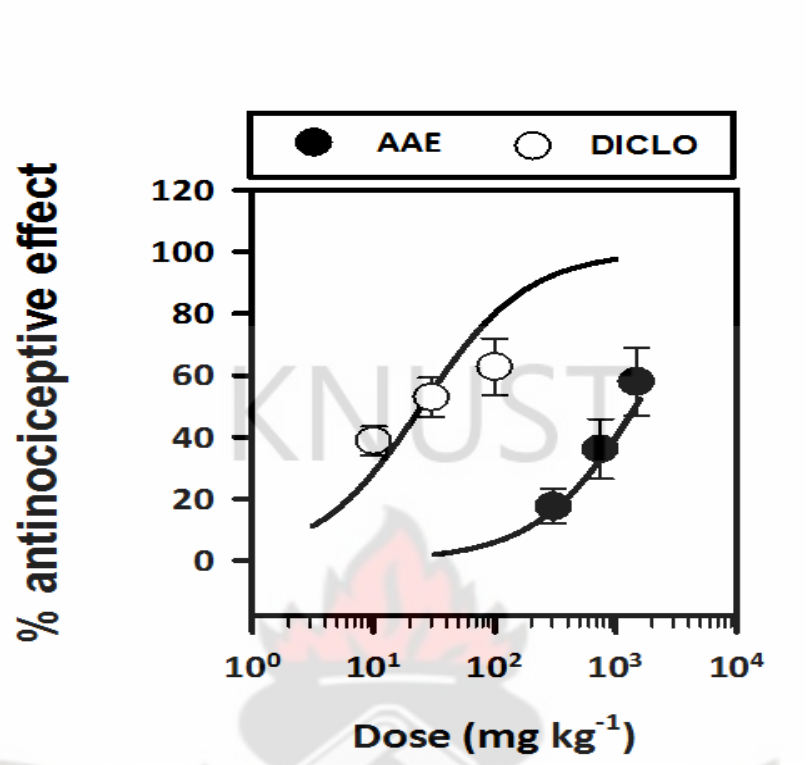

Figure 7.4 Dose response curves of AAE and diclofenac on the % antinociceptive effect in the acetic acid induced writhing test in rats. Each point represents mean ± S.E.M (n=8).

7.4 DISCUSSION

Antiaris toxicaria was effective in all the pain models used. Acute pain models were employed in order to evaluate possible centrally and peripherally mediated effects.

The formalin test is useful for the screening of novel compounds, since it encompasses inflammatory, neurogenic, and central mechanisms of nociception (Ellis *et al.*, 1998). Analgesics are known to decrease the incidence of licking or biting induced by intraplantar injection of formalin into the paw of the rodent (Courteix *et al.*, 1998).

AAE inhibited both phases of the formalin test representing effect on both the neurogenic and inflammatory phases of pain. It is considered the most predictive of acute pain and a valid model for clinical pain (Dubuisson and Dennis, 1977). Formalin test is therefore a good predictor of analgesics that may be effective in neuropathic pain and pain not sensitive to conventional therapy (Lopes *et al.*, 2009). The first phase

which lasts for ten minutes is believed to be caused by direct action of formalin on transient receptor potential ankyrin subtype 1 receptors (TRPA 1) which are cation channels on sensory C-fibres (McNamara *et al.*, 2007). The second phase lasts from 11 to 60 minutes and is associated with the combination of an inflammatory reaction in the peripheral tissue causing a release of nociceptive mediators such as serotonin, histamine, bradykinin and prostaglandins which subsequently cause sensitization of the central neurons leading to changes in central processing of pain (da Rocha *et al.*, 2011). Inflammatory pain has also been associated with inhibition of the anterior cingulate cortex (ACC) which decreases inflammatory nociceptive response of the prolonged, tonic, inflammatory portion of the formalin test manifested as reduction in paw licking/biting behaviour (Donahue *et al.*, 2001). AAE may have acted peripherally possibly by the inhibition of TRPA 1 receptor activation and centrally, by inhibition of the nociceptive effects of pain mediators such as prostaglandins and bradykinin producing antinociception in the formalin test. Morphine is an opioid analgesic used as control which blocked both phases of the formalin in accordance with literature (Trongsakul *et al.*, 2003).

The acetic acid writhing test investigates visceral antinociceptive activity of drugs. Colic pain is a common example of visceral pain. It also a model of inflammatory pain, bearing resemblance to the second phase of the formalin test. Acetic acid injection results in the local release of pro-inflammatory substances such as prostaglandins, substance P, bradykinin, and/or cytokines (Raja *et al.*, 1988) which activate and sensitize peripheral nociceptive afferent neurons. AAE's effectiveness in this test may indicate analgesic properties due to possible inhibition of the synthesis and/or release of pro-inflammatory substances peripherally, similar to the actions of diclofenac (Panthong *et al.*, 2007; Panthong *et al.*, 2007).

The seeds of *A. toxicaria* are used traditionally as analgesic whiles the stem is used for epilepsy management. Some anticonvulsants such as gabapentin and lamotrigine have exhibited analgesic effects in the formalin test (Sawynok *et al.*, 2001; Vissers *et al.*, 2003; Paudel *et al.*, 2011; Kilic *et al.*, 2012). Clinicians also use anticonvulsant such as phenytoin in the management of neuropathic pain (Birse *et al.*, 2012). Hence, analgesic property obtained in this study will be advantageous in the therapeutic use of *A. toxicaria*.

7.5 CONCLUSION

The aqueous extract of the stem bark of *Antiaris toxicaria* therefore, possesses antinociceptive properties and justifies its traditional use for pain treatment.

Chapter 8

GENERAL TOXICITY

8.1 INTRODUCTION

Toxicological evaluation of medicinal plants need be carried out to determine their overall safety for use. Owing to the increase in number of medicinal plant users as opposed to the paucity of scientific information on their safety, concerns regarding toxicity of these remedies arise (Saad *et al.*, 2006). *Antiaris toxicaria*, like any other plant contains numerous bioactive principles which may cause beneficial and/or adverse effects. There has, therefore, been a recommendation that all natural products used in therapeutics be subjected to safety tests (Franzotti *et al.*, 2000; Khalil *et al.*, 2006). In acute studies, the shorter duration of exposure allows monitoring of effects of a substance at high doses over a short period. Acute exposure to drugs is of major international concern for instance in the treatment of accidental or intentional overdose. Sub acute studies involve a much more extended period of exposure, mostly 14 days (Eaton, 2005).

Methods for testing followed the Organization for Economic Cooperation and development (OECD) guidelines for Acute Oral toxicity testing using the Up-and-down procedure (Test Number 425).

8.2 MATERIALS AND METHODS

8.2.1 Animals

Male ICR mice (20-25 g) and Sprague-Dawley rats (120-200 g) were obtained from the Noguchi Memorial Institute for Medical Research and were cared for in the Departmental Animal House. Animals were handled as described in previous chapters.

8.2.2 Acute Toxicity

Animals were randomly selected and divided into four groups of five mice/ rats in each. The extract (300, 1000 and 3000 mg kg^{-1}) was orally administered to the group of animals respectively. The control group received 10 ml kg^{-1} *p.o.* of distilled water.

Animals were observed over 24 h after treatment for any change in behaviour or death. Animals were fasted overnight prior to testing.

8.2.3 Sub-acute Toxicity

The extract was administered daily for 14 consecutive days. Group A, the control, received 10 ml kg^{-1} *p.o* of distilled water daily. Group B, C and D were treated with extract (300, 1000 and 3000 mg kg^{-1} *p.o.*) respectively daily. The animals were monitored closely for signs of toxicity. Sacrificing of animals occurred on the fifteenth day by cervical dislocation. Blood was obtained by cardiac puncture. 1.5 ml of blood was collected into a test tube containing 2.5 µg of ethylenediamine tetra acetic acid (EDTA) as an anticoagulant for haematological assay and 3.5 ml of blood into Vacutainer™ tubes containing separating gel. Serum was collected and stored at – 80ºC until assayed for biochemical parameters. Organs harvested included liver, kidney, spleen, brain, stomach and spinal cord.

8.2.4 Haematological parameters assessment

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

8.2.5 Serum biochemical parameters assessment

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

8.2.6 Organ weight assessment

Organs including the spleen, liver, kidney, stomach, brain and spinal cord were excised quickly, trimmed of fat and connective tissue and weighed on a balance. Body weight of the rats was taken on days 0 and 14. The organ-to-body weight index (OBI) was calculated as

$$OBI = \frac{Organ\ weight\ of\ animal}{Body\ weight\ of\ animal} \times 100$$

8.2.7 Histopathological examination

The liver, kidney, spleen, stomach, brain and spinal cord were harvested for histopathological examination. Tissues were fixed in 10% buffered formalin (pH 7.2). Dehydration was done with a series of ethanolic solutions, embedded in paraffin wax and processed for histological analysis. Sections (2 µm thick) were cut and stained with haematoxylin-eosin for examination. The stained tissues were observed through an Olympus microscope (BX-51) and photographed by a chare-couple device (CCD) camera.

8.2.8 Analysis of data

Data were presented as mean±S.E.M and significant differences between means determined by one-way analysis of variance (ANOVA) followed by Newman-Keuls' *post hoc* test. Analyses were carried out with GraphPad Prism for Windows version 5.0 (GraphPad Software, San Diego, CA, USA).

8.3 RESULTS

8.3.1 Acute Toxicity

8.3.1.1 *Mice*

No death was recorded over the 24 h period. During observation, the animals did not exhibit any sign of decreased mobility, respiratory depression or convulsions. They, however, showed signs of straub tail effect.

Table 8.1Effects of *Antiaris toxicaria* in acute toxicity studies in mice.

	MORTALITY	EFFECTS
DOSE (mg kg^{-1})	D/T	
0	0/7	No change
300	0/7	Straub tail effect
1000	0/7	Straub tail effect
3000	0/7	Straub tail effect

D=Deaths recorded; T=Total number of animals observed.

8.3.1.2 ***Rats***

No animal exhibited any sign of decreased mobility, respiratory depression or convulsions. They, however, showed signs of straub tail effect and analgesia at all the doses used. No death was recorded.

Table 8.2 Effects of *Antiaris toxicaria* in acute toxicity studies in rats.

	MORTALITY	EFFECTS
DOSE (mg kg^{-1})	D/T	
0	0/7	No change
300	0/7	Straub tail effect
1000	0/7	Straub tail effect Analgesia
3000	0/7	Straub tail effect Analgesia

D=Deaths recorded; T=Total number of animals observed

8.3.2 Sub Acute Toxicity

All the animals survived throughout the 14 days. Besides the straub tail effect and analgesia to tail pinch test, no other toxicity signs were observed. There were no significant changes in body weight.

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8.3.3 Effect of extract on haematological parameters

8.3.3.1 *Mice*

None of the parameters showed any significant change.

Table 8.3 Effects of *Antiaris toxicaria* on haematological parameters in mice.

		AAE DOSE (mg kg^{-1})		
Test	Control	300	1000	3000
WBC (K/µL)	7.09±0.14	7.27±0.14	7.024±0.28	7.21±0.37
RBC (M/µL)	9.21±0.06	9.10±0.33	8.546±0.45	8.33±0.32
HGB (g/dL)	13.62±0.19	12.92±0.57	13.44±0.32	13.88±0.59
HCT (%)	43.46±0.82	43.62±0.73	41.48±0.38	43.12±0.86
MCV (fL)	45.34±1.84	43.84±1.07	42.82±0.91	45.14±1.25
MCH (pg)	16.64±0.204	16.90±0.47	16.40±0.19	16.24±0.24
MCHC (g/dL)	32.14±0.72	32.14±1.06	32.20±2.91	32.06±1.02
PLT (K/µL)	1236±68.71	1327±30.98	1238±209	1096±47.70
RDW (%)	22.20±0.32	22.02±0.93	21.68±1.44	22.06±0.97
MPV (fL)	8.10±0.19	7.43±0.53	8.14±0.60	7.46±1.23

Data are presented as mean±SEM (n=5).

8.3.3.2 ***Rats***

Haemoglobin was significantly (*P=0.0470, $F_{3, 16}$=3.311*; Table 8.4) decreased as well as MPV (*P=0.0294, $F_{3, 16}$=4*; Table 8.4) by the middle dose. All other parameters remained unaffected.

Table 8.4 Effects of *Antiaris toxicaria* on haematological parameters in rats.

		AAE DOSE (mg kg^{-1})		
Test	Control	300	1000	3000
WBC (K/µL)	9.73±2.42	5.41±0.72	6.69±0.59	8.96±1.22
RBC (M/µL)	9.21±0.50	7.91±0.43	7.95±0.53	8.26±0.17
HGB (g/dL)	15.94±0.60	14.16±0.61	13.68±0.62*	14.56±0.16
HCT (%)	48.94±2.39	45.98±2.28	43.52±2.08	46.30±0.29
MCV (fL)	53.00±1.14	57.92±1.70	55.96±2.06	55.58±0.73
MCH (pg)	17.0±0.23	17.70±0.27	17.30±0.36	17.46±0.17
MCHC (g/dL)	31.22±0.27	30.99±0.84	30.86±0.58	31.20±0.20
PLT (K/µL)	1117±158.30	1144±139.40	1171±244.5	954.20±28.75
RDW (%)	21.72±0.75	20.76±0.83	20.94±1.05	20.92±0.42
MPV (fL)	8.12±0.11	7.72±0.21	7.48±0.14*	7.58±0.10*

Data are presented as mean±SEM (n=5). *$P<0.05$ compared to vehicle-treated group (One-way analysis of variance followed by Newman-Keuls' *post hoc* test).

8.3.4 **Effect of extract on serum biochemical parameters**

8.3.4.1 ***Mice***

All parameters remained unaffected significantly by extract treatment.

Table 8.5 Effects of *Antiaris toxicaria* on serum biochemical parameters in mice.

		AAE DOSE (mg kg^{-1})		
Test	Control	300	1000	3000
AST (U/L)	212.80±11.71	248.10±39.49	268±57.81	243.80±49.10
ALT (U/L)	61.28±3.87	54.18±9.10	56.88±2.73	53.86±9.67
ALP (U/L)	206.50±16.91	208.90±35.22	227.20±30.44	219.20±49.13
GGT (U/L)	3.58±0.77	4.140±0.81	3.58±0.66	3.64±0.72
Total protein (g/L)	66.76±2.50	65.94±4.64	66.50±2.54	60.02±2.87
Albumin (g/L)	51.78±3.50	47.22±1.93	49.52±2.71	45.86±2.66
Globulin	20.14±1.70	16.40±1.69	17.90±1.64	18.62±0.82
Direct Bilirubin (µmol/L)	0.61±0.06	0.596±0.04	0.61±0.04	0.63±0.07
Total Bilirubin (µmol/L)	66.76±2.50	65.94±4.64	66.50±2.54	60.02±2.87
Urea (*m*mol/L)	51.78±3.50	47.22±1.93	49.52±2.71	45.86±2.66
Creatinine (µmol/L)	20.14±1.70	16.40±1.69	17.90±1.64	18.62±0.82
BUN/Creatinine Ratio	0.61±0.06	0.60±0.04	0.61±0.04	0.63±0.07

Data are presented as mean±SEM (n=5).

8.3.4.2 ***Rats***

Analysis revealed significant increases in AST ($P=0.0264$, $F_{3, 16}= 4.008$; Table 8.6) and ALT ($P=0.0021$, $F_{3, 16}= 7.688$; Table 8.6). The extract also decreased significantly urea

(*P=0.0262, $F_{3, 16}$= 4.017*; Table 8.6) and serum creatinine (*P=0.0015, $F_{3, 16}$= 8.278*; Table 8.6).

Table 8.6 Effects of *Antiaris toxicaria* on serum biochemical parameters in rats.

		AAE DOSE (mg kg^{-1})		
Test	Control	300	1000	3000
AST (U/L)	161.40±36.41	250.30±18.32*	185.50±11.78	155.00±9.70
ALT (U/L)	62.66±2.32	79.24±10.36	107.90±2.86**	99.50±9.77**
ALP (U/L)	147.30±12.08	267.40±57.15	301.80±34.76	285.60±75.51
GGT (U/L)	4.26±0.60	3.56±0.52	3.72±1.36	3.08±0.35
Total protein (g/L)	70.42±2.74	68.22±2.99	64.73±2.57	64.76±1.41
Albumin (g/L)	45.50±2.00	46.89±1.39	45.24±3.32	45.60±0.70
Globulin	22.73±3.24	21.29±3.61	16.94±3.89	18.18±0.91
Direct Bilirubin (µmol/L)	0.55±0.04	0.21±0.10	0.26±0.06	0.39±0.13
Total Bilirubin (µmol/L)	1.18±0.04	1.16±0.07	1.24±0.07	1.12±0.06
Urea (*m*mol/L)	6.77±0.63	6.37±0.23	7.23±1.09	4.24±0.32*
Creatinine (µmol/L)	53.80±0.86	46.40±2.42*	41.00±2.74**	43.00±1.10**
BUN/Creatinine Ratio	59.02±5.30	65.70±5.13	81.22±10.74	45.80±3.31

Data are presented as mean±SEM (n=5). **P<0.05, **P<0.01* compared to vehicle-treated group (One way analysis of variance followed by Newman-Keuls' *post hoc* test).

8.3.5 **Effect of extract on organ weights**

8.3.5.1 ***Mice***

The extract had no significant effect on the parameters.

Table 8.7 Effects of *Antiaris toxicaria* on organ-to-body ratio in mice.

		AAE DOSE (mg kg^{-1})		
Test	Control	300	1000	3000
Liver	4.75±0.27	4.83±0.18	4.90±0.18	5.16±0.15
Kidney	0.53±0.03	0.54±0.02	0.52±0.02	0.55±0.02
Spleen	0.33±0.02	0.33±0.01	0.33±0.01	0.34±0.01
Stomach	0.99±0.07	1.08±0.12	0.95±0.10	1.03±0.03

Data are presented as mean±SEM (n=5).

8.3.5.2 ***Rats***

All organs isolated showed no significant relative weight change.

Table 8.8 Effects of *Antiaris toxicaria* on organ-to-body ratio in rats

		AAE DOSE (mg kg^{-1})		
Test	Control	300	1000	3000
Liver	3.83±0.18	4.68±0.22	3.90±0.36	3.38±0.14
Kidney	0.29±0.02	0.34±0.02	0.32±0.02	0.26±0.02
Spleen	0.28±0.01	0.34±0.02	0.28±0.03	0.25±0.03
Stomach	0.62±0.04	0.70±0.05	0.76±0.07	0.75±0.04
Brain	0.85±0.04	1.05±0.08	0.90±0.08	0.89±0.11
Spinal cord	0.16±0.01	0.19±0.01	0.16±0.02	0.18±0.02

Data are presented as mean±SEM (n=5).

8.3.6 **Histopathological results for mice and rats**

No extract treatment- related changes were observed in this study. For all animals in the treated and control groups, the morphological structure of the liver was normal with no necrosis or disruption of the central vein was found (Plate 8.1; Plate 8.5). The glomeruli of the kidney were found to be normal for each group. There was no thickening of the glomerulus or the epithelia of the renal capsule. The proximal and distal convoluted tubules appeared normal (Plate 8.2; Plate 8.6). Stomach sections showed normal grooves in the mucosa, zymogenic cells and parietal cells (Plate 8.3; Plate 8.7). The spleens were normal for both control and treated animals showing no signs of splenomegaly (Plate 8.4; Plate 8.8). Brain and spinal cord sections from rats showed no signs of cell swelling in both white and gray matter. Cytoplasmic vacuolation was absent in the white matter as well as cell shrinkage and necrosis (Plate 8.9 to Plate 8.14).

8.3.6.1 ***Mice***

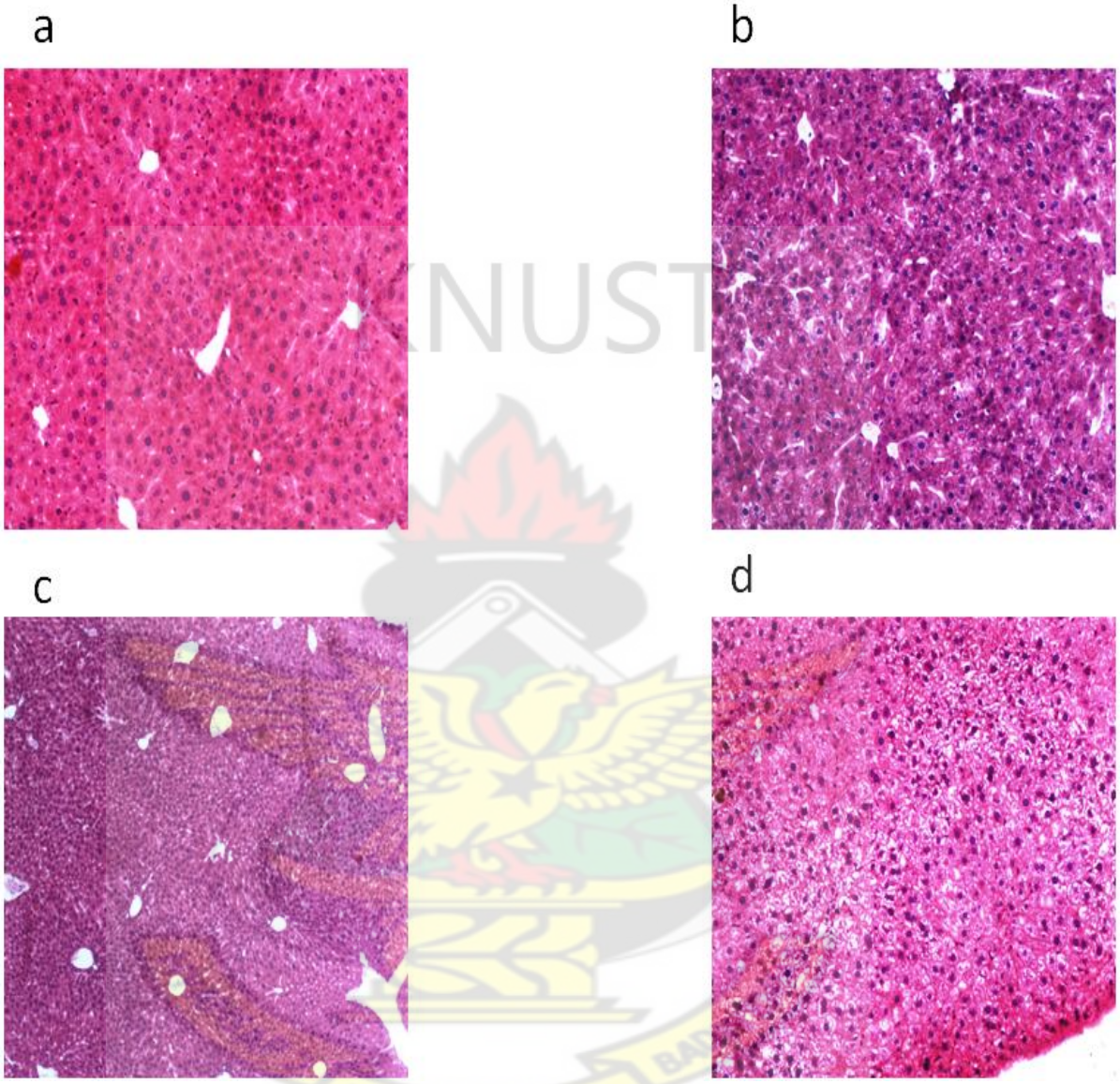

Plate 8.1 Photomicrographs of transverse sections of the liver of mice a) control, b) AAE 300 mg kg^{-1}, c) AAE 1000 mg kg^{-1} and d) AAE 3000 mg kg^{-1} after 14 days of repeated dose treatment (H & E, x 100).

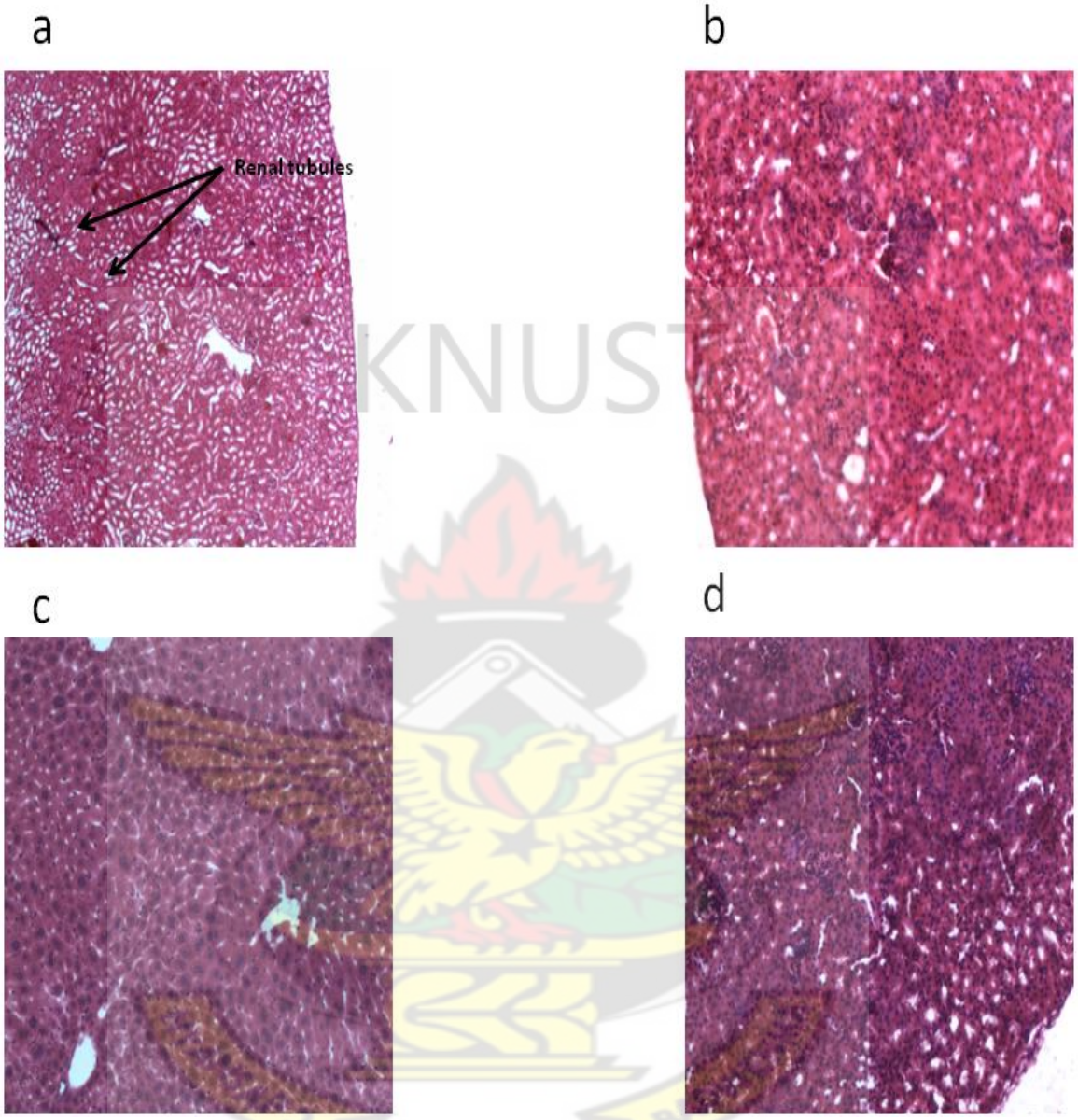

Plate 8.2 Photomicrographs of transverse sections of the kidney of mice a) control, b) AAE 300 mg kg^{-1}, c) AAE 1000 mg kg^{-1} and d) AAE 3000 mg kg^{-1} after 14 days of repeated dose treatment (H & E, x 100).

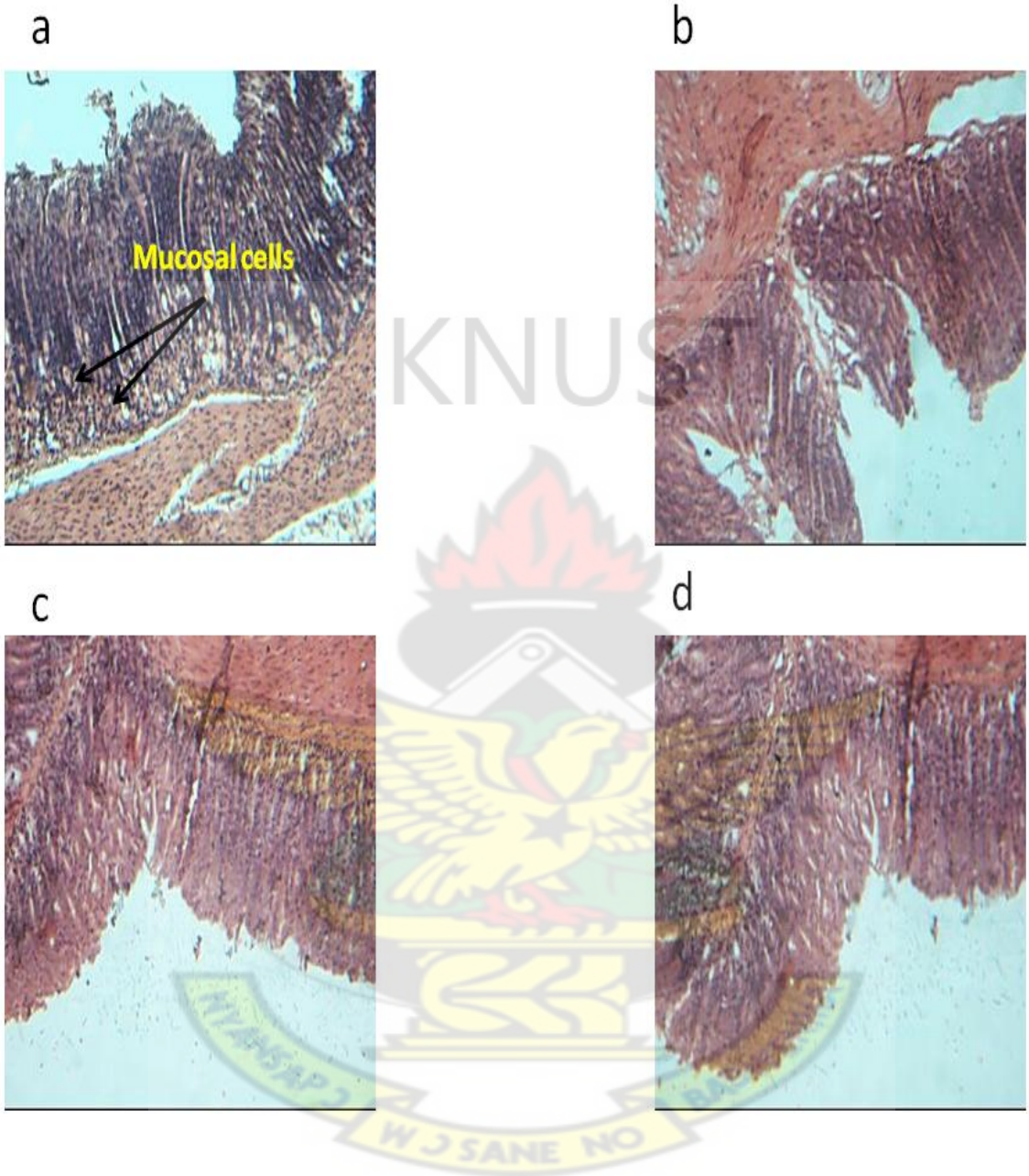

Plate 8.3 Photomicrographs of transverse sections of the stomach of mice a) control, b) AAE 300 mg kg^{-1}, c) AAE 1000 mg kg^{-1} and d) AAE 3000 mg kg^{-1} after 14 days of repeated dose treatment (H & E, x 400).

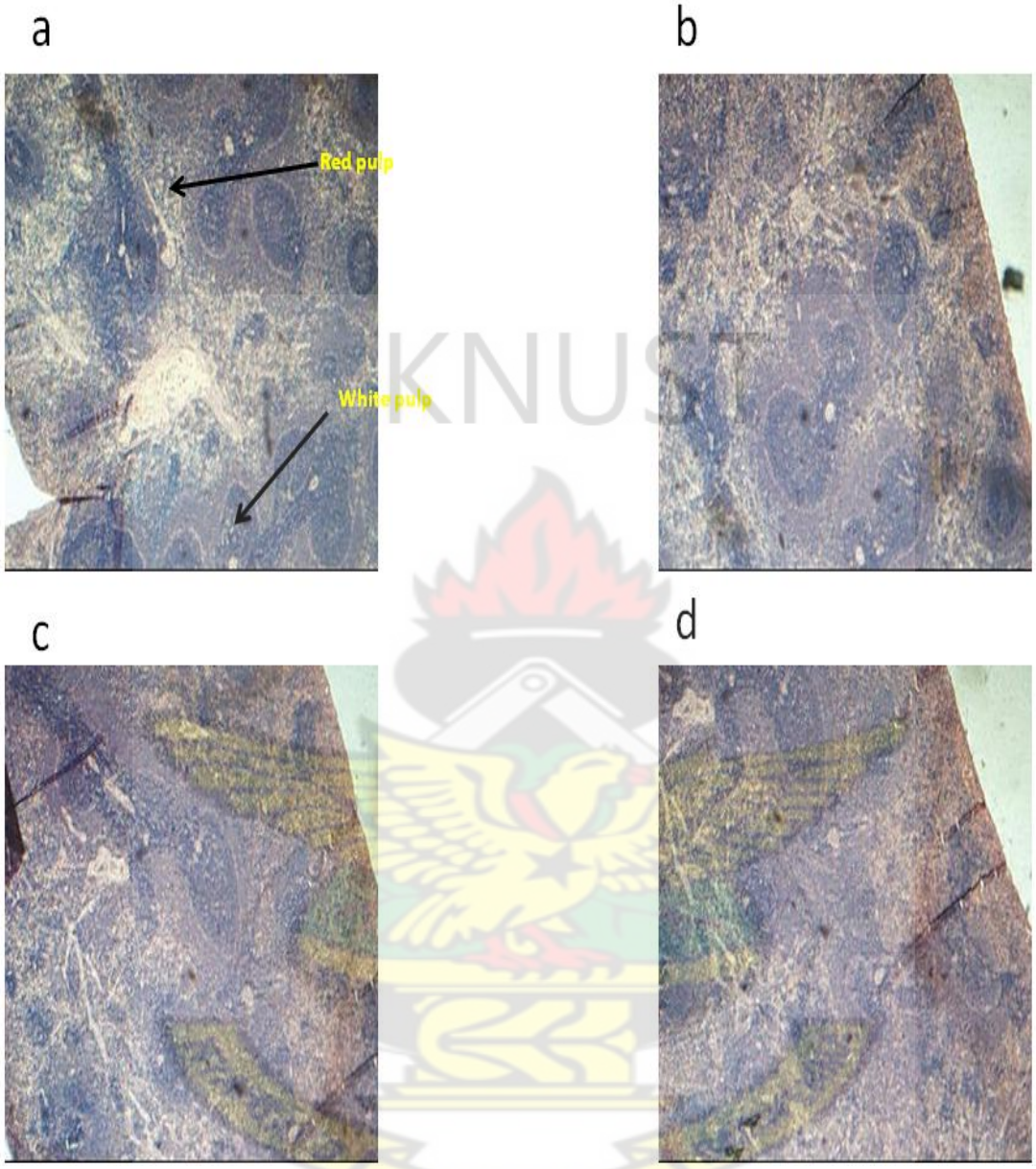

Plate 8.4 Photomicrographs of transverse sections of the spleen of mice a) control, b) AAE 300 mg kg^{-1}, c) AAE 1000 mg kg^{-1} and d) AAE 3000 mg kg^{-1} after 14 days of repeated dose treatment (H & E, x100).

8.3.6.2 ***Rats***

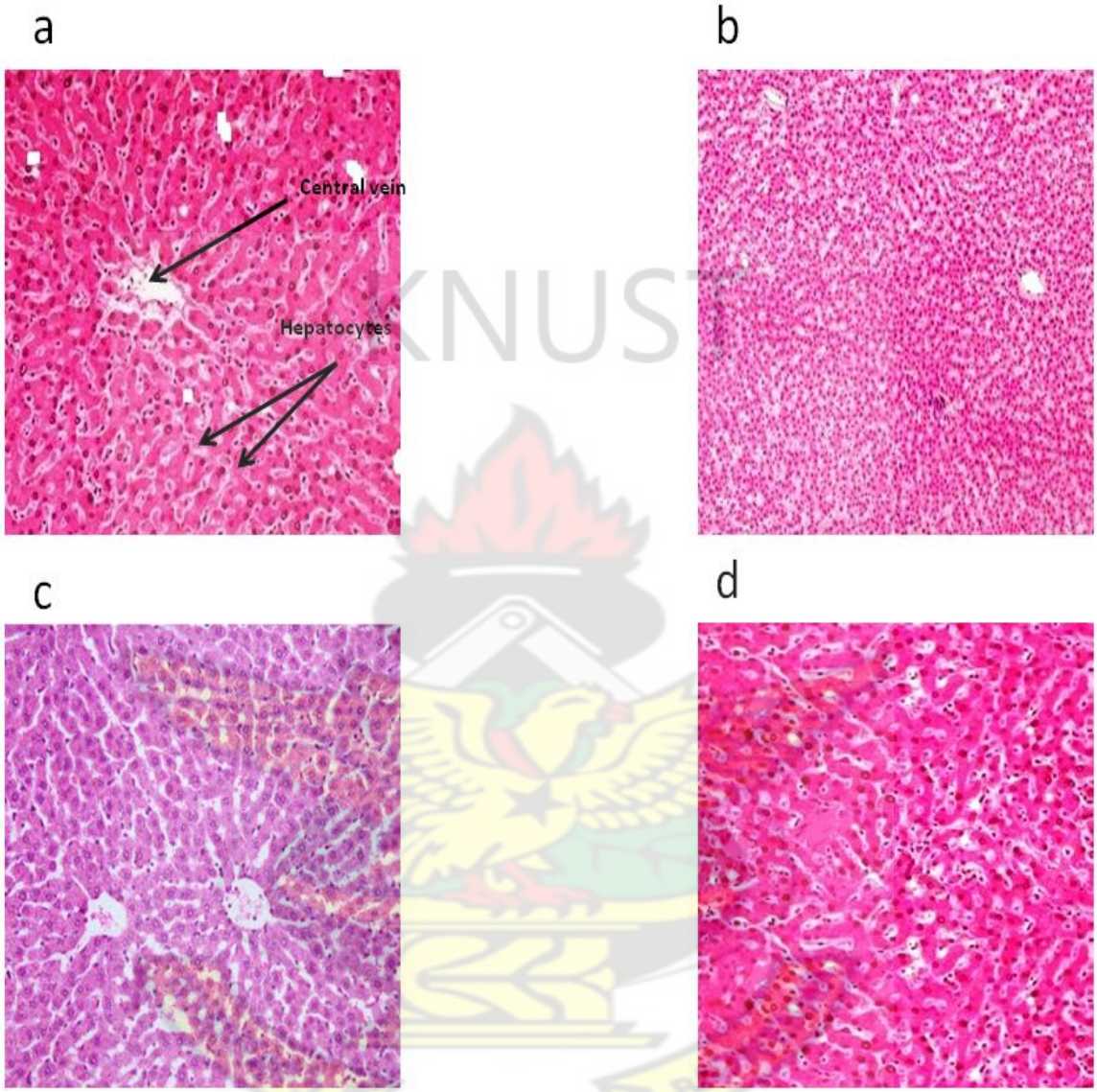

Plate 8.5 Photomicrographs of transverse sections of the liver of rats a) control, b) AAE 300 mg kg^{-1}, c) AAE 1000 mg kg^{-1} and d) AAE 3000 mg kg^{-1} after 14 days of repeated dose treatment (H & E, x 100).

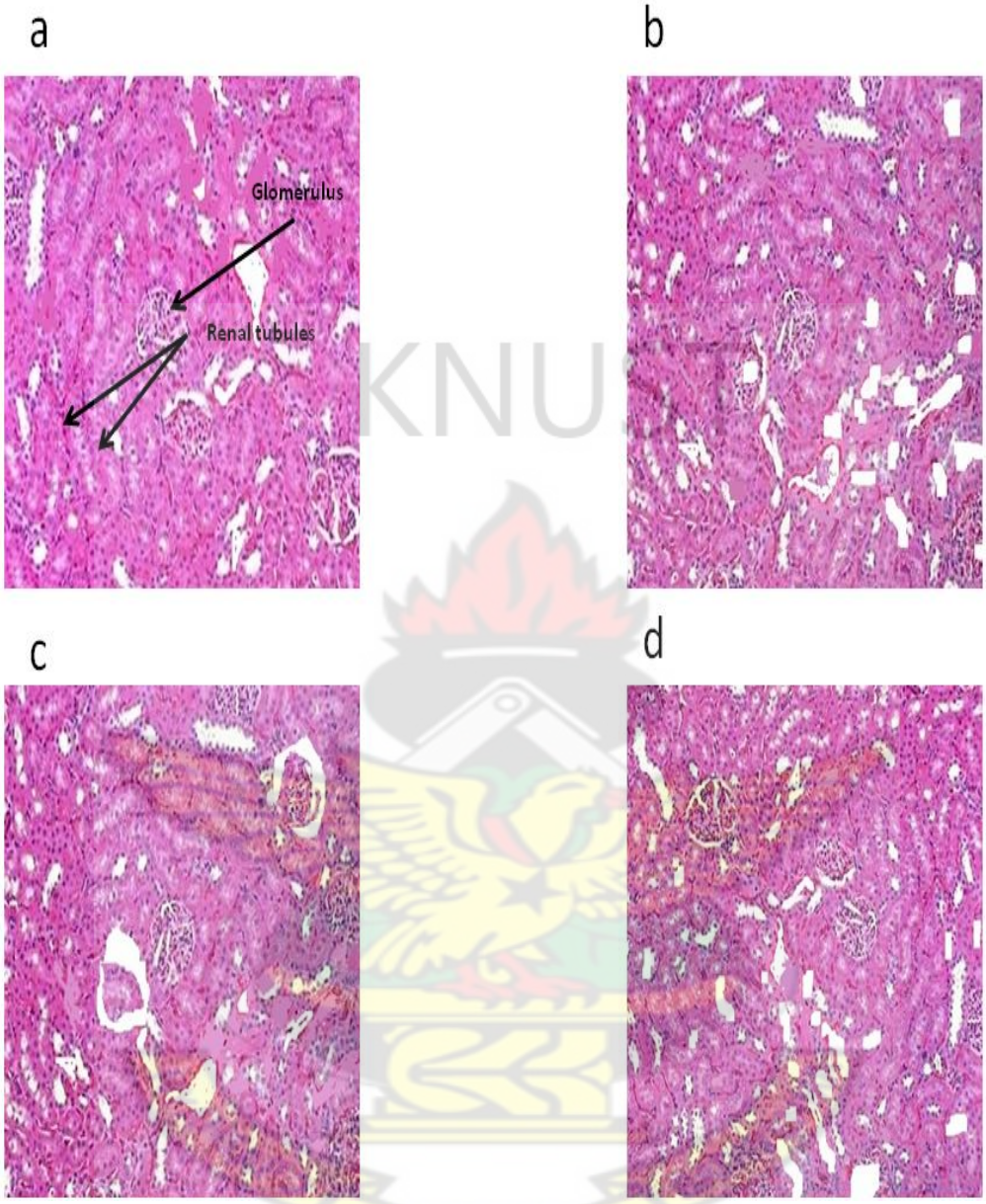

Plate 8.6 Photomicrographs of transverse sections of the kidney of rats a) control, b) AAE 300 mg kg^{-1}, c) AAE 1000 mg kg^{-1} and d) AAE 3000 mg kg^{-1} after 14 days of repeated dose treatment (H & E, x 100).

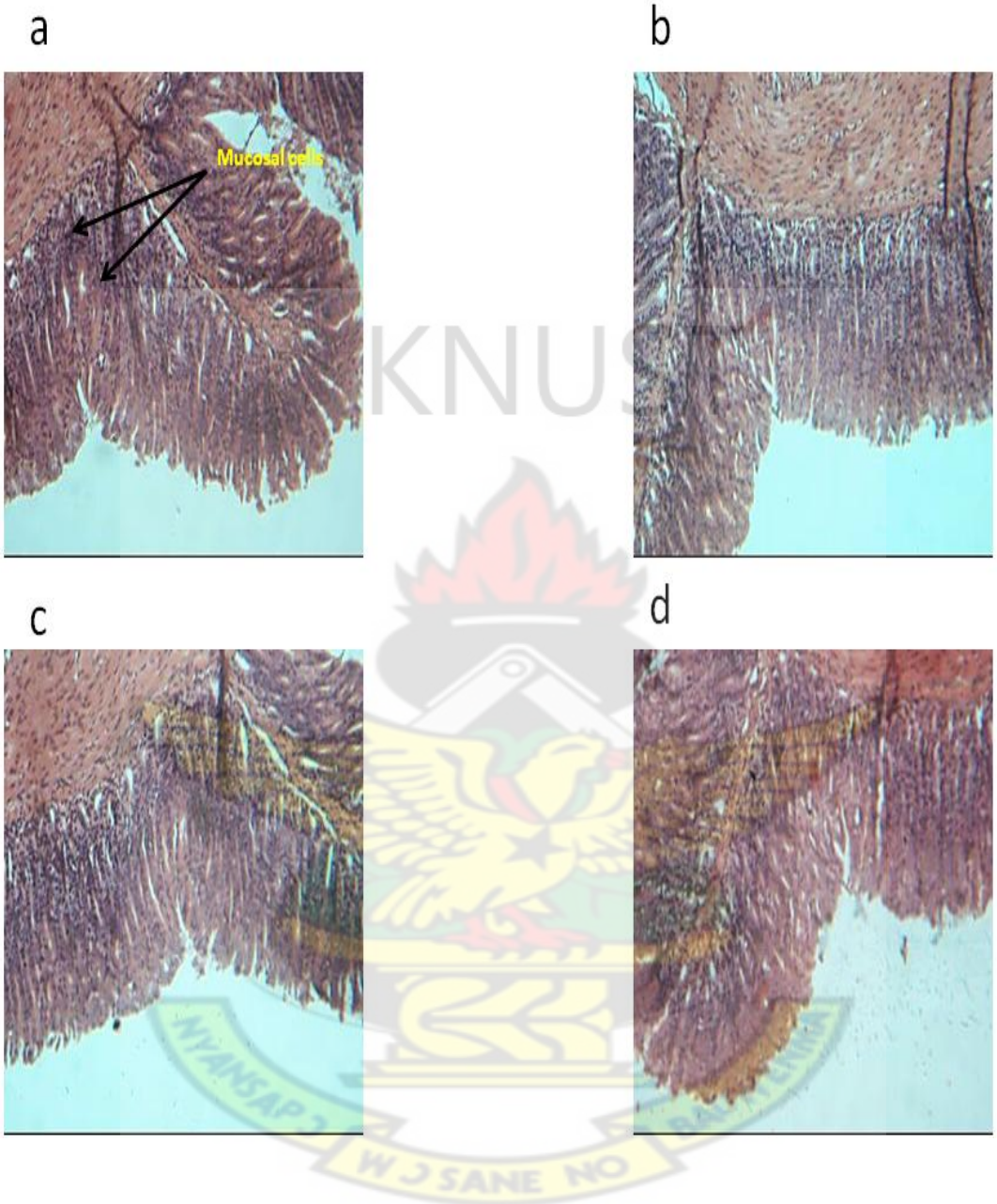

Plate 8.7 Photomicrographs of transverse sections of the stomach of rats a) control, b) AAE 300 mg kg^{-1}, c) AAE 1000 mg kg^{-1} and d) AAE 3000 mg kg^{-1} after 14 days of repeated dose treatment (H & E, x 400).

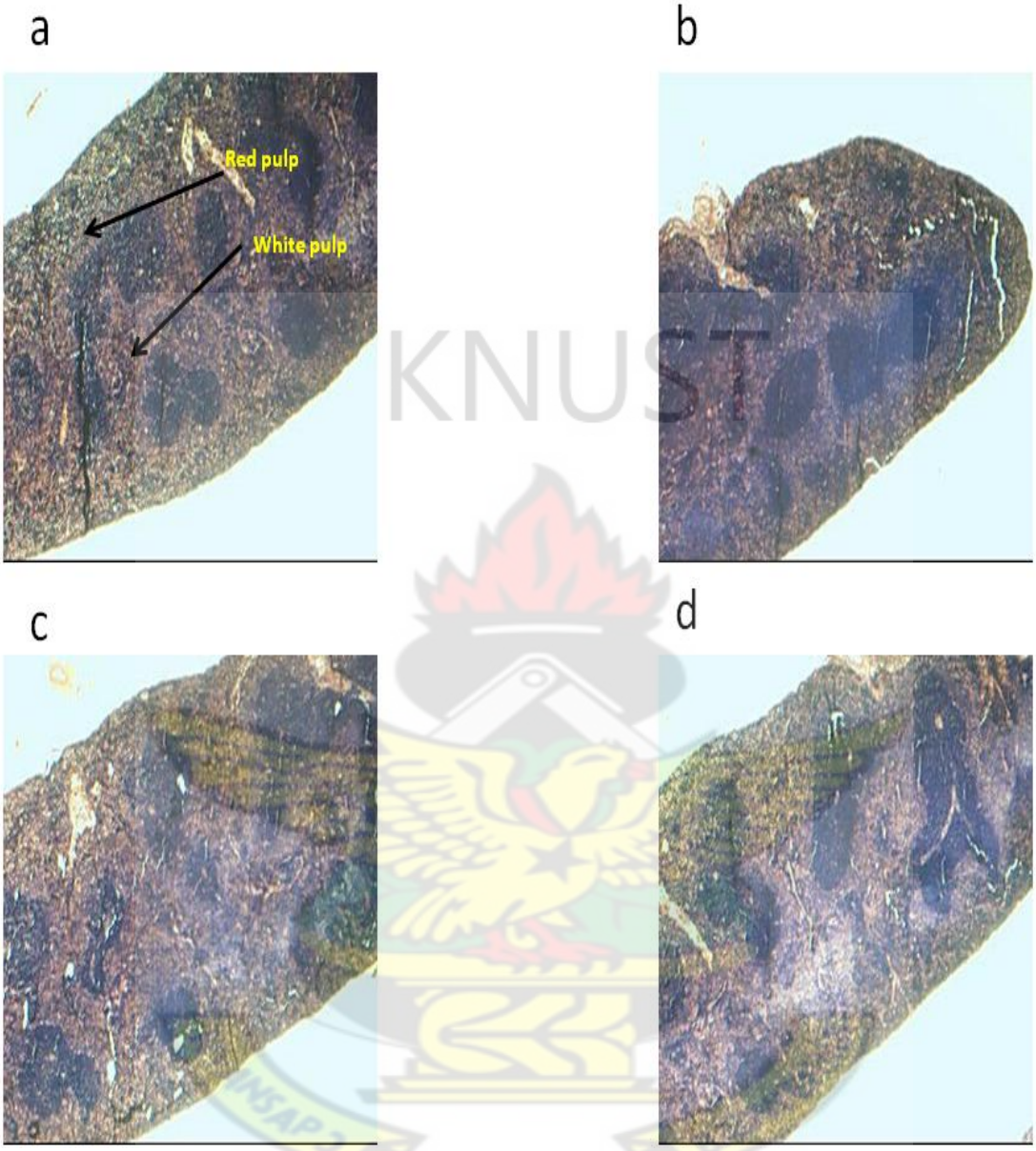

Plate 8.8 Photomicrographs of transverse sections of the spleen of rats a) control, b) AAE 300 mg kg^{-1}, c) AAE 1000 mg kg^{-1} and d) AAE 3000 mg kg^{-1} after 14 days of repeated dose treatment (H & E, x 100).

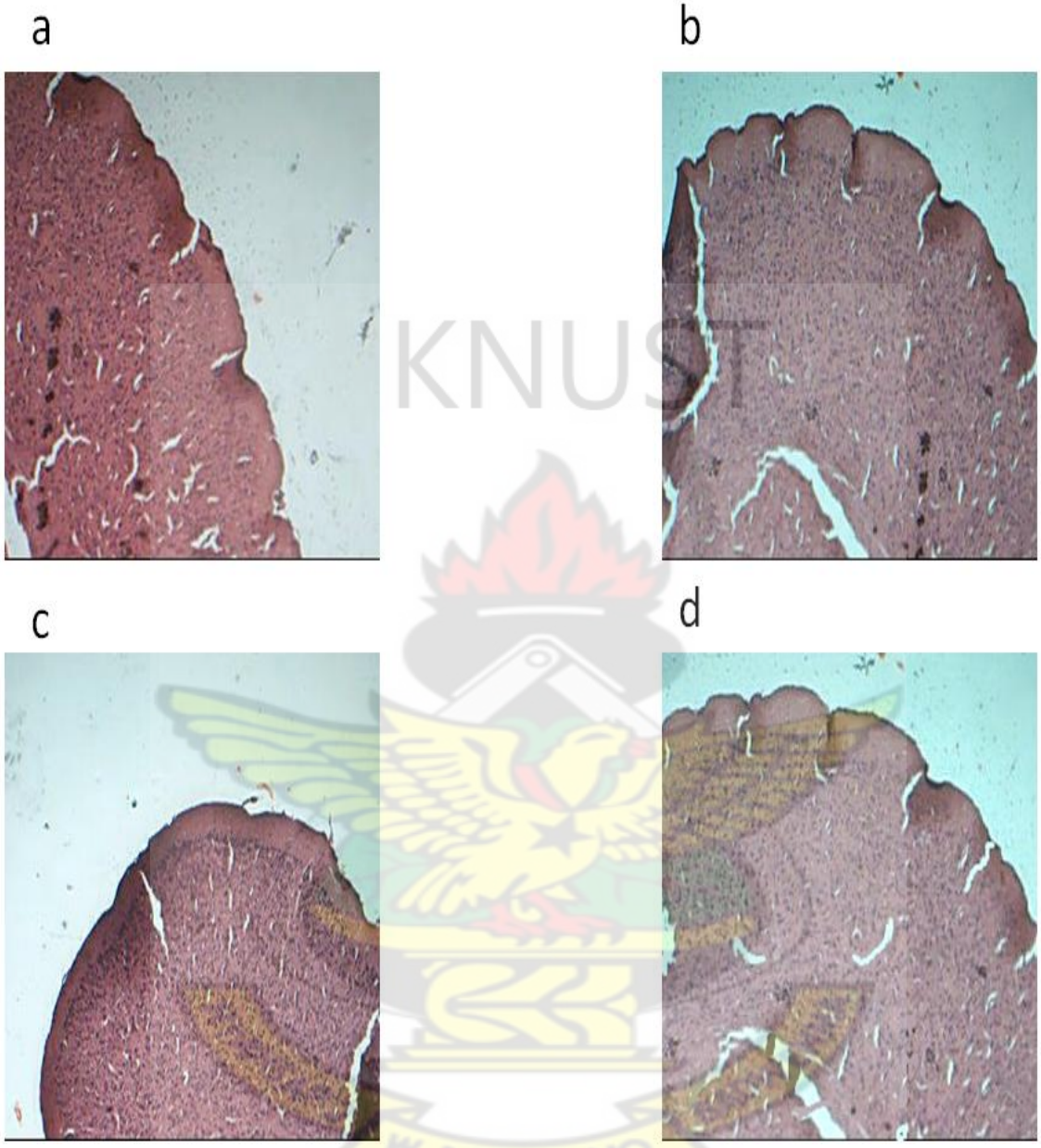

Plate 8.9 Photomicrographs of transverse sections of the frontal lobe of rat brain a) control, b) AAE 300 mg kg^{-1}, c) AAE 1000 mg kg^{-1} and d) AAE 3000 mg kg^{-1} after 14 days of repeated dose treatment (H & E, x 100).

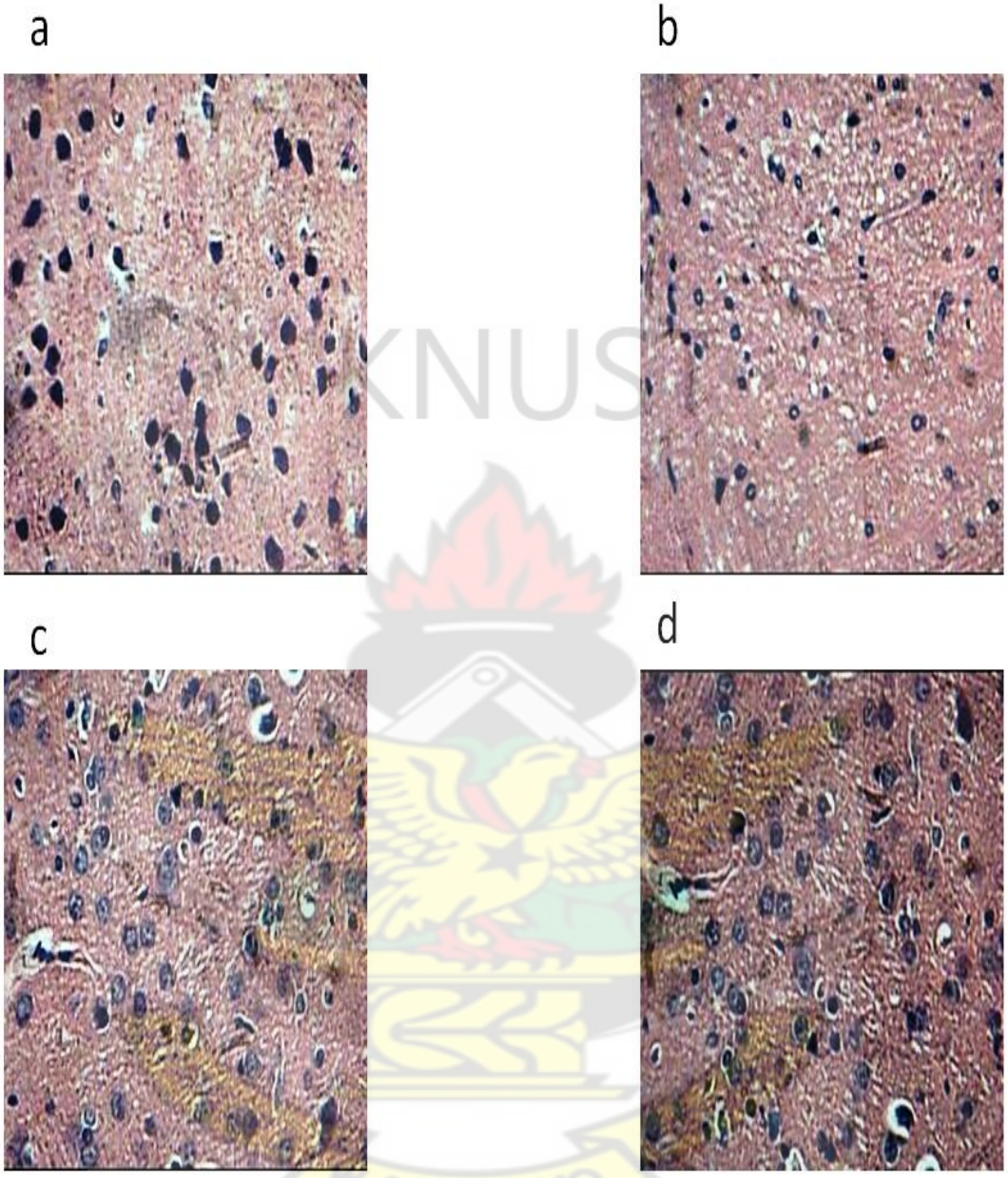

Plate 8.10 Photomicrographs of transverse sections of the temporal lobe of rat brain a) control, b) AAE 300 mg kg^{-1}, c) AAE 1000 mg kg^{-1} and d) AAE 3000 mg kg^{-1}after 14 days of repeated dose treatment (H & E, x 400).

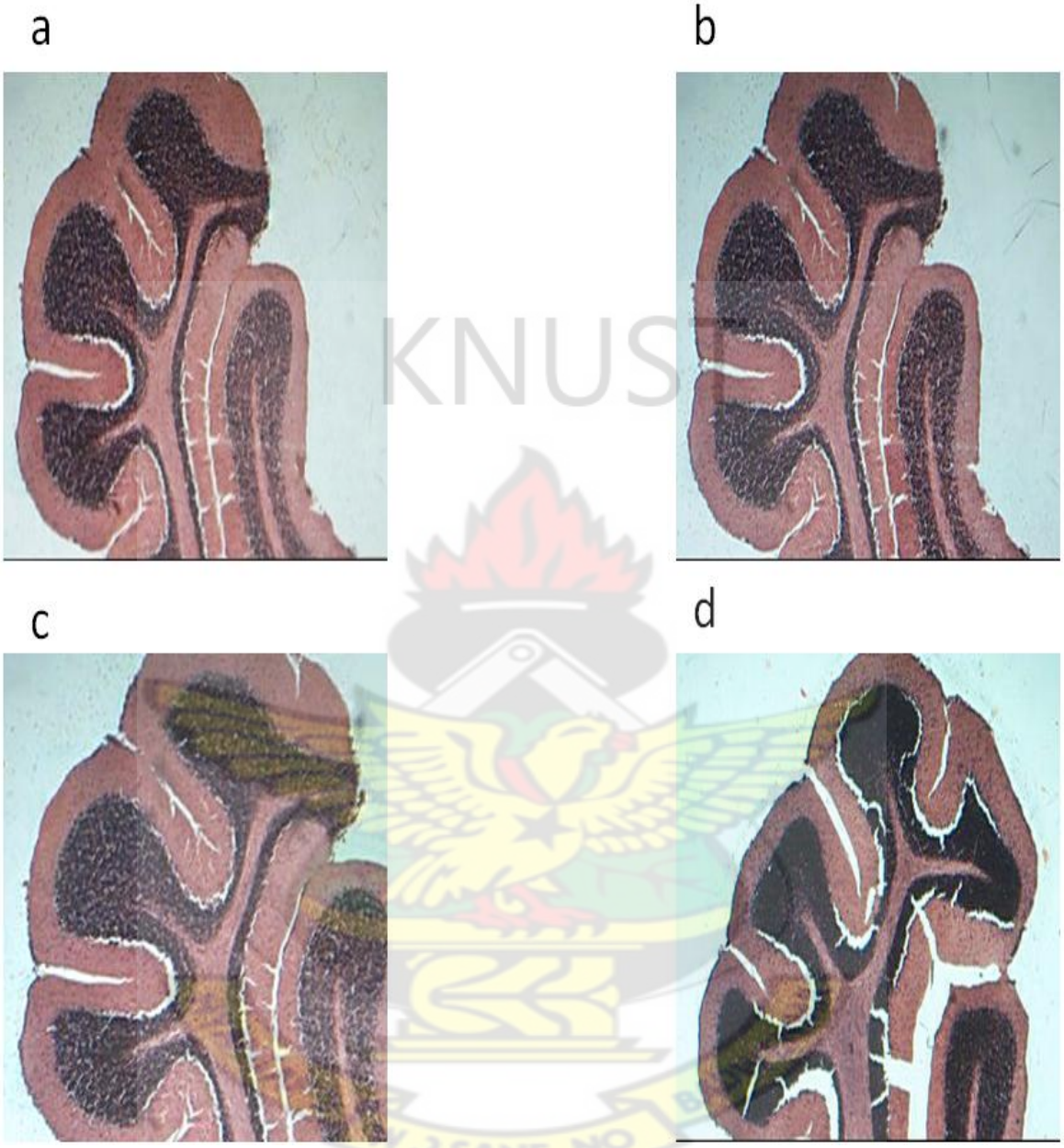

Plate 8.11 Photomicrographs of transverse sections of the cerebellum of rat brain a) control, b) AAE 300 mg kg^{-1}, c) AAE 1000 mg kg^{-1} and d) AAE 3000 mg kg^{-1}after 14 days of repeated dose treatment (H & E, x 100).

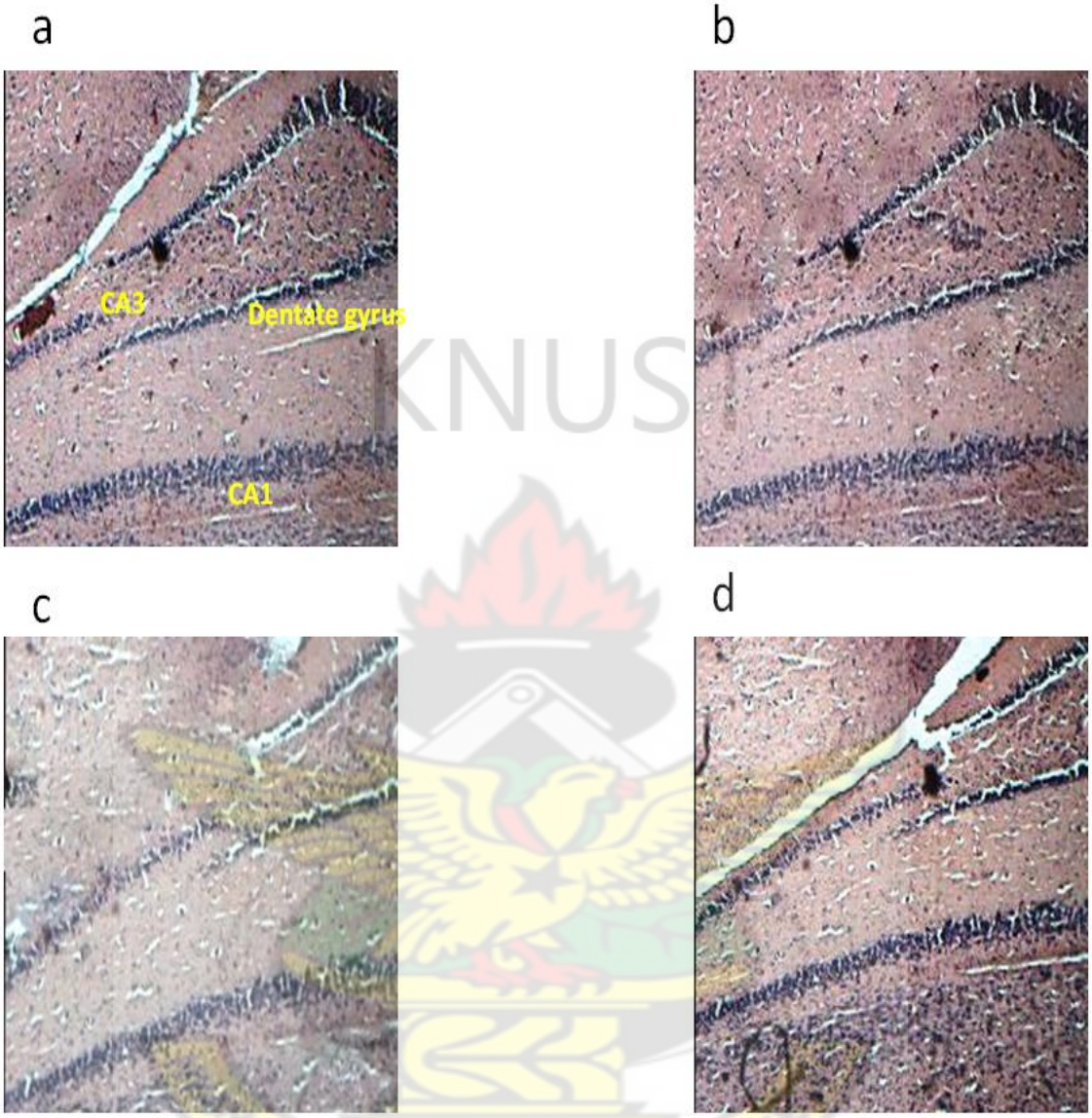

Plate 8.12 Photomicrographs of coronal sections of rat brain showing the hippocampus a) control, b) AAE 300 mg kg^{-1}, c) AAE 1000 mg kg^{-1} and d) AAE 3000 mg kg^{-1}after 14 days of repeated dose treatment (H & E, x 100).

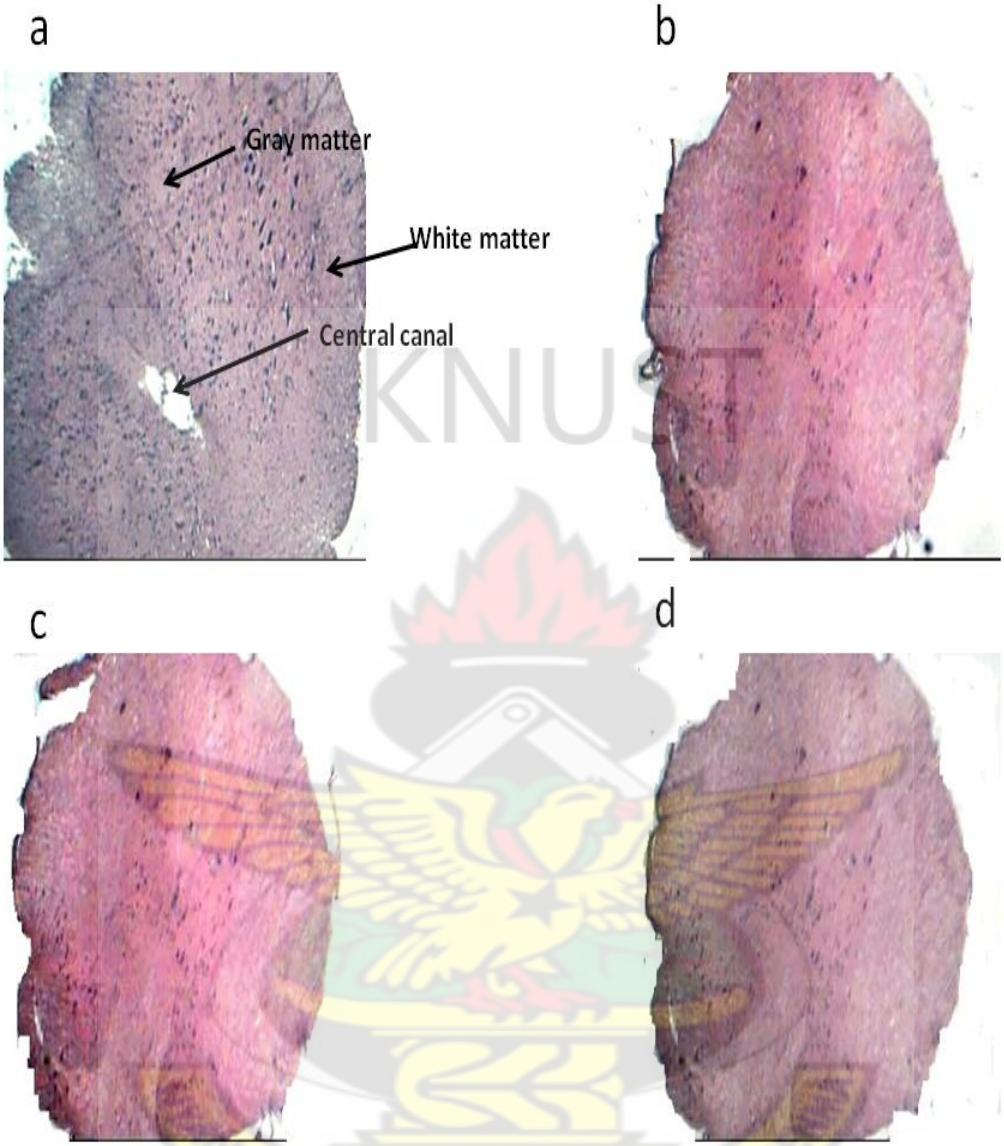

Plate 8.13 Photomicrographs of transverse sections of rat spinal cord a) control, b) AAE 300 mg kg^{-1}, c) AAE 1000 mg kg^{-1} and d) AAE 3000 mg kg^{-1}after 14 days of repeated dose treatment (H & E, x 100).

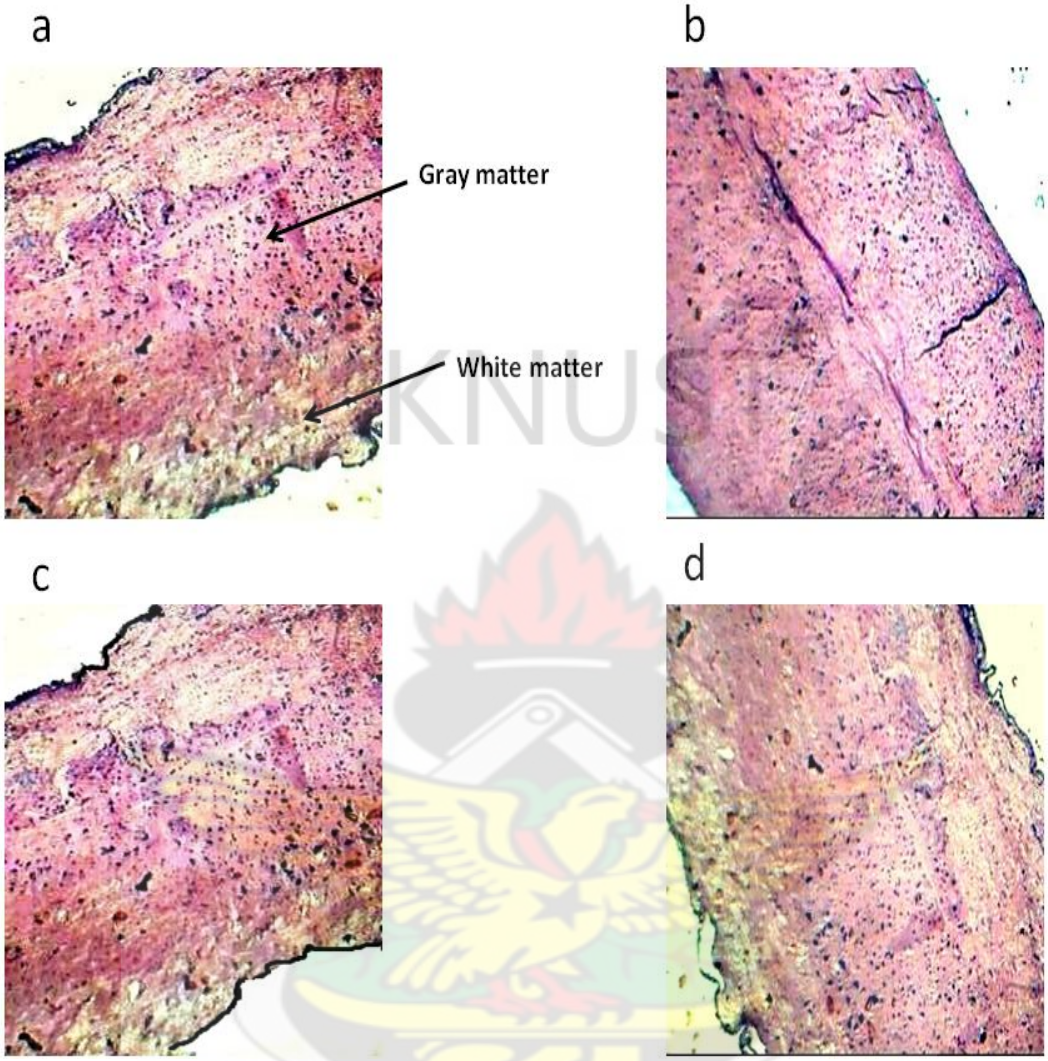

Plate 8.14 Photomicrographs of longitudinal sections of rat spinal cord a) control, b) AAE 300 mg kg^{-1}, c) AAE 1000 mg kg^{-1} and d) AAE 3000 mg kg^{-1}after 14 days of repeated dose treatment (H & E, x 100).

8.4 DISCUSSION

AAE was administered to ICR mice and Sprague Dawley rats at 300-3000 mg kg^{-1} body weight per day for 14 days and some significant adverse toxicological effects due to the treatment were observed. No deaths were recorded, indicating that the LD_{50} for the extract is above 3000 mg $kg^{-1.}$

Body weight changes have been used as an indicator of toxicity of drugs and chemicals (El Hilaly et al., 2004). No significant changes were observed in the general behaviour, body weight and food intake of animals in the treated groups as compared to the untreated group after daily treatment for 14 days. This suggests that oral doses administered had no effect on the normal growth of the animals.

The blood is one of the most sensitive targets for toxic compounds making it an important index of physiological and pathological assessment in man and animals (Mukinda and Syce, 2007). It is the main medium of transport for many substances in the body. Blood components are therefore exposed to many foreign substances (Timbrel, 2000). Damage to these components may result in a variety of effects such as a reduction in the oxygen carrying capacity of the blood and a fall in immunity. *A. toxicaria* showed a decrease in haemoglobin levels at the 1000 mg kg^{-1} dose in rats. A lower than normal haemoglobin concentration may indicate anaemia. Anaemia may be defined as a decline in blood haemoglobin (Hb) level. Three major mechanisms are recognised for its cause: blood loss (e.g. excessive bleeding), inefficient erythropoiesis (e.g. bone marrow disease and nutritional deficiencies), and increased red blood cell (RBC) destruction (e.g. drug-induced RBC haemolysis) (Naeim, 2008). There is more often than not an association between these mechanisms and certain morphologic characteristics reflecting the size (normocytic, microcytic, or macrocytic) of the RBC and/ or the Haemoglobin content (normochromic, hypochromic, or hyperchromic) of the erythrocytes (Naeim, 2008). Four blood parameters measured correlate with the size, haemoglobin content, and degree of anisocytosis in the RBCs. These are the Mean Corpuscular Volume(MCV), Mean Corpuscular Haemoglobin (MCH), Mean Corpuscular Haemoglobin Concentration (MCHC) and Red cell Distribution Width (RDW). All these other parameters were normal, however (Naeim, 2008).

Mean Platelet Volume (MPV) is a measurement of the average size of platelets in the blood. This measurement provides an indicator as to whether the bone marrow is producing platelets normally (Threatte, 1993). It is a more subtle measurement that enables the detection of a problem before it shows up as a low platelet count. Since newly produced platelets tend to be larger than older platelets, a reduced mean platelet volume measurement suggests that fewer new platelets are being produced even though the platelet count is normal (Threatte, 1993). This implies that already circulating cells are not affected by the extract. This was however not the situation in mice. This may be an indication of an anti-haematopoiesis activity. On the other hand, the rest of the haematological parameters showed no significant differences between the control and the treated groups ruling out anti-haematopoietic effect.

The liver metabolizes most drugs and chemicals predisposing it to toxicity. The biochemical parameters AST and ALT showed significant treatment-related increases in rats. The transaminases (AST and ALT) are well-known as good indicators of liver function and toxicity (Rahman et al., 2001; El Hilaly et al., 2004) with AST being less specific than ALT as an indicator of liver function .Wolf *et al* (1973) have identified that any damage to parenchymal hepatocytes is marked by elevations of both transaminases in the blood (1973). Therefore, the significant increases in ALT and AST activities strongly suggest damage to hepatocytes. This was nonetheless not supported by histopathological changes in the liver.

Serum creatinine and blood urea nitrogen (BUN) are often employed in evaluation of renal function. Serum creatinine increases only with nephron damage but the BUN is also affected by dehydration, hepatic metabolism of protein and reduced glomerular filtration rate (Mohanram and Toto, 2005). Blood urea is a measure of urea production and renal excretion. Levels depend on protein intake and as well as breakdown, which also, depends on diet, liver function as well as kidney function. The mean ratio of serum creatinine to the BUN should be approximately 1:10 (Mohanram and Toto, 2005). The decreased creatinine and blood urea levels may be due to decreased muscle mass or atrophy or inadequate dietary protein, kidney filtration or secretion (Mayersohn et al., 1983).

Histopathological changes in organs including cell necrosis, fatty infiltration of cells, and inflammation among other parameters are indicative toxicological changes. When

cells fail to metabolize fatty acid, accumulation of fat within cytoplasmic vacuoles gives rise to fatty change. Cell necrosis is cell death which may arise due to irreversible injury (Wheater, 1990). Histopathological assessment of the liver, kidney, spleen, stomach, brain and spinal cord showed no signs of toxicity related to extract treatment. Other biochemical markers showed no significant changes in animals exposed to the extract over 14 days

8.5 CONCLUSION

Based on this study, it can be concluded that the extract should be used cautiously since it possesses toxic effects which may be specie-dependent. Caution should however be exercised when extrapolating the results to man.

Chapter 9

GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

9.1 GENERAL DISCUSSION

This study has demonstrated that the aqueous stem bark extract of *Antiaris toxicaria* has significant anticonvulsant effect. It also demonstrated antidepressant-like and anxiolytic properties and relative safety of the extract has also been established. The aqueous stem bark of *Antiaris toxicaria* has significant anticonvulsant effects possibly mediated via GABAergic mechanisms and activation of potassium ion channels. The effectiveness of the plant's extract in the experimental convulsion models used probably suggests that the plant could be used in both *petit* and *grand mal* types of epilepsy. Since epilepsy is believed to be triggered due to an imbalance between excitatory and inhibitory mechanisms of the CNS, both arms were investigated in this study. GABA and glutamate are the major inhibitory and excitatory neurotransmitters in the CNS, respectively. The extract was therefore studied against various models involving these neurotransmitters. The study revealed that AAE and diazepam inhibited pentylenetetrazole (PTZ)-induced seizures. The pentylenetetrazole (PTZ) test models human generalized and absence seizures (Loscher and Schmidt, 1988). Pentylenetetrazole has been used in identifying pharmaceuticals that act by reducing seizure susceptibility. The exact mechanism of action of PTZ at the cellular neuronal level is still uncertain but is generally believed to produce seizures by inhibiting gamma-aminobutyric acid (GABA) inhibitory neurotransmission (De Sarro *et al.*, 2003). Picrotoxin is GABA-receptor antagonist that is much more specific in its action. It produces seizures by blocking the chloride-ion channels linked to $GABA_A$ receptors, preventing the entry of chloride ions into neurons. The extract exhibited potent effect in the picrotoxin-induced convulsions lending more credence to the hypothesis that the extract might be working via GABAergic mechanisms. This was further confirmed using flumazenil as antagonist. Flumazenil is known to be a specific antagonist at the diazepam site of the $GABA_A$ receptor. The extract may therefore be proposed to be

acting at the same subunit as diazepam. It was however not proven as to whether or not it was gabamimetic. Standard anticonvulsant agents such as diazepam act via similar mechanism which was also confirmed. The kindling test helps to identify agents that are antiepileptogenic. The extract showed effect in this model. Another inhibitory neurotransmitter of the CNS is glycine which is the main transmitter inhibited in the induction of convulsions by strychnine. Since, the extract showed no effect in this model, involvement of glycine on the anticonvulsant effect of AAE can be ruled out totally. The kainate and 4-aminopyridine models are mechanistic models of epilepsy. Kainic acid acts on the kainate ionotropic glutamate receptors in order to elicit convulsions. The extract showed somewhat potent inhibition against convulsions in this model suggesting that one of AAE's mechanisms of action is via inhibition at the kainate receptor. It therefore reduced the action of glutamate hence decreasing glutamatergic-mediated excitatory reflexes in the CNS. In the 4-aminopyridine test, AAE significantly increased the latency to convulsions pointing to activation of potassium ion channels. 4-Aminopyridine is a potassium ion channel antagonist known to stimulate the release of neurotransmitters, including glutamate, in numerous biological tissue preparations (Hu *et al.*, 1991; Tapia *et al.*, 1999). Since, the duration of seizures was dose-dependently reduced, it can be concluded that AAE further inhibited the sustained excitotoxic effect of glutamate. It can be said that the dose of the extract offers significant protection against death from 4-aminopyridine-induced convulsions as evident from the log-rank test

Pilocarpine is a cholinergic agonist and is widely used in studies of epilepsy to model experimentally induced limbic seizures (Turski *et al.*, 1987). Seizures and SE induced by pilocarpine is known to produce alterations which are behaviourally and electroencephalographically similar to temporal lobe epilepsy (TLE). Similar to kainate induced seizures, the extract has shown efficacy against experimental TLE in rodents suggesting it may be very effective in such a condition.

AAE also produced antinociceptive effects in the formalin; acetic acid induced writhing test and tail withdrawal test.

The extract gave an indication of possible analgesic activity by showing reduced response to tail pinch test in the Irwin test. This effect was first evaluated using the tail withdrawal assay where potent analgesia was established. This test involves

the use of a thermal stimulus and an increase in the reaction time is generally considered to be an important parameter for evaluating central antinociceptive activity (Rujjanawate *et al.*, 2003). The tail withdrawal response is known to be spinally mediated and the method is sensitive to centrally-acting analgesic drugs (Gupta *et al.*, 2005) and that was evident by the analgesic effect of morphine in this test. This therefore implicated central mechanisms of action in the analgesic effect of the extract. AAE produced the Straub tail response in mice and rats in this study which is often measured in response to opioids, mediated by the μ_2-receptor (Nath *et al.*, 1994; Houshyar *et al.*, 2000). It may therefore possess actions similar to centrally acting analgesics such as morphine.

The subcutaneous injection of formalin was used to evaluate neurogenic and inflammatory pain in the formalin test (Tjolsen *et al.*, 1992). The nociceptive response to formalin occurs in a biphasic pattern; Phase 1 corresponds to acute nociceptive neurogenic pain, and is sensitive to analgesic drugs acting via the opioidergic system. Phase 2 corresponds to an inflammatory pain, dependent on several inflammatory mediators and is very sensitive to non-steroid anti-inflammatory drugs (NSAIDs) such as the cyclooxygenase inhibitors (Couto *et al.*, 2011). Centrally-acting analgesics inhibit both phases while peripherally acting drugs inhibit only the second phase (Abram and Olson, 1994; Yamamoto and Nozaki-Taguchi, 2002; Yamamoto *et al.*, 2002). Once both first and second phase were inhibited by AAE, it can be inferred that its effect is attributable to peripherally acting as well as centrally acting pain mediators.

Acetic acid writhing assay models human clinical somatic pain conditions and is sensitive to peripherally-acting analgesic drugs (Couto *et al.*, 2011). The intraperitoneal administration of acetic acid induced a stereotypical behaviour in mice (abdominal contractions, twisting of dorsoabdominal muscles in addition to a reduction in motor activity and coordination) (Le Bars *et al.*, 2001). There is the release of arachidonic acid via prostaglandin biosynthesis and sympathetic nervous system mediators (Koo *et al.*, 2006). AAE and diclofenac were effective in the acetic acid-induced writhing assay, each producing a dose-dependent suppression of the writhing response. The acetic acid-induced writhing can be seen as a general non-selective pain model, since acetic acid indirectly induces the release of endogenous mediators, stimulating the peripheral nociceptor and

sensitive neurons that are sensitive to the inflammatory mediators. It may also be regarded as a model of inflammatory pain (Ribeiro *et al.*, 2000; Couture *et al.*, 2001).

The effect of *A toxicaria* is similar to that of diclofenac which acts not only through anti-inflammatory pathways, but has a peripheral antinociceptive effect which is associated with ATP-sensitive K^+ channel (Alves and Duarte, 2002; Panthong *et al.*, 2007; Panthong *et al.*, 2007).

Given that some anticonvulsant are used clinically in the management of neuropathic pain (Birse *et al.*, 2012), the analgesic property obtained in this study will be advantageous in the therapeutic use of *A. toxicaria*.

AAE has also demonstrated sedative effects by enhancing pentobarbitone-induced sleep in mice without skeletal muscle relaxant properties (as demonstrated in the rotarod and beam traversal tests). Time spent on the rotating rod in the rotarod test is apparently affected by both sedation and loss of muscle tone (Green *et al.*, 2001). Some drugs can cause a reduction in muscle tone through central mechanisms independent of sedation (Green *et al.*, 2001). Sedative properties observed in the sleeping time test appeared not to affect the rotarod and beam traversal tests. This makes the extract advantageous over drugs that act via GABAergic mechanisms such as benzodiazepines, as these are known to possess muscle relaxant properties. Reduced spontaneous activity was obtained at lower doses of AAE which may be attributable to its sedative properties observed. The paradoxical increase in locomotor activity at higher doses may be as a result of increased explorative tendencies brought on by anxiolysis (Turski *et al.*, 1982).

Enzyme induction test carried out using phenobarbitone resulted in a significant decrease in sedative effect produced by the extract. Therapeutic agents which induce hepatic enzymes may stimulate their own metabolism by autoinduction. As such, for compounds active in their parent form, induction may increase elimination and hence reduce the desired pharmacological effect. There may also be enhanced formation of the active metabolite(s) for prodrugs resulting in an increased pharmacological effect and hence toxicity (Dickins, 2004; Lin, 2006). With the first instance being the most likely case with phenobarbitone and the extract, an important possible clinical consequence may be drug–drug interactions leading to loss of the desired pharmacological effect of the extract when co-administered with an enzyme inducing therapeutic agent. Some enzyme inducers

may enhance specific adverse effects. For instance, CYP2E1 induction is believed to increase the severity of hepatotoxicity not only by increasing the formation of reactive metabolites of numerous compounds, but also by generating reactive oxygen species (oxidative stress) during its catalytic cycle.

Antiepileptic drugs (AEDs) are the interface between neurology and psychiatry as they are used to treat both epilepsy and psychiatric disorders (Ketter *et al.*, 2003; Landmark, 2008). The choice of AED made by the neurologist in the treatment of epilepsy is most often based on the classic benefits (seizure control efficacy), risks (adverse side effects), and alternatives (availability, formulary, costs). However, consideration must be given to psychiatric comorbidities and which AED might best serve the patient in both maximizing seizure control and minimizing psychiatric symptoms (García-Morales *et al.*, 2008).

It has been established that Psychiatric disorders are more prevalent in people with epilepsy than in the general population (Hermann *et al.*, 2000; Marsh and Rao, 2002). Anxiety has been recognized as the most common psychiatric comorbidity (Beyenburg *et al.*, 2005). Anxiety disorders are not always pure psychological or psychosocial phenomena, but can be a direct result of neurobiological processes. The relationship between anxiety, fear, and epilepsy was recognized by Hughlings Jackson (Jackson, 1879). He recognized fear as part of a seizure itself rather than a reaction to what was about to occur. Anxiety disorders represent a clinically important comorbid disorder in epilepsy patient (Vazquez and Devinsky, 2003). Currently used pharmacological options for treating anxiety disorders include several drug classes including benzodiazepines (Scicutella and Ettinger, 2002). Anxiety symptoms are common in patients with epilepsy but treatment remains suboptimal (Vazquez and Devinsky, 2003).

A number of anticonvulsants e.g. diazepam find use in anxiety states mainly because of their actions on GABA and glutamate. Since, the extract had showed prior action on these neurotransmitters, anxiolytic properties were investigated and found to be consistent with literature. An anticonvulsant that doubles as an anxiolytic is therefore an advantage.

Just as anxiety is associated with epilepsy, depression has been shown to be a common comorbid condition accompanying epilepsy. In epileptic patients, depression is the strongest predictor of poor quality of life (Lehrner *et al.*, 1999).

The extract exhibited clear antidepressant effect strengthening further its usefulness in the management of epilepsy as well as in pain. Some anticonvulsants e.g. clonazepam have been investigated and are licensed for use in depressive conditions (Calabrese *et al.*, 2008; Santos *et al.*, 2008; Kaufman, 2011). The extract showed antidepressant effects in the models employed. The mechanism of action as antidepressant was elucidated and shown to be mediated through serotoninergic pathway which was confirmed using the 5-hydroxytryptophan potentiation test. This test strongly indicates actions mediated by inhibition of serotonin reuptake (Pandey *et al.*, 2008). The extract showed relaxation of carbachol-induced contractile activity on the isolated uterus preparation confirming its β-adrenoceptor activity (Liu *et al.*, 1998). β-adrenoceptor agonists have been shown to possess antidepressant effects but have not been successfully introduced into clinical practice owing to the myriad of side effects such as tachycardia or alteration of locomotor activity (Simiand *et al.*, 1992). Changes in locomotor activity were observed in the neuropharmacological screening making the extract quite similar in this side effect profile to standard β-adrenoceptor agonists. Tachycardia was, however, not observed at the doses used giving the extract a little edge over the standard drugs.

Clinical effectiveness of antidepressants as analgesics has been confirmed in preclinical studies using various models of nociceptive and chronic pain (Zarrindast *et al.*, 2000; Marchand *et al.*, 2003; Rojas-Corrales *et al.*, 2003; Anjaneyulu and Chopra, 2004; Duman *et al.*, 2004). They are believed to interact with the pain pathways in several ways. This effect is separated from the anti-depressive action (Arnold *et al.*, 2005).

Oral administration of AAE at 3000 mg kg^{-1} failed to cause any deaths in mice treated over a 24 h period hence, estimating the LD_{50} as above 3000 mg kg^{-1}. *A. toxicaria* showed a decrease in haemoglobin levels at the 1000 mg kg^{-1} dose in rats. This may be associated with anaemia, defined as a decline in blood haemoglobin (Hb) level. This may be due to loss of blood (e.g. excessive bleeding), inefficient erythropoiesis (e.g. bone marrow disease and nutritional deficiencies), and increased red blood cell (RBC) destruction (e.g. drug-induced RBC haemolysis).

Mean Platelet Volume (MPV) measures the average size of platelets in the blood providing an indicator as to whether the bone marrow is producing platelets

normally (Threatte, 1993). Since newly produced platelets tend to be larger than older platelets, a reduced mean platelet volume measurement suggests that fewer new platelets are being produced even though the platelet count is normal (Threatte, 1993).

AST and ALT were elevated in rats. AST and ALT are good indicators of liver function and toxicity (Rahman et al., 2001; El Hilaly et al., 2004). Therefore, the significant increases in ALT and AST activities strongly suggest damage to hepatocytes which is most likely related to treatment with the extract. The Serum creatinine and blood urea nitrogen (BUN) were both lower than that for untreated rats which may imply decreased muscle mass or atrophy or inadequate dietary protein, kidney filtration or secretion (Mayersohn et al., 1983).

Histopathological assessment of the liver, kidney, spleen, stomach, brain and spinal cord however showed no signs of toxicity related to any found in the haematological and serum biochemical reports. Also, the toxic effects seen in the rats were not present in mice indicating that toxicity of the extract is specie-dependent. No organ weight changes were observed.

The effects produced by the extract have been proposed to be via action on some ionotropic and metabotropic receptors and their related pathways. Figure 9.1 summarizes the proposed mechanisms of action of *Antiaris toxicaria* aqueous stem bark extract.

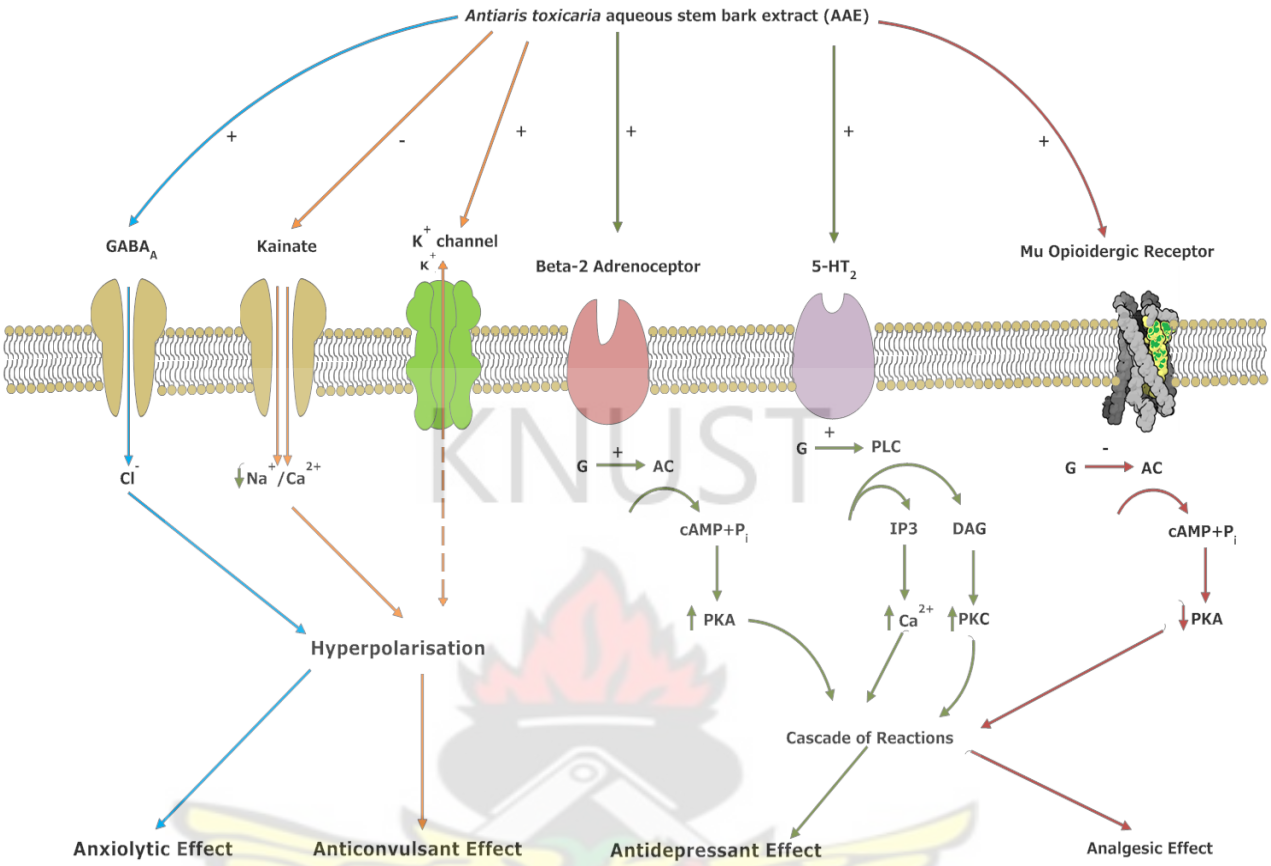

Figure 9.1Proposed mechanisms of action of *Antiaris toxicaria* aqueous extract. AAE activates $GABA_A$ neurotransmission causing an increase in chloride (Cl^-) conductance and consequently neuronal hyperpolarization. It also inhibits kainate receptor activation to decrease sodium and calcium ion conductance. Activation of potassium ion channels also produces hyperpolarization. The hyperpolarization results in anticonvulsant and anxiolytic effect. AAE activates beta-2 adrenoceptor receptors, 5-HT and opioid pathways leading to a cascade of signal transduction mechanisms which are responsible for the observed antidepressant effect and analgesic properties.

9.2 CONCLUSIONS

Results presented here indicate that the aqueous extract of *Antiaris toxicaria* stem bark exhibits anticonvulsant, anxiolytic-like, antidepressant-like and analgesic effects.

- Activity on the GABAergic system has been established without an effect on motor coordination. This contributes to its anticonvulsant property in concert with enhancement of potassium ion conductance and inhibition of kainate receptor activation.
- It may enhance activity of the serotonergic system as shown in the tail suspension test. It also possesses β-adrenoceptor activity and opioidergic activity. These actions are responsible for the antidepressant and analgesic properties.

9.3 RECOMMENDATIONS

- The extract should be fractionated and the active constituent(s) responsible for the neuropharmacological effects observed should be isolated and characterized.
- The extract should be tested in other antinociceptive models and the precise mechanisms by which *Antiaris toxicaria* produces analgesic activity should be established.
- Chronic toxicity studies should be carried out on the extract.

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APPENDIX

COMPOSITION OF DE JALON SOLUTION

COMPONENT	QUANTITY
NaCl	90 g
10 % KCl	42 ml
Glucose	5 g
$NaHCO_3$	5 g
$CaCl_2$ (Molar)	2.7 ml
Distilled water qs	10 L

TABLE OF ED_{50} AND E_{max} VALUES FOR VARIOUS TESTS

Test	Extract		Standard	
	ED_{50}	E_{max}	ED_{50}	E_{max}
Formalin Test				
			Morphine	
Phase 1	418.10	100	0.39	100
Phase 2	648.90	100	1.06	100
4-Aminopyridine seizures				
			Sodium valproate	
	298.50	100	258.80	100
PTZ-induced kindling				
			Diazepam	
	276.70	88.83	0.052	60.36
MEST				
			Carbamazepine	
Duration	238.8	53.55	14.60	104.60
Latency	24460	~ 20	6.76	115
Picrotoxin-induced seizures				
			Diazepam	
Latency	338.00	21.88	0.17	105.50
Duration	227.10	100.00	0.03	97.29

Frequency	179.20	79.13	0.08	107.50
PTZ-induced seizures				
			Diazepam	
Latency	1407.00	~30.00	0.11	109.70
Duration	484.20	100.00	0.01	100.90
Frequency	683.80	97.85	0.01	101.70
Strychnine				
Latency	~484.90	~5.84		
Duration	137.10	7.78		
FST				
			Fluoxetine	
Mobility	351.30	100.00	8.39	100.00
			Imipramine	
			4.52	100.00
TST				
			Fluoxetine	
Mobility	340.90	100.00	3.32	100.0
			Imipramine	
PILO-induced seizures			2.08	100.00
			Diazepam	
	80.06	100.00	1.67	100.00
Writhing Assay				
			Diclofenac	
	1757.00	100.00	25.07	90.00

DETAILED OBSERVATIONS IN THE IRWIN TEST

Dose (mg/kg)	0							100							300							1000						
Time(min)	15	30	60	120	180	24h	48h	15	30	60	120	180	24h	48h	15	30	60	120	180	24h	48h	15	30	60	120	180	24h	48h
Lethality	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Convulsions	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tremor	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Straub tail	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	+	+	+	+	-	-	-
Sedation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Excitation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Abnormal gait (rolling)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Abnormal gait (tiptoe)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Jumps	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Motor incoordination	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Loss of balance	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Fore-paw treading	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Writhes	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Piloerection	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Stereotypies (sniffing)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Stereotypies (chewing)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Stereotypies (head movement	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Head twitches	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Scratching	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Respiration	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Aggressiveness	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Fear	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Reactivity to touch	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Muscle tone	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Loss of writhing reflex	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ptosis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Exophthalmos	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Loss of grasping	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Akinesia	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Catalepsy	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Loss of traction	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Loss of corneal reflex	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Analgesia	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-
Defaecation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Salivation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lacrimation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Urination	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Change in Rectal temperature	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Dose (mg/kg)	3000						
Time(min)	15	30	60	120	180	24h	48h
Lethality	-	-	-	-	-	-	-
Convulsions	-	-	-	-	-	-	-
Tremor	-	-	-	-	-	-	-
Straub tail	+	+	+	+	-	-	-
Sedation	-	-	-	-	-	-	-
Excitation	-	-	-	-	-	-	-
Abnormal gait(rolling)	-	-	-	-	-	-	-
Abnormal gait(tiptoe)	-	-	-	-	-	-	-
Loss of balance	-	-	-	-	-	-	-
Fore-paw treading	-	-	-	-	-	-	-
Writhes	-	-	-	-	-	-	-
Piloerection	-	-	-	-	-	-	-
Stereotypies (sniffing)	-	-	-	-	-	-	-
Stereotypies (chewing)	-	-	-	-	-	-	-
Stereotypies (head movements)	-	-	-	-	-	-	-
Head twitches	-	-	-	-	-	-	-
Scratching	-	-	-	-	-	-	-
Respiration	-	-	-	-	-	-	-
Aggressiveness	-	-	-	-	-	-	-
Fear	-	-	-	-	-	-	-
Reactivity to touch	-	-	-	-	-	-	-
Muscle tone	-	-	-	-	-	-	-
Loss of writhing reflex	-	-	-	-	-	-	-
Ptosis	-	-	-	-	-	-	-
Exophthalmos	-	-	-	-	-	-	-
Loss of grasping	-	-	-	-	-	-	-
Akinesia	-	-	-	-	-	-	-
Catalepsy	-	-	-	-	-	-	-
Loss of traction	-	-	-	-	-	-	-
Loss of corneal reflex	-	-	-	-	-	-	-
Analgesia	+	+	+	-	-	-	-

Defaecation	-	-	-	-	-	-	-
Salivation	-	-	-	-	-	-	-
Lacrimation	-	-	-	-	-	-	-
Urination	-	-	-	-	-	-	-
Change in Rectal temperature	-	-	-	-	-	-	-

AAE (100, 300, 1000 and 3000 mg kg^{-1}, *p.o.*; n=6). Observations were performed at 15, 30, 60, 120, 180 min, 24 h and 48 h after administration. + indicates presence of effect and – shows effect was absent.