ANTICONVULSANT AND RELATED NEUROPHARMACOLOGICAL EFFECTS OF A HYDRO-ETHANOLIC WHOLE PLANT EXTRACT OF *SYNEDRELLA NODIFLORA* (L.) GAERTN (FAM: ASTERACEAE)

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DECLARATION

The experimental work described in this report was carried out at the Department of Pharmacology, KNUST and the Department of Applied Therapeutics, Faculty of Pharmacy, Health Science Center, Kuwait University. This work has not been submitted for any other degree.

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ABSTRACT

Synedrella nodiflora (L.) Gaertn. (Family: Asteraceae) is an annual herb which grows to about 60-120 cm high and occurs throughout the West African region. In Ghanaian traditional medicine, the whole plant is used for the treatment of epilepsy. This study presents the anticonvulsant, sedative, muscle relaxant, antinociceptive, antioxidant, the effect on anxiety and the safety of a hydro-ethanolic extract of the whole plant of *Synedrella nodiflora*.

Four murine models of experimental epilepsy were employed for the anticonvulsant screening of the extract, namely; pentylenetetrazole-, picrotoxin- and pilocarpine- induced seizure and penetylenetetrazole- induced kindling. The ability of the extract to cause sedation was investigated using the pentobarbitone- induced sleep test. The neuromuscular effects of the extract were also determined *in vivo* using the rota-rod test and *in vitro* with the chick biventer cervicis preparation. Moreover, formalin induced pain and acetic acid induced writhing assay were the tests employed to assess the antinociceptive effects of the extract. The antioxidant effects of the extract was also determined by measuring the total phenols, antioxidant capacity, 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) scavenging assay, reducing power assay and inhibition of linoleic acid peroxidation. The elevated plus maze, the light/dark test and the Versamax animal monitor were used to investigate the effect of the extract on anxiety in rodents. The acute toxicity test was also done to assess the safety of the extract as herbal medicine.

The extract, SNE, showed significant anticonvulsant effect against seizures induced by PTZ by dose dependent increase in both the onset of the myoclonic jerks and latency to myoclonic seizures and a reduction in the duration of seizures. However this effect was not significant compared to the vehicle treated group and not dose dependent. SNE significantly and dose-dependently delayed the latencies to myoclonic jerks and tonic-clonic seizures induced by picrotoxin. SNE also reduced the duration of seizures significantly. SNE also significantly reduced the total frequency of seizures. SNE, also, dose-dependently reduced the total duration of seizures induced by pilocarpine in the mice treated. SNE significantly suppressed the PTZ-kindled seizure at all the dose levels used. SNE (100-1000 mg kg^{-1}) dose-dependently inhibited

lipid peroxidation in the PTZ-kindled rats. SNE also, dose-dependently, increased the duration of sleep induced by pentobarbitone in mice. The ability of SNE to attenuate PTZ-, picrotoxin-pilocarpine- induced seizures, PTZ- induced kindling, enhanced sedation of pentobarbitone suggests the possible involvement of GABAergic, anti-cholinergic, or antioxidant mechanism(s).

SNE at 1000 mg kg^{-1} produced a significant reduction in the time spent on the rota-rod at 24 rpm over the two-hour period thus suggesting motor impairment at this dose. Baclofen at 10 mg kg^{-1} significantly reduced the duration spent by pre-treated mice on the rota-rod. SNE also antagonised acetylcholine- induced contractions in the chick biventer cervicis preparation, thus a neuromuscular blocker. These results suggest a skeletal muscle relaxant effect by *S. nodiflora possibly* via centrally mediated mechanism(s) involving neuromuscular blockade.

In the formalin-induced pain and the acetic acid- induced writhing, morphine and diclofenac were used as standard opioid and NSAID respectively. The hydro-ethanolic extract of *S. nodiflora* (100-1000 mg kg^{-1}, p.o) and morphine (1-10 mg kg^{-1}) dose-dependently decreased both phases of the formalin-induced nociceptive behavior. The antinociceptive effects of *S. nodiflora* (300 mg kg^{-1}, p.o) on the first and second phases of formalin-induced pain were significantly blocked by caffeine but not by naloxone. In the acetic acid-induced writhing test, diclofenac and *S. nodiflora* significantly reduced the number of writhes dose-dependently. Also, the effect of the *S. nodiflora* (300 mg kg^{-1}) was blocked by caffeine (3 mg kg^{-1}) but the analgesic effect of diclofenac was significantly enhanced. The observed effects of caffeine on the central and peripheral analgesic effects of *S. nodiflora* in the formalin and acetic acid-induced writhing suggest the possible involvement of adenosinergic mechanism(s).

The extract (0.1-3.0 mg ml^{-1}) was found to contain phenolic compounds which could be responsible for the antioxidant properties. The extract also exhibited antioxidant properties by reducing Fe^{3+} to Fe^{2+} in the reducing power test, scavenged DPPH free radicals and effectively inhibited linoleic acid autoxidation.

In the EPM paradigm, *S. nodiflora* extract (10-300 mg kg^{-1}) exhibited anxiogenic–like activity by dose-dependently decreasing the number of entries into both the open and closed arms, no significant effect on the percent number of entry into the open arms and a decrease in the time spent in the open arm in comparison to the vehicle-treated group. Similarly, *S. nodiflora* extract (SNE) dose-dependently decreased the number of head dips and stretch-attend postures and the duration of grooming. In the LD test, SNE also exhibited anxiogenic-like effect by significantly and dose-dependently reducing the number of entry into the light compartment, the number of transitions and not significantly, the time spent in the light area. In the EPM and LD test, diazepam (0.1-1.0 mg kg^{-1}), a reference anxiolytic drug, produced a directly opposite response to that exhibited by SNE. The extract, in the VAMS, decreased the locomotor activity of pretreated-mice dose dependently and significantly. SNE reduced the distance travelled and time spent at the center of the observation cage indicating an anxiogenic-like effect. The extract's ability to cause a general reduction in locomotor activity suggests sedative effects rather than anxiogenesis.

Oral administration of SNE (1000-10,000 mg kg^{-1}) yielded no mortality in the treated mice over a 24 h of observation. Thus the LD_{50} was approximated to be greater than 10,000 mg kg^{-1}. Thus the extract can be said of as being less toxic. Also high protective indexes produce in the PTZ-, picrotoxin- and pilocarpine-induced seizures by the extract suggest that it has a safe therapeutic profile.

In conclusion, the hydro-ethanolic extract of *S. nodiflora* has anticonvulsant effect in the acute and chronic seizure models of epilepsy used and indicates a possible GABAergic mechanism(s); exhibits central analgesic effect possibly mediated through adenosinergic mechanism and a peripheral anti-inflammatory activity and a potent antioxidant and free radical scavenger, a muscle relaxant and has anxiogenic-like and sedation effects.

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ABBREVIATIONS

AEDs	Antiepileptic Drugs
EC_{50}	Concentration of drug which elicits 50% of the maximum response
ED_{50}	Dose of drug which elicits 50% of the maximum response
IC_{50}	Concentration that produces 50% of maximum inhibition
GABA	Gamma Amino Butyric acid
IL	Interleukin
i.p.	Intraperitoneal
KNUST	Kwame Nkrumah University of Science and Technology
LT	Leucotriene
LXs	Lipoxins
MDA	Malondialdehyde
SE	Status Epilepticus
SNE	*Synedrella nodiflora* extract (hydro-ethanolic)
TLE	Temporal Lobe Epilepsy
NMDA	*N*-methyl *D*-aspartate
NSAIDs	Nonsteroidal anti-inflammatory drugs

PG	Prostaglandin
p.o.	Per os

Chapter 1

INTRODUCTION

1.1 OVERVIEW OF EPILEPSY

Epilepsy, or the "falling sickness", not only has a much older history than any of the other individual nervous or mental disorders, but it has also occupied people's minds to a much larger extent than the majority of ailments to which the genus *Homo sapiens* is susceptible to (Kinnier-Wilson and Reynolds, 1990). Ancient conception of epilepsy as a supernatural disorder due to invasion of the body by demons is very evident, sometimes with individual names for the demons associated with particular seizure types (Pirkner, 1929).

The ancient Hebrews regarded convulsive seizures and epilepsy as sacrosanct with religious, magical or divine causes, and called it *morbus sacer*, the 'sacred illness' or the 'holy illness' (McGrew, 1985). The Greek physician Hippocrates wrote the first book about epilepsy, '*On the Sacred Disease*' around 400 BC (Barrow and Fabing, 1966). He recognized that epilepsy was a brain dysfunction and argued against the ideas that seizures were a curse from the gods and that people with epilepsy could predict the future. This assertion was fully defended by some Hippocratic writers in the fourth century B.C. and, but some of them, being humoralists, diagnosed its cause as an excess of phlegm in the brain rushing into the blood vessels of the body. Another writer in the same tradition ascribed seizures to a mixture of blood and air (Kanner, 1930). This tradition persisted until the 17th century, though the effort to counter it with rational explanations also began very early. During the 18th centuries, the Hippocratic belief that epilepsy is caused by a physical disorder of the brain but not as a result of a supernatural curse gained acceptance (Risse, 1988). The fear that epilepsy was contagious was "confirmed," leading to the creation of the first asylums to house psychiatric and epileptic patients during the early 19th century. By mid 20th century, Americans viewed epilepsy as both a social and medical disease. Individuals were isolated and legislated against in employment and laws regarding driving, marrying, reproducing, and becoming parents. Families hid the presence of epilepsy, fearing stigmatization and limited marriage opportunities for relatives. By 1966 only three states (West Virginia, North Carolina, and

Virginia) in the United States had eugenic marriage laws against epilepsy, while 13 states still had eugenic sterilization laws against people with epilepsy (Barrow and Fabing, 1966).

Epilepsy is a known neurological disorder characterized by seizures, which are sudden, unprovoked, transitory, and recurrent episodes of abnormal hypersynchronous neuronal discharge. Often there is no recognisable cause, although it may develop after brain damage, such as trauma, infection or tumour growth, or other kinds of neurological disease, including various inherited neurological syndromes. Epilepsy is the most common primary neurological disorder known, it is estimated that over 50 million people worldwide are epileptic (1-2 % of the world's population), out of which 40 million are thought to be living in developing countries (McNamara *et al.*, 1993).

Epilepsy is treated mainly with drugs, though brain surgery may be used for severe cases. Current antiepileptic drugs are effective in controlling seizures in about 70% of patients, but their use is often limited by side-effects. There is evidence that in many countries more than 50% of people with epilepsy (even about 90% of people with epilepsy in developing countries) are not receiving appropriate treatment (Meinardi *et al.*, 2001; Scott *et al.*, 2001). From the first written record in 4000 BC until the present day, myths and superstitions have been associated with epilepsy. This is one of the reasons that, in many parts of the world, living conditions for people with epilepsy are far worse than might be expected, given the state of knowledge about epilepsy and the means available to stop seizures and restore complete participation in society. In addition, as long as a person is likely to have seizures, the family and close friends also suffer the burden of this disorder (Global Campaign Against Epilepsy- out of the shadows, 1998). Consequently, people with epilepsy continue to be stigmatized and have a lower quality of life than people with other chronic illnesses. However, bridging the treatment gap and reducing the burden of epilepsy is not straightforward and faces many constraints. Cultural attitudes, a lack of prioritization of epilepsy as a public health condition, poor health system infrastructure, and inadequate supplies of antiepileptic drugs all conspire to hinder appropriate treatment. Much remains to be done but it is hoped that current efforts such as this research will lead to better treatment of people with epilepsy in developing countries.

Traditional systems of medicine are popular in developing countries and up to 80% of the patients in these countries rely on traditional medicines or folk remedies for their primary health care needs (Akerele, 1988). Several plants used for the treatment of epilepsy in different systems of traditional medicine have shown activity when tested on modern bioassays for the detection of anticonvulsant activity (Raza *et al.*, 1999) and many such plants remain to be scientifically investigated (Sander and Shorvon, 1996). This avenue seems promising especially to the developing world in its quest to solve the problem of inadequate treatment of epilepsy due to insufficient supply and low affordability of antiepileptic drugs. Thus the search for new antiepileptic dugs from plant source is warranted.

Synedrella nodiflora (family Asteraceae) is a common weed of waste places in Ghana. Traditionally, the whole plant is boiled and the aqueous extract drunk as required for the treatment of epilepsy, whiles the leaves are used for the treatment of hiccup and threatened abortion (Mshana *et al.*, 2000). The present study is aimed at evaluating the anticonvulsant, analgesic and other related neuropharmacological properties of the whole plant extract of *S. nodiflora*.

1.2 SYNEDRELLA NODIFLORA (L.) GAERTN. ASTERACEAE

1.2.1 Common names

Ga- Kpakpo-debli

Twi – *mamponfo-apow* (the people of Mampong are civilized)

Akan – *nkwadupo tutummerika kohwe epo* (run quickly, go look at the sea)

French: herbe a feu (Berhaut)

English: Cinderella weed, nodeweed, pig grass, porter bush, synedrella

Spanish: cerbatana

Tongan: pakaka. pakopaka

1.2.2 Description

S. nodiflora is an erect herb growing to a height of 60-120cm. It is branched dichotomously with 5-15 cm long and 2-9 cm wide ovate or elliptic leaves, which are crenate-serrate, acute, abruptly narrowed at base to the 1-5 cm long and the petiole mostly pubescent on both surfaces, usually 3-nerved. It has 10-20- flowers, which are 8-10 mm long; involucral bracts 2-seriate, usually only 4 or 5, nearly 1 cm long; all corollas yellow, about 3.5-4 mm long; ligules bifid; achenes of 2 kinds, those of ray-florets winged, glabrous, those of disc-florets unwinged, often puberulent, all nearly black and 4-5 mm long (Stone, 1970). It propagates through its seeds.

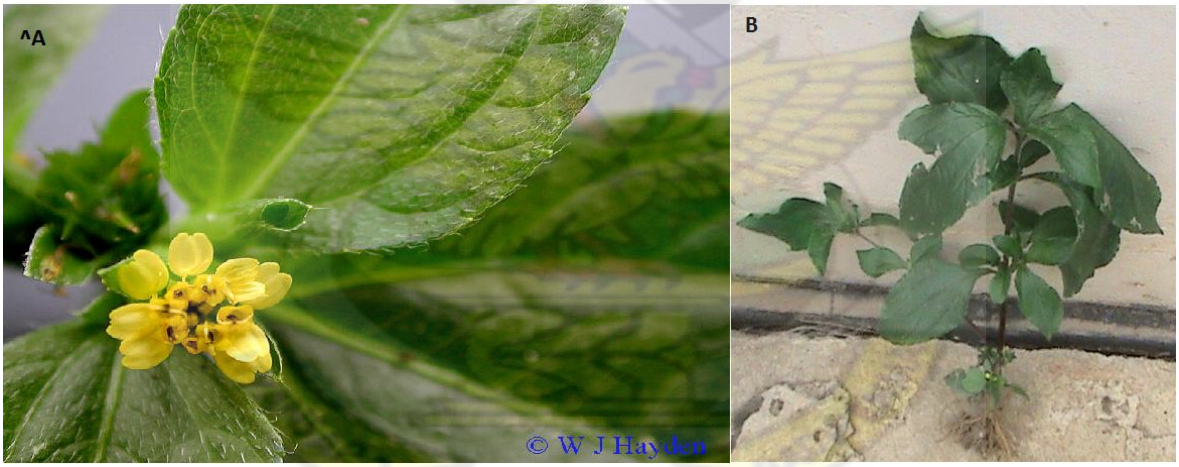

Figure 1: The leaves and flowers (A) and the whole plant (B) of *S. nodiflora*

1.2.3 Ecology and geographical distribution

It is a common plant of hot environment, either in humid or semi-dry areas. It is abundantly distributed in abandoned areas, a major weed in gardens, and an intermediate weed along roadsides and in crops and plantations. The plant grows best where the soil is moist and fertile and where there is plenty of light (Swarbrick, 1997).

In Ghana, it is usually found as a weed of waste places and found along river and stream banks and also along roadsides (Mshana *et al.*, 2000). In New Guinea, it is a common weed of cultivation and a feed for livestock (Henty and Pritchard, 1975). In Tonga, it is also a weed in plantations and waste areas (Yuncker, 1959).

The plant can also be found in other parts of the world including Guam, the southern pacific islands (Fiji, Niue and Tonga islands) and the Hawaiian islands. In Guam and Hawai, it is found abundantly around houses and along roadsides (Stone, 1970; Wagner *et al.*, 1999). In Fiji, the plant is found from near sea level to about 200 m as a naturalized weed in open areas, villages, cultivated areas, and along roads (Smith, 1991). In Niue, it is a very common plantation weed that is also found less commonly in many waste places (Sykes, 1970).

1.2.4 Traditional Uses

S. nodiflora is used widely in Africa and Asia for various purposes. In Ghana, the whole plant is boiled and the aqueous extract drank for the treatment of epilepsy, the leaves are used for threatened abortion, hiccup, laxative and a feed for livestocks (Dalziel, 1931; Mshana *et al.*, 2000). The plant is also used by subsistence farmers of Ghana as post- harvest protectants (Cobbinah *et al.*, 1999).

The plant is also used for various purposes in other African countries. It is known that some indigenous tribes in Nigeria use the whole plant for the treatment of cardiac troubles and to stop wound bleeding (Idu and Onyibe, 2007). The foliage is readily eaten by livestock in Cameroon (Irvine, 1961). In the traditional medicines of some tribes in Ivory Coast, it is reported that the plant is prepared as a paste for use in topical embrocation for oedemas and the leonine form of leprosy; and the liquid obtained from the leaves crushed to a paste with those of *Cyathula prostrate* (amaranthaceae), seeds of *Aframomum melegueta* (zingiberaceae), salt and kaolin is drunk as a sovereign remedy for heart troubles (Kerharo and Bouquet, 1950). In Congo, the leaf sap is used for gum and mouth infections (Bouquet, 1969).

In other parts of the world, where the plant is found, its traditional uses have been reported. In Indonesia the young foliage is eaten as a vegetable and the leaf sap together with other

materials, is applied for stomachache, and the plant is used in embrocation for rheumatism (Burkill, 1985) In Malaysia, the leaves are used for poulticing sore legs and for the treatment of headache, and the sap is instilled into the ear for earache.(Burkill, 1985)

1.2.5 Review of Previous Studies on the Biological Activities of S. nodiflora

Anti-inflammatory and analgesic activities

Abad and colleague researchers reported that the whole plant extract of *S. nodiflora* inhibited both the acute and chronic phases of the Mizushima's adjuvant-carrageenan induced inflammation (ACII). Additionally, the extract exhibited potent anti-inflammatory activity daily throughout the experiment and were as effective as the reference drugs, phenylbutazone (80 $mgkg^{-1}$) and indomethacin (3 $mgkg^{-1}$) (Abad *et al.*, 1996).

In 1996, Forestieri and colleagues also reported on a survey conducted to evaluate the anti-inflammatory, analgesic, antipyretic and toxicity of different extracts (decoction, petroleum ether, ethanol and aqueous) of seven medicinal plants used in African medicine among which was the whole plant of *S.nodiflora*. In the study, *S. nodiflora* extracts were less toxic and inhibited acetic acid induced writhing in the rodents used. Also, the decoction of *S. nodiflora* showed a low analgesic effect in the hot plate test and no anti-inflammatory and antipyretic activities in all extracts tested (Forestieri *et al.*, 1996).

Insecticidal and Vertebrate toxicity

Belmain and others also reported that the leaf extract of *S. nodiflora*, among other plants investigated, was able to control storage pests but had no toxic effect in vertebrates (Belmain *et al.*, 2001).

In 2005, it was again reported that various solvent extracts of the aerial parts of *S. nodiflora* possess insecticidal effects on the fourth instar larvae of *S. litura. S. litura* is a serious pest of most cultivated crops and has developed resistance against many synthetic pesticides (Martin Rathi J. and Gopalakrishnan, 2005).

Fermentation and Methanogenic Characteristics

The decomposition pattern and methanogenic activities of the dry leafy mass of *S. nodiflora*, as a feedstock used in India, have been reported (Chanakya *et al.*, 1997).

1.3 EPILEPSY

1.3.1 Overview

Epilepsy is the most common primary neurological disorder known, affecting 0.4–0.8% of the population and up to 50 million people worldwide (McNamara *et al.*, 1993). It is estimated that the condition affects approximately 50 million people, around 40 million of them living in developing countries The incidence of epilepsy in low-income countries may be as high as 190 per 100 000 people. Consequently, in the context of the large and rapidly increasing populations in these countries, epilepsy is a significant health and socioeconomic burden requiring urgent attention (McNamara *et al.*, 1993).

Epilepsy is a disorder of the central nervous system characterized by seizures, which are sudden, unprovoked, transitory and recurrent episodes of abnormal hypersynchronous neuronal discharge (McNamara *et al.*, 1993). Stemming from the Greek word *epilamvanein* meaning ‘‘to be grasped’’, the term epilepsy is to some degree a misnomer, referring more accurately to a group of disorders with diverse origins and manifestations, collectively known as the epilepsies. The essential feature of the epilepsies is the appearance of behavioural changes, termed seizures. Seizures are transient alteration of behavior due to the disordered, synchronous, and rhythmic firing of populations of brain neurons interspersed by periods of normal electrical activity (Stables *et al.*, 2002*)*.Seizures can be "nonepileptic" when evoked in a normal brain by treatments such as electroshock or chemical convulsants or "epileptic" when occurring without evident provocation. Epileptic seizures often cause transient impairment of consciousness, leaving the individual at risk of bodily harm and often interfering with education and employment. The available drugs used in the therapy of seizure inhibit seizures, but none can cure epilepsy. Compliance with medication is a major problem because of the need for long-term therapy together with unwanted side effects of many drugs. Although many

treatments are available, much effort is being devoted to novel approaches. Many of these approaches center on elucidating the genetic causes and the cellular and molecular mechanisms by which a normal brain becomes epileptic, insights that promise to provide molecular targets for both symptomatic and preventive therapies.

1.3.2 Diagnosis of Epilepsy

The electroencephalogram (EEG) is the primary tool for recording electrical activity of the human brain. Small metal disk electrodes are attached to the scalp at specified locations. When sufficiently amplified, voltage changes generated in neocortical neurons are recorded on the EEG as waveforms of various frequencies, amplitudes, and morphology. EEG patterns vary according to the patients' age, state of alertness, and genetic background. The EEG records activity from near the brain's surface, primarily from the neocortex. Electrical activity from the depth of the brain (eg, brainstem, thalamus, deep temporal lobe) may not be recorded reliably by routine EEG. If surface recording is inadequate, special recording techniques might be required, such as surgical placement of electrodes directly on the brain or implanted into it (Stafstrom, 1998).

1.3.3 Classification of Epilepsy

The clinical classification of epilepsy defines two major seizure categories, namely *partial* and *generalised*, though there is an overlap and many varieties of each. Either form is classified as *simple* (if consciousness is not lost) or *complex* (if consciousness is lost). Other classifications are based on the type of seizure, the presence or absence of neurologic or developmental abnormalities and electroencephalographic (EEG) findings (Commission on Classification and Terminology of the International League Against Epilepsy, 1981). For instance, the syndrome of juvenile myoclonic epilepsy is characterized by the onset of myoclonic seizures, generalized tonic–clonic seizures, and less frequently absence seizures in adolescents who have normal intellectual function, with EEG findings of rapid, generalized spike-wave and polyspike-wave discharges (Benbadis, 2001).

In generalized epilepsies, the predominant type of seizures begins simultaneously in both cerebral hemispheres. Many forms of generalized epilepsy have a strong genetic component; in

most, neurologic function is normal. In partial epilepsies, by contrast, seizures originate in one or more localized foci, although they can spread to involve the entire brain. Most partial epilepsies are believed to be the result of one or more central nervous system insults, but in many cases the nature of the insult is never identified.

1.3.4 Pathophysiology

The susceptibility for generating an epileptic seizure differs from patient to patient. Some may have a lower threshold for epileptic seizures and are therefore more likely to develop this condition. If provoked sufficiently any brain can elicit a seizure. Therefore, a person may experience a single seizure as a sign of transient cerebral overload. Only if seizures become recurrent and are not provoked by systemic disease, is the person diagnosed with epilepsy. Seizure phenomenology varies from patient to patient and more than one seizure type can occur within the same patient. No relationship exists between the clinical signs expressed and the underlying etiology. Irrespective of the fact that epilepsy can be caused by a variety of intracranial structural, cellular or molecular conditions and manifests itself in different ways, the epileptic seizure always reflect abnormal hypersynchronous electrical activity of neurons, caused by an imbalance between excitation and inhibition in the brain. The main representative of excitation in the brain is the excitatory postsynaptic potential (EPSP) whereas that of inhibition is the inhibitory postsynaptic potential (IPSP) (Fisher, 1995). The neuronal membrane potential is regulated by an accurate balance between EPSPs and IPSPs. If this balance is compromised, an epileptic seizure can be generated. More than 100 neurotransmitters or neuromodulators have been shown to play a role in the process of neuronal excitation. Excitatory amino acids, especially L-glutamate, act at more than half the neuronal synapses in the brain, hereby playing a major role in the spread of seizure activity (Johnston, 1996). There is an increased release of glutamate in the brain associated with epileptic activity. Gamma-aminobutyric acid (GABA) is the major inhibitory neurotransmitter of the CNS. GABAergic inhibition can be pre-synaptic (release of GABA from the GABAergic nerve terminal into presynaptic nerve terminals causing a reduction of neuro-transmitter release) or postsynaptic (caused by the interaction of GABA with specific

postsynaptic receptors). GABA released from GABAergic nerve terminals binds to two distinct types of GABA receptors $GABA_A$ and $GABA_B$ receptors to produce neuronal inhibition (Macdonald, 1997). GABA is catabolized postsynaptically by GABA-transaminase. Dysfunction of the GABA-system can be caused by defects of synaptic GABA release, or of the postsynaptic GABA receptor. Under normal conditions the excitatory post-synaptic potentials are followed immediately by GABAergic inhibition. Neuronal hypersynchronization occurs if excitatory mechanisms dominate; either initiated by increased excitation or decreased inhibition. As the abnormal neuronal hypersynchronous activity continues, more and more neurons will be activated (high frequency depolarization/repolarization), generating the epileptic seizure. The abnormal hypersynchronization gives rise to the characteristic abnormalities that can be registered in the electroencephalogram. The existence of excitatory connections between pyramidal neurons generating epileptic bursts through a positive-feedback mechanism in epileptogenic areas and the fact that neurons in some epilepsy prone regions, (e.g. the structures of the limbic system in the temporal lobe, especially the hippocampal CA3 region), possess the capability to generate "intrinsic bursts", dependent on voltage dependent calcium currents or persistent sodium currents has been identified as key features of epileptogenic circuits (Jefferys, 1994).

Sex hormones influence the regulation of GABAergic transmission in the CNS. Animal models have shown that the infusion of estrogens lowers the threshold for experimentally provoked seizures, and that this effect of estrogen is intensified if a cortical lesion is already present. Progesterone has been shown to possess an inhibitory effect on spontaneous and experimentally provoked seizures (Herzog, 1991; Hopkins, 1995). Progesterone probably works through a direct activation of the GABA complex to enhance the effect of GABA. Additionally, progesterone possesses the capability of inhibiting glutamatergic activity. The convulsant/anticonvulsant effects of estrogens and progesterones, respectively, are demonstrated in women with catamenial epilepsy (seizure clustering around the time of menses). There is an increase in seizure frequency, immediately proceeding the menstrual period, which correlates to a decrease in progesterone level, and an additional increase in

seizure frequency immediately preceding the time of ovulation, correlating with a high estrogen level with no simultaneous increase in progesterone. At the end of ovulation, simultaneously with an increase in the progesterone level, the seizure frequency decreases (Herkes *et al.*, 1993; Rosciszewska *et al.*, 1986). The relationship between sex hormones and epileptic seizures has not been investigated in natural occurring epilepsy in dogs and cats, but these mechanisms might exist in bitches/mares experiencing clusters of seizures associated with hormonal fluctuations.

In summary, the brain can use existing, normal circuitry to generate and spread seizure activity if the system is perturbed in such a way to favor excitation over inhibition. In the case of recurrent, unprovoked seizures (epilepsy), neuronal function is persistently abnormal. This chronic hyperexcitability can result from a number of mechanisms. For example, reduced inhibition can result from death or dysfunction of inhibitory neurons, genetic conditions in which GABA synthesis is impaired or GABA receptors have an abnormal subunit composition, or early in life when the development of inhibitory connections lags behind excitatory ones. Enhanced excitation might occur if N-methyl-D-aspartate (NMDA) receptors are overactivated due to the presence of excessive glutamate or glycine or if ionic channels that are responsible for repolarization are genetically aberrant (Stafstrom, 1998).

Structural Alterations

One of the great mysteries in neuroscience is how the brain becomes permanently altered to create the substrate for chronic epilepsy. Sometimes an etiology or structural cause can be determined, but often no explanation is found. One type of epilepsy, TLE, can be a consequence of structural alterations to the hippocampus, one of the most epileptogenic areas of the brain. Hippocampal injury, which can be caused by status epilepticus, could produce persistent hyperexcitability long after the status episode has ceased. This chronic hyperexcitability is due to the combined effects of several structural alterations: neuronal death, gliosis or “mesial temporal sclerosis,” and the growth of new, abnormal axonal connections (“sprouting”) (Stafstrom, 1998).

Dentate granule neurons receive all incoming activity entering the hippocampus.They ordinarily fire only single action potentials and innervate hippocampal pyramidal neurons, which fire single action potentials in response to dentate input. Status epilepticus typically causes death of pyramidal cells (due to overactivation of NMDA receptors and excessive Ca^{2+} entry), but spares dentate neurons. Therefore, axons of dentate neuron 1 are left without a postsynaptic target, so they wind back to innervate their own dendrites and those of neighboring dentate neurons, forming an "autoexcitatory," reverberating excitatory circuit. This results in dentate neuron 2 receiving excessive excitatory input and firing multiple action potentials, causing surviving pyramidal neurons to do the same. Rather than being unique only to the hippocampus, sprouting might comprise a more general mechanism by which brain circuits become hyperexcitable (Stafstrom, 1998).

Enhanced Excitability in the Immature Brain

The immature brain is particularly predisposed to seizures, with seizure incidence being highest during the first decade and especially during the first year of life. Several physiologic features favor a relative hyperexcitable state early in life. Ca^{2+} and Na^{+} channels, which mediate neuronal excitation, develop relatively early. Excitatory synapses tend to form before inhibitory synapses. NMDA receptors are overexpressed transiently early in postnatal development, when they are needed for critical developmental processes. The branching pattern of axons in the immature brain is markedly more complex than later in life, forming an exuberant network of excitatory connections. The ability of glial cells to clear extracellular K+ also increases with age. Finally, GABA, perhaps paradoxically, may be an excitatory transmitter early in development. Therefore, the excitatory/ inhibitory balance in the brain changes dramatically during development. The disadvantage of these physiologic adaptations is that the brain is especially vulnerable to hyperexcitability during a critical window of development. Nevertheless, these neurophysiologic idiosyncrasies of early brain development provide the opportunity for producing novel, age-specific therapies (Stafstrom, 1998).

1.4 ANTIEPILEPTIC DRUGS (AEDs)

1.4.1 Management of Epilepsy

Seizure disorders most often are treated with pharmacotherapy. Optimal AED thereapy may completely control seizures in 60-95% of patients (Dreifuss, 1989; Pellock, 1989). Surgical operations involving the removal of the epileptic foci may be an option in resistance of seizures to antiepileptic drugs (AEDs). The choice of an appropriate antiepileptic drug depends upon accurate classification of seizures and diagnosis of the epileptic syndrome. Once diagnosis of epileptic seizures is established, the decision to treat with AEDs is based upon the likelihood of recurrence (Lott, 1999).

1.4.2 Historical background

The modern treatment of seizures dates as far back as in 1850, initially with the use of bromides, based on the theory that epilepsy was caused by an excessive sex drive. In 1910, phenobarbital, which then was used to induce sleep, was found to reduce seizures and became the drug of choice for many years. A number of medications similar to phenobarbital were developed, including primidone. In 1940, phenytoin (PHT) was found to be an effective drug for the treatment of epilepsy, and since then it has become a major first-line antiepileptic drug (AED) in the treatment of partial and secondarily generalized seizures (Ochoa and Richie, 2009) (http://emedicine.medscape.com/article/1187334-overview#aw2aab6b3)

In 1968, carbamazepine (CBZ) was approved, initially for the treatment of trigeminal neuralgia; later, in 1974, it was approved for partial seizures. Ethosuximide has been used since 1958 as a first-choice drug for the treatment of absence seizures without generalized tonic-clonic seizures. Valproate was licensed in Europe in 1960 and in the United States in 1978, and now is widely available throughout the world. It became the drug of choice in primary generalized epilepsies and in the mid 1990s was approved for treatment of partial seizures. These anticonvulsants were the mainstays of seizure treatment until the 1990s, when newer AEDs with good efficacy, fewer toxic effects, better tolerability, and no need for blood level

monitoring were developed. The new AEDs have been approved in the United States as add-on therapy only, with the exception of topiramate and oxcarbazepine; lamotrigine is approved for conversion to monotherapy (Ochoa and Richie, 2009) (emedicine.medscape.com).

1.4.3 Treatment of epilepsy

A grasp of knowledge of the mechanism of action and pharmacokinetics of AEDs is important in clinical practice for desired therapeutic outcomes (see Figure 1).

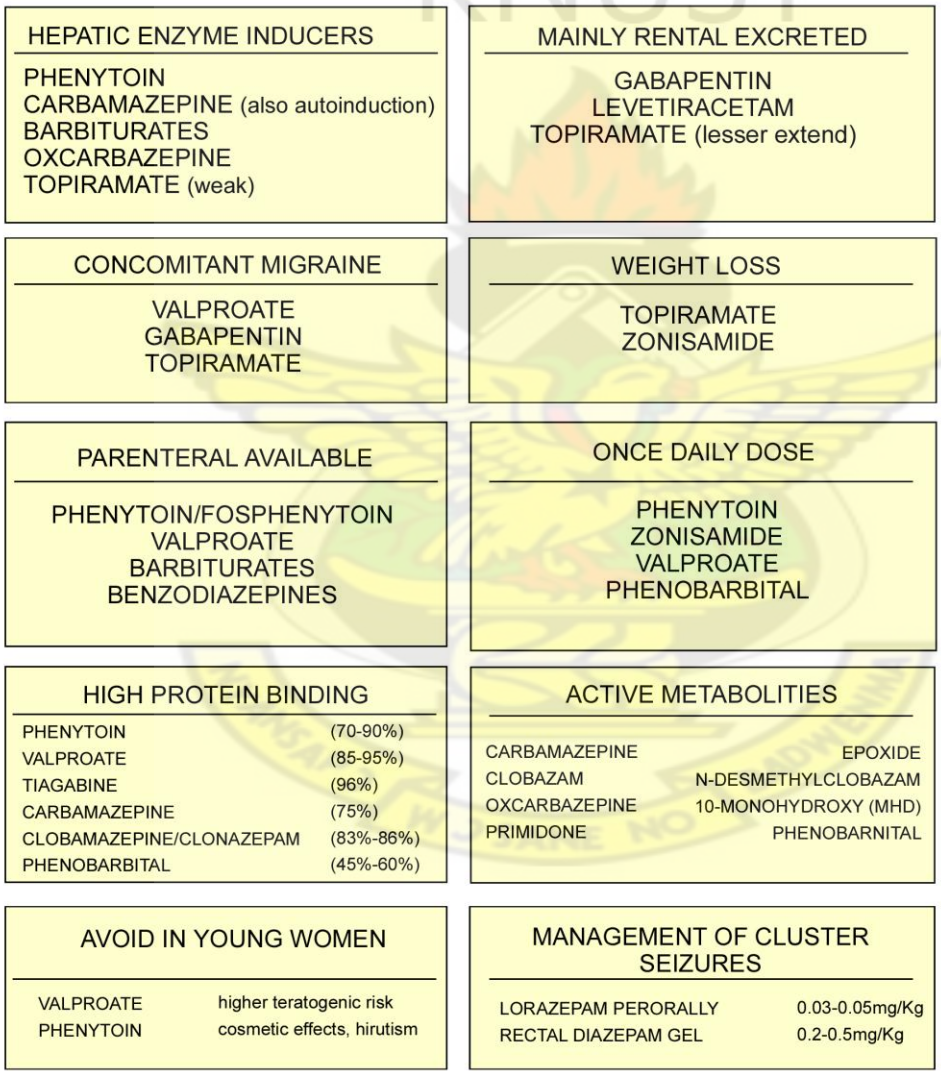

Figure 2 Pearls of antiepileptic drug use and management.

Source: http://emedicine.medscape.com/article/1187334-overview#aw2aab6b3

Many structures and processes are involved in the development of a seizure, including neurons, ion channels, receptors, glia, and inhibitory and excitatory synapses. The AEDs are designed to modify these processes to favor inhibition over excitation in order to stop or prevent seizure activity (see Figure 2).

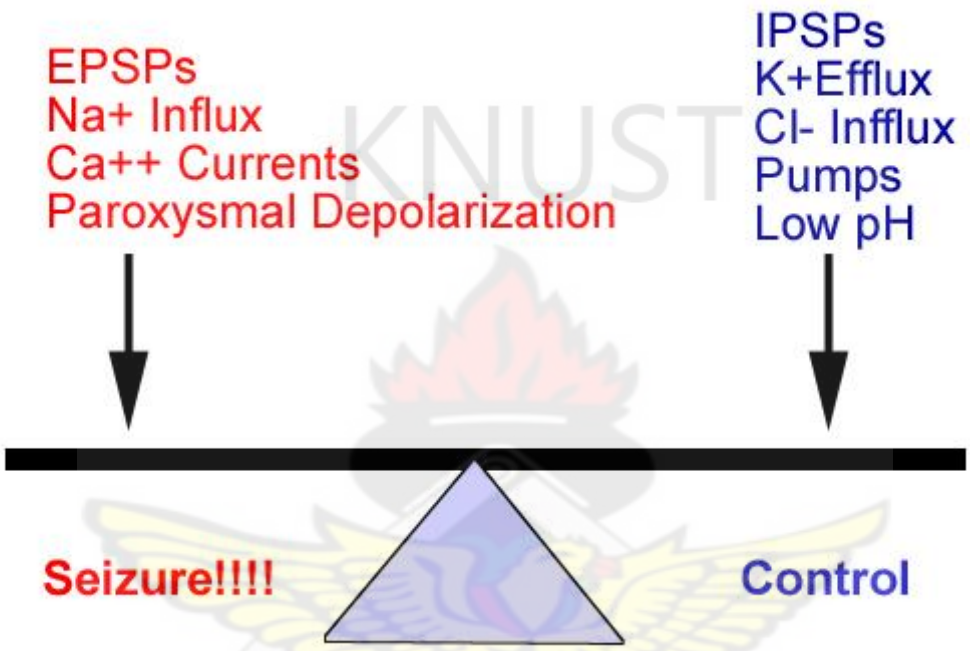

Figure 3: The dynamic target of seizure control in the management of epilepsy is achieving balance between the factors that influence the excitatory postsynaptic potential (EPSP) and those that influence inhibitory postsynaptic potential (IPSP).

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The AEDs can be grouped according to their main mechanism of action, although many of them have several actions and others have unknown mechanisms of action. The major groups include sodium channel blockers, calcium current inhibitors, gamma-aminobutyric acid (GABA) enhancers, glutamate blockers, carbonic anhydrase inhibitors, hormones, and drugs with unknown mechanisms of action(Ochoa and Richie, 2009) (see Figure 3).

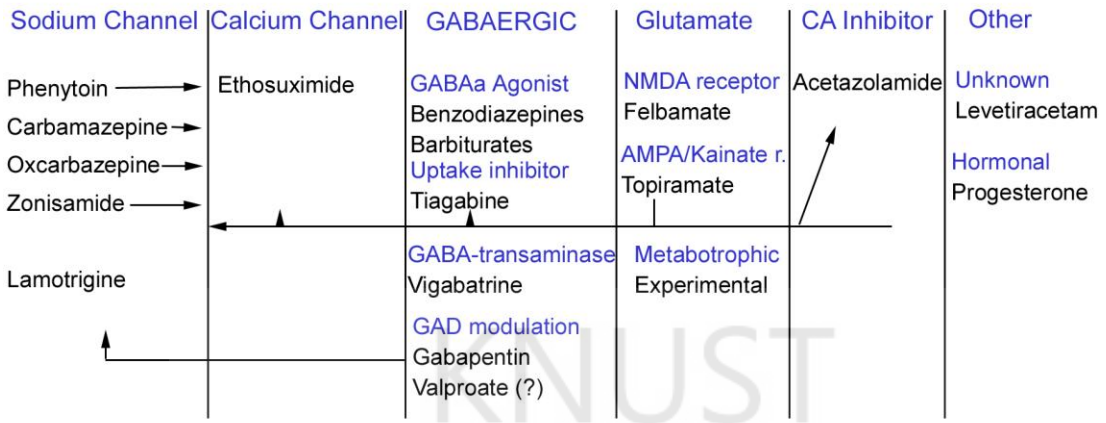

Figure 4: Antiepileptic drugs can be grouped according to their major mechanism of action. Some antiepileptic drugs work by acting on a combination of channels and/or some unknown mechanism of action.

Source: http://emedicine.medscape.com/article/1187334-overview#aw2aab6b3

1.4.4 Mechanisms of Action of AEDs

1.4.4.1 Sodium currents/channels

The firing of an action potential by an axon is accomplished through sodium channels. Each sodium channel dynamically exists in three states, which are as follows:

- A resting state during which the channel allows passage of sodium into the cell
- An active state in which the channel allows increased influx of sodium into the cell
- An inactive state in which the channel does not allow passage of sodium into the cell

During an action potential, these channels exist in the active state and allow influx of sodium ions. Once the activation or stimulus is terminated, a percentage of these sodium channels become inactive for a period of time known as the refractory period. With constant stimulus or rapid firing, many of these channels exist in the inactive state, rendering the axon incapable of propagating the action potential. AEDs that target these sodium channels prevent the return of these channels to the active state by stabilizing the inactive form of these channels. In doing so, repetitive firing of the axons is prevented (Ochoa and Richie, 2009) (see figure 4).

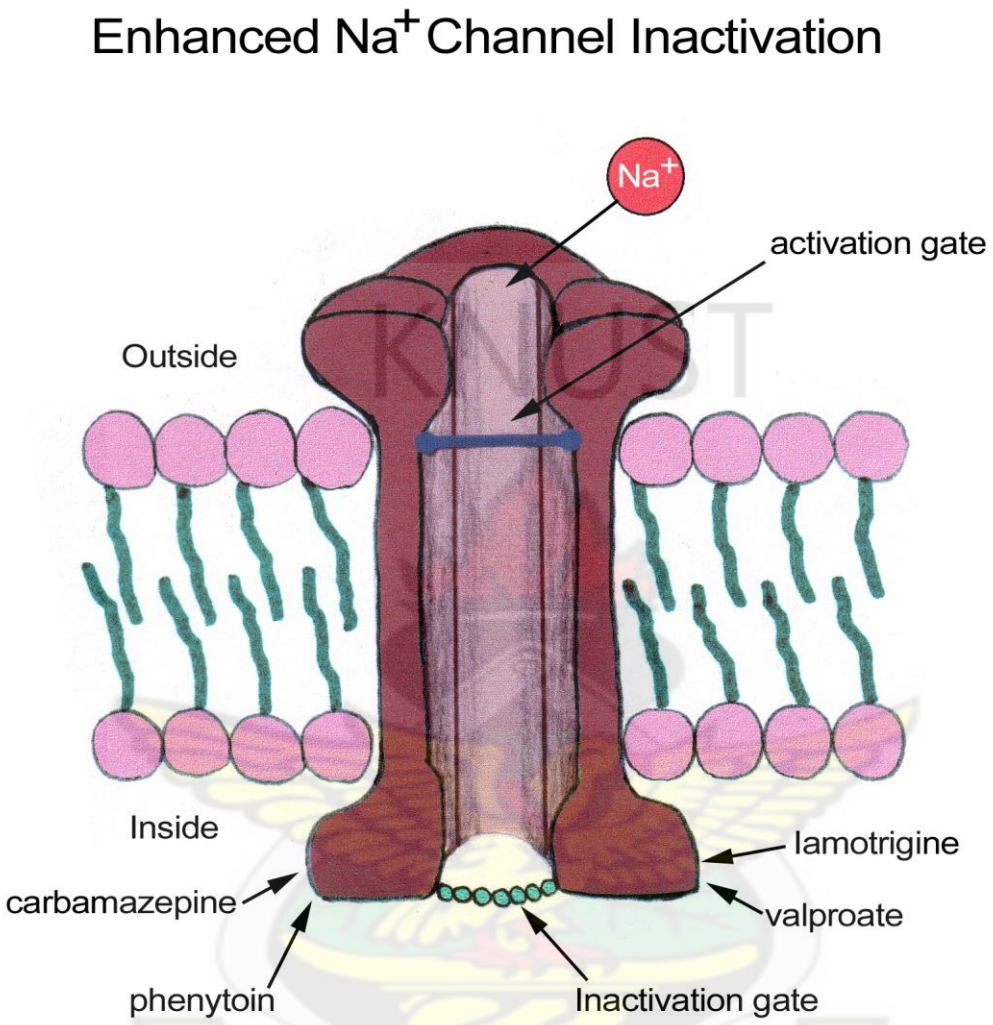

Figure 5: Some antiepileptic drugs stabilize the inactive configuration of the sodium (Na^+) channel, preventing high-frequency neuronal firing.

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1.4.4.2 Calcium channels

Calcium channels exist in 3 known forms in the human brain: L, N, and T. These channels are small and are inactivated quickly. The influx of calcium currents in the resting state produces a partial depolarization of the membrane, facilitating the development of an action potential after rapid depolarization of the cell. They function as the "pacemakers" of normal rhythmic brain

activity. This is true particularly of the thalamus. T-calcium channels have been known to play a role in the three per second spike-and-wave discharges of absence seizures. AEDs that inhibit these T-calcium channels are particularly useful for controlling absence seizures (Ochoa and Richie, 2009) (see figure 5).

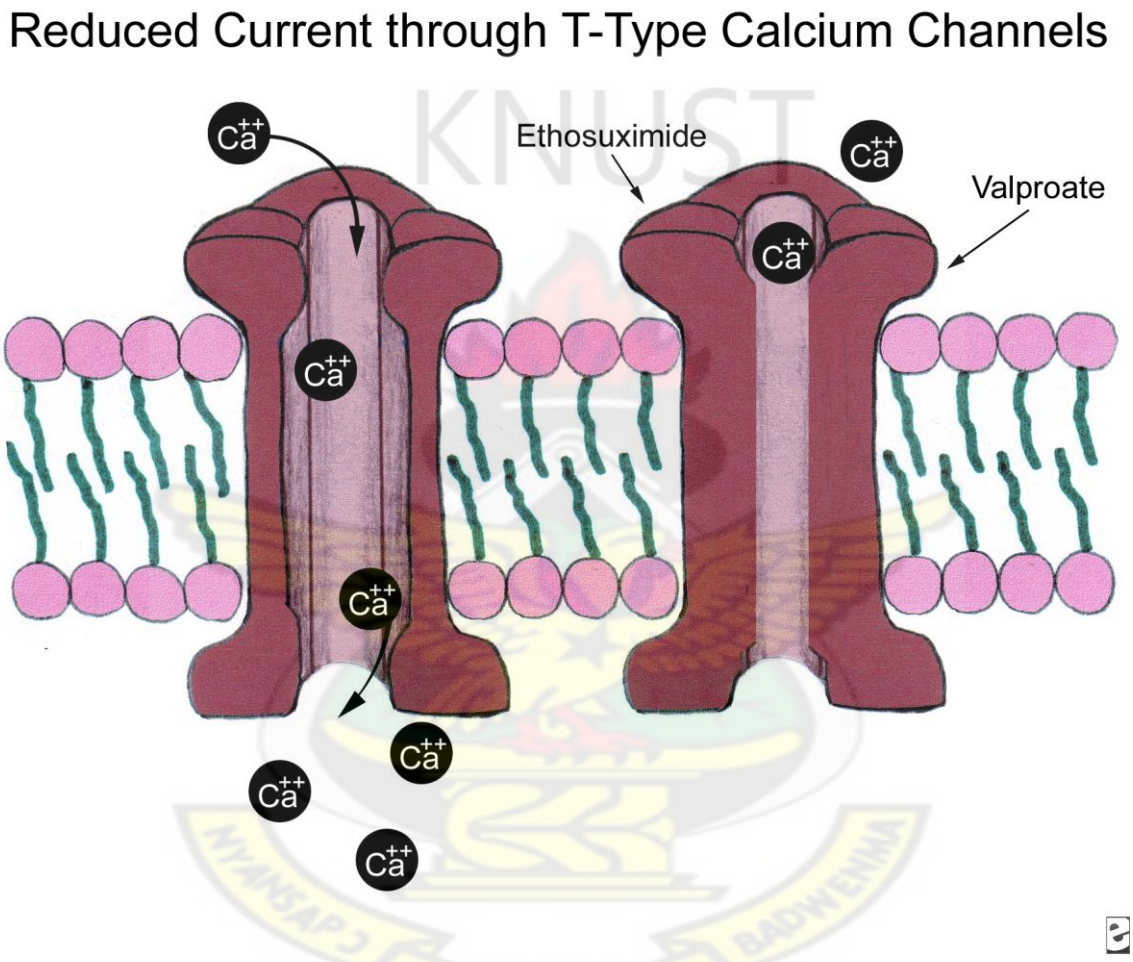


Figure 6: Low-voltage calcium (Ca2+) currents (T-type) are responsible for the rhythmic thalamocortical spike and wave patterns of generalized absence seizures. Some antiepileptic drugs lock these channels, inhibiting the underlying slow depolarizations necessary to generate spike-wave bursts.

Source: http://emedicine.medscape.com/article/1187334-overview#aw2aab6b3

1.4.5 GABA-A receptors/channels

When GABA binds to a GABA-A receptor, the passage of chloride, a negatively charged ion, into the cell is facilitated via chloride channels. This influx of chloride increases the negativity of the cell (ie, a more negative resting membrane potential). This causes the cell to have greater difficulty reaching the action potential. GABA is produced by decarboxylation of glutamate mediated by the enzyme glutamic acid decarboxylase (GAD). Some drugs may act as modulators of this enzyme, enhancing the production of GABA and down-regulating glutamate (Ochoa and Richie, 2009).

Some AEDs function as an agonist to this mode of chloride conductance by blocking the reuptake of GABA (ie, tiagabine) or by inhibiting its metabolism mediated by GABA transaminase (i.e, vigabatrin), resulting in increased accumulation of GABA at the postsynaptic receptors (Ochoa and Richie, 2009) (see Figure 6).

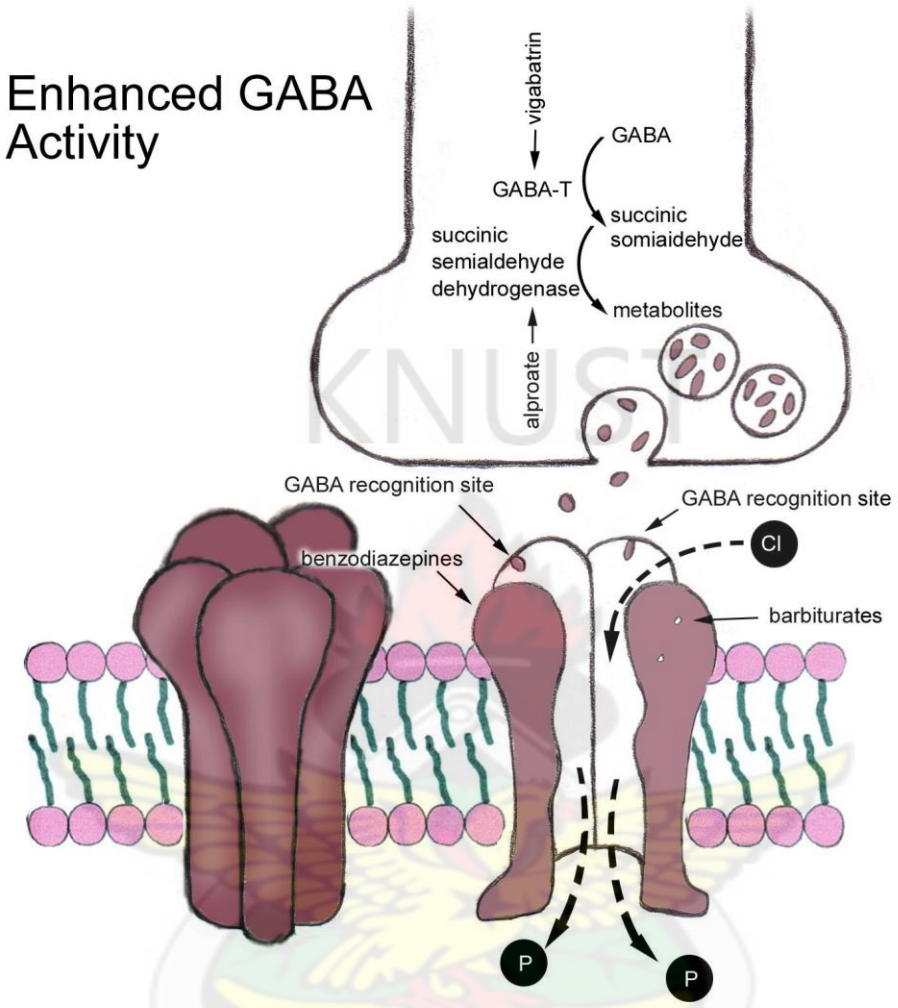


Figure 7: The GABA-A receptor mediates chloride (Cl^-) influx, leading to hyperpolarization of the cell and inhibition. Antiepileptic drugs may act to enhance Cl^- influx or decrease GABA metabolism.

Source: http://emedicine.medscape.com/article/1187334-overview#aw2aab6b3

1.4.6 Glutamate receptors

Glutamate receptors bind glutamate, an excitatory amino acid neurotransmitter. Upon binding glutamate, the receptors facilitate the flow of both sodium and calcium ions into the cell, while potassium ions flow out of the cell, resulting in excitation. The glutamate receptor has 5 potential binding sites and causes different responses depending on the stimulated or blocked

site. These sites are the alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) site, the kainate site, the *N* -methyl-D-aspartate (NMDA) site, the glycine site, and the metabotropic site that has 7 subunits (GluR 1-7). AEDs that modify these receptors are antagonistic to glutamate (see Figure 7-8) (Ochoa and Richie, 2009).

Table 1: Glutamate, the main excitatory neurotransmitter in the CNS, binds to multiple receptor sites that differ in activation and inactivation time courses, desensitization kinetics, conductance, and ion permeability. The 3 main glutamate receptor subtypes are *N*-methyl-D-aspartate (NMDA), metabotropic, and non-NMDA (alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid [AMPA] and kainate receptors). Antiepileptic drugs known to possess this mechanism of action are listed.

NMDA	AMPA/KAINATE	METABOTROPHIC
Felbamate Levetiracetam (glycine?)	Tropiramate	Under research

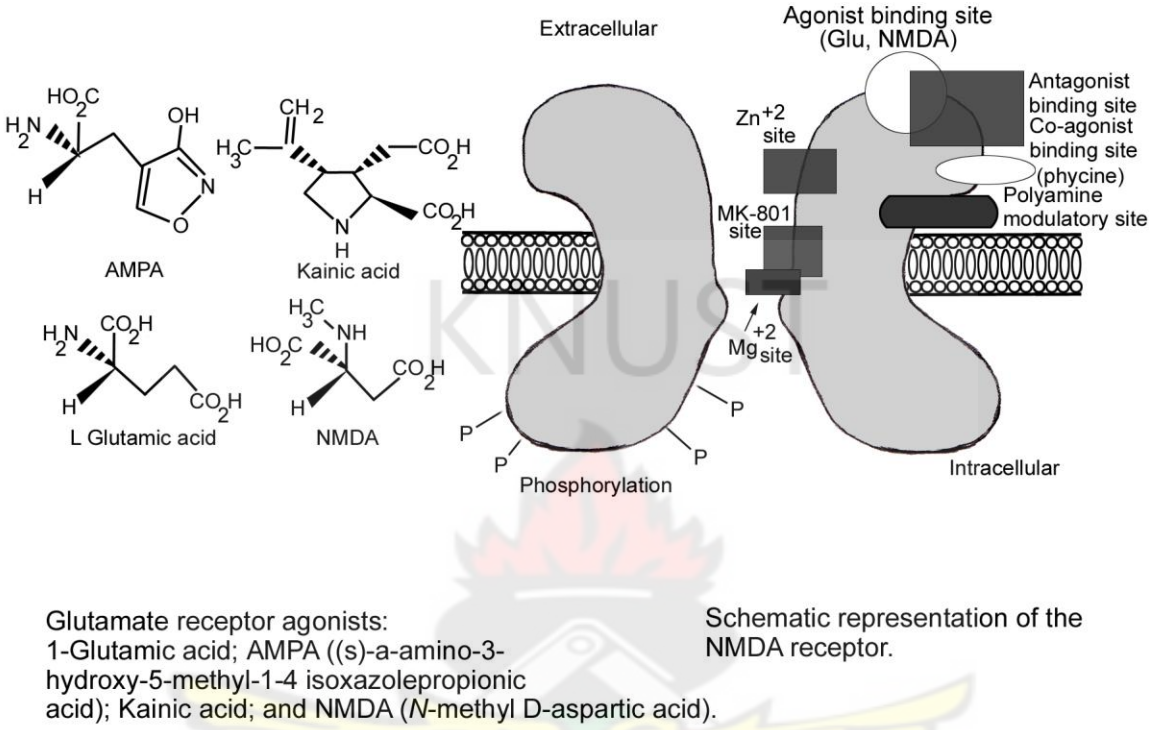


Figure 8: Schematic representation of the *N*-methyl-D-aspartate (NMDA) receptor.

Source: http://emedicine.medscape.com/article/1187334-overview#aw2aab6b3

1.4.7 Sex hormones

Progesterone is a natural anticonvulsant that acts by increasing chloride conductance at GABA-A receptors and attenuates glutamate excitatory response. It also alters messenger RNA for GAD and GABA-A receptor subunits. On the other hand, estrogen acts as a proconvulsant by reducing chloride conductance and acting as an agonist at NMDA receptors in the CA1 region of the hippocampus (Ochoa and Richie, 2009).

1.4.8 Carbonic anhydrase inhibition

Inhibition of the enzyme carbonic anhydrase increases the concentration of hydrogen ions intracellularly and decreases the pH. The potassium ions shift to the extracellular compartment to buffer the acid-base status. This event results in hyperpolarization and an increase in seizure threshold of the cells. Acetazolamide has been used as an adjunctive therapy in refractory seizures with catamenial pattern (i.e., seizure clustering around menstrual period). Topiramate and zonisamide also are weak inhibitors of this enzyme; however, this is not believed to be an important mechanism for their antiepileptic efficacy (Ochoa and Richie, 2009).

1.4.9 Synaptic vesicle protein 2A binding

SV2A is ubiquitously expressed in the brain, but its function has not been clearly defined. SV2A appear to be important for the availability of calcium-dependent neurotransmitter vesicles ready to release their content (Xu and Bajjalieh, 2001). The lack of SV2A results in decreased action potential-dependent neurotransmission, while action potential-independent neurotransmission remains normal (Crowder *et al.*, 1999; Janz *et al.*, 1999). The role in epilepsy is confirmed by the finding of SV2A knock-out mice developing strong seizure phenotype a few weeks after birth. The anticonvulsant potency of SV2A ligands is correlated with their binding affinity in the audiogenic seizure-prone mice (Lynch *et al.*, 2004; Noyer *et al.*, 1995). Levetiracetam binds the SV2A.

1.4.10 Sodium Channel Blockers

Sodium channel blockade is the most common and the mostwell-characterized mechanism of currently available AEDs. AEDs that target these sodium channels prevent the return of the channels to the active state by stabilizing the inactive form. In doing so, repetitive firing of the axons is prevented. The presynaptic and postsynaptic blockade of sodium channels of the axons causes stabilization of the neuronal membranes, blocks and prevents posttetanic potentiation, limits the development of maximal seizure activity, and reduces the spread of seizures (Ochoa and Richie, 2009).

1.4.10.1 Carbamazepine

Carbamazepine (CBZ) is a major first-line AED for partial seizures and generalized tonic-clonic seizures. It is a tricyclic compound and initially was used primarily for the treatment of trigeminal neuralgia, but its value in the treatment of epilepsy was discovered quite by chance. The main mode of action of CBZ is to block sodium channels during rapid, repetitive, sustained neuronal firing and to prevent posttetanic potentiation. It has been approved in the United States for the treatment of epilepsy since 1974. However, it has been used for epilepsy since 1968 (Ochoa and Richie, 2009; Suzette *et al.*, 2004).

1.4.10.2 Phenytoin

Since its introduction in 1938, phenytoin (PHT) has been a major first-line AED in the treatment of partial and secondary generalized seizures in the United States. It blocks movements of ions through the sodium channels during propagation of the action potential, and therefore blocks and prevents posttetanic potentiation, limits development of maximal seizure activity, and reduces the spread of seizures. It also demonstrates an inhibiting effect on calcium channels and the sequestration of calcium ions in nerve terminals, thereby inhibiting voltage-dependent neurotransmission at the level of the synapse. An antiepileptic effect also is seen on calmodulin and other secondary messenger systems, the mechanisms of which are unclear. The adverse-effect profile (eg, gingival hyperplasia and coarsening of the facial features in women) makes its use less desirable than CBZ in some patients (Ochoa and Richie, 2009).

1.4.10.3 Fosphenytoin

Fosphenytoin sodium injection is a prodrug intended for parenteral administration. Its active metabolite is PHT. It is safer and better tolerated than PHT and can be infused 3 times faster than intravenous (IV) PHT (Ochoa and Richie, 2009).

1.4.10.4 Oxcarbazepine

Oxcarbazepine (OXC) is a recently developed analog of CBZ. OXC was developed in an attempt to hold on to the benefits of CBZ while avoiding its auto-induction and drug interaction properties. Licensed in over 50 countries, including the United States, OXC now is considered one of the first-line therapies in some countries. OXC does not produce the epoxide metabolite, which is largely responsible for the adverse effects reported with CBZ. Like CBZ, OXC blocks the neuronal sodium channel during sustained rapid repetitive firing (Ochoa and Richie, 2009).

1.4.10.5 Lamotrigine

Lamotrigine (LTG) is a triazine compound that is chemically unrelated to any of the other AEDs. It was developed as an antifolate agent based on a theory that the mechanism of some AEDs is related to their antifolate property. It was approved in the United States in 1994. Its major mechanism of action is blocking voltage-dependent sodium-channel conductance. It has been found to inhibit depolarization of the glutaminergic presynaptic membrane, thus inhibiting release of glutamate. It has a weak antifolate effect that is unrelated to its antiseizure efficacy (Ochoa and Richie, 2009; Suzette *et al*., 2004).

1.4.10.6 Zonisamide

Zonisamide (ZNS) was synthesized as a benzisoxazole in 1974. It is chemically unrelated to any of the other AEDs. It is a small-ringed structure related to sulfonamide antibiotics with pH-dependent solubility in water. Although ZNS was approved by the US Food and Drug Administration (FDA) in March 2000 for the indication of partial seizures in patients older than 12 years as adjunctive therapy to other AEDs, it has been approved and studied in Japan for more than 10 years. The major mechanism of action of ZNS is reduction of neuronal repetitive firing by blocking sodium channels and preventing neurotransmitter release. It also exerts influence on T-type calcium channels and prevents influx of calcium. ZNS also exhibits neuroprotective effects through free radical scavenging (Ochoa and Richie, 2009).

1.4.11 GABA Receptor Agonists

A seizure reflects an imbalance between excitatory and inhibitory activity in the brain, with an increment of excitation over inhibition. The most important inhibitory neurotransmitter in the brain is GABA (Ochoa and Richie, 2009). For a summary of the AEDs with effects on GABA, see table 2.

Table 2: GABA drugs and their known sites of action.

$GABA_A$ agonist	UPTAKE BLOCKER	GABA-T INHIBITOR	GAD modulator?	PRODRUG
Barbiturates Benzodiazepines Progesterone Ganaloxone	Tiagabine	Vigabatrine	Gabapentin Valproate	Progabide

GABA has 2 types of receptors, GABA-A and GABA-B. When GABA-A receptor is stimulated, chloride channels open to allow the influx of negative ions (ie, chloride) into the neuron and cause hyperpolarization, moving the membrane potential further from the cell-firing threshold. The GABA-B receptor is linked to a potassium channel (Ochoa and Richie, 2009; Suzette *et al.*, 2004).

The GABA system can be enhanced by direct binding to GABA-A receptors, by blocking presynaptic GABA uptake, by inhibiting the metabolism of GABA by GABA transaminase, and by increasing the synthesis of GABA (see figure 8) (Ochoa and Richie, 2009).

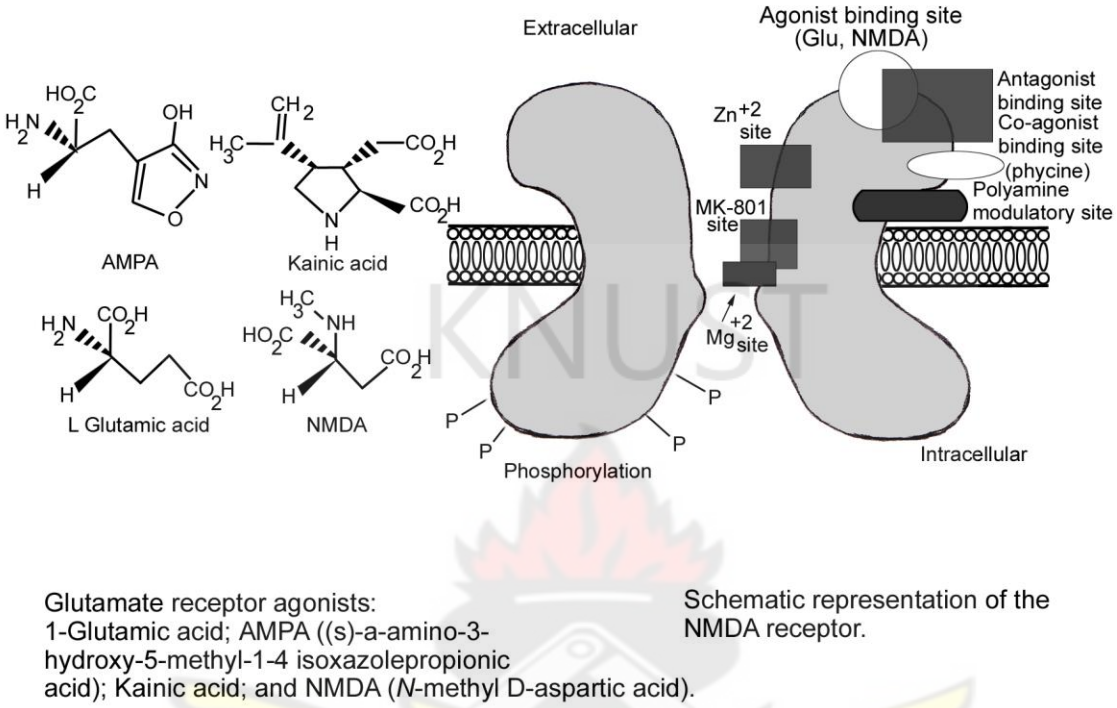


Figure 9: Schematic representation of the *N*-methyl-D-aspartate (NMDA) receptor.

Source: http://emedicine.medscape.com/article/1187334-overview#aw2aab6b3

The GABA-A receptors have multiple binding sites for benzodiazepines, barbiturates, and others substances such as picrotoxins, bicuculline, and neurosteroids. These drugs bind to different receptor sites to exert their action, but the clinical implications of each receptor site are not well understood (Ochoa and Richie, 2009; Suzette *et al.*, 2004).

The benzodiazepines most commonly used for treatment of epilepsy are lorazepam, diazepam, clonazepam, and clobazam. The first 2 drugs are used mainly for emergency treatment of seizures because of their quick onset of action, availability of IV forms, and strong anticonvulsant effects. Their use for long-term treatment is limited because of the development of tolerance (Ochoa and Richie, 2009; Suzette *et al.*, 2004).

The two barbiturates mostly commonly used in the treatment of epilepsy are phenobarbital and primidone. They bind to a barbiturate-binding site of the benzodiazepine receptor to affect the duration of chloride channel opening. They have been used widely throughout the world. They are very potent anticonvulsants, but they have significant adverse effects that limit their use. With the development of new drugs, the barbiturates now are used as second-line drugs for the treatment of chronic seizures (Ochoa and Richie, 2009; Suzette *et al.*, 2004).

1.4.11.1 Clobazam

This benzodiazepine has a 1,5 substitution instead of the usual 1,4-diazepine. This change results in an 80% reduction in its anxiolytic activity and a 10-fold decrease in its sedative effects. It has been licensed in Europe since 1975 but is not available in the United States. In addition to its agonist action at the GABA-A receptor, clobazam may affect voltage-sensitive conductance of calcium ions and the function of sodium channels (Ochoa and Richie, 2009).

1.4.11.2 Clonazepam

Clonazepam, a 1,4-substituted benzodiazepine, is one of the first benzodiazepines used for epilepsy. Clonazepam has higher affinity for the GABA-A receptor site than diazepam and binds to GABA-A receptors that do not bind with other benzodiazepines. It may have some action on sodium-channel conductance (Ochoa and Richie, 2009; Suzette *et al.*, 2004).

1.4.11.3 Phenobarbital

This is the most commonly prescribed AED of the 20th century. It is a very potent anticonvulsant with a broad spectrum of action. Currently, its use is limited because of its adverse effects. It is a free acid, relatively insoluble in water. The sodium salt is soluble in water but unstable in solution. It has a direct action on GABA-A receptors by binding to the barbiturate-binding site that prolongs the duration of chloride channel opening. It also reduces

sodium and potassium conductance and calcium influx and depresses glutamate excitability (Ochoa and Richie, 2009).

1.4.11.4 Primidone

This drug is metabolized to PHB and phenylethylmalonamide (PEMA). The main action is through the derived PHB. The real clinical effect of primidone or PEMA is unknown and controversial (Ochoa and Richie, 2009; Suzette *et al.*, 2004).

1.4.12 GABA Reuptake Inhibitors

At least 4 specific GABA-transporting compounds help in the reuptake of GABA; these carry GABA from the synaptic space into neurons and glial cells, where it is metabolized. Nipecotic acid and tiagabine are inhibitors of these transporters; this inhibition makes increased amounts of GABA available in the synaptic cleft. GABA prolongs inhibitory postsynaptic potentials (IPSPs) (Ochoa and Richie, 2009).

1.4.12.1 Tiagabine

Tiagabine (TGB) is a derivative of the GABA uptake inhibitor nipecotic acid. It acts by inhibition of the GABA transporter-1 (GAT-1). This inhibitory effect is reversible. TGB is lipid soluble and thus is able to cross the blood-brain barrier. It was introduced into clinical practice in 1998. Measurements in human and experimental models have confirmed that extracellular GABA concentrations increase after administration of TGB. Studies have shown little or no effect at other receptor systems (NICE, 2004; Ochoa and Richie, 2009; Suzette *et al.*, 2004).

1.4.13 GABA Transaminase Inhibitor

GABA is metabolized by transamination in the extracellular compartment by GABA-transaminase (GABA-T). Inhibition of this enzymatic process leads to an increase in the extracellular concentration of GABA. Vigabatrin (VGB) inhibits the enzyme GABA-T (Ochoa and Richie, 2009).

1.4.13.1 Vigabatrin

In the 1970s, GABA was recognized as an important inhibitory neurotransmitter in the CNS. Favoring the balance toward the GABA system was a major target of drug research, and soon VGB was developed. The drug was licensed worldwide, except in the United States because of its toxicity. It is a close structural analog of GABA, binding irreversibly to the active site of GABA-T. Newly synthesized enzymes take 4-6 days to normalize the enzymatic activity. In vivo studies in human and animal subjects have shown that VGB significantly increases extracellular GABA concentrations in the brain. VGB has no other known action (Ochoa and Richie, 2009).

1.4.14 AEDs with a Potential GABA Mechanism of Action

The enzyme GAD converts glutamate into GABA. Currently, valproate and gabapentin are known to have some effects on this enzyme to enhance the synthesis of GABA in addition to other potential mechanisms of action. Valproate also blocks the neuronal sodium channel during rapid sustained repetitive firing. Gabapentin has a weak competitive inhibition of the enzyme GABA-T. As with other AEDs, whether these mechanisms of action alone are responsible for the antiseizure efficacy of these drugs is unclear (Ochoa and Richie, 2009).

1.4.14.1 Gabapentin

Gabapentin (GBP) was developed to have a structure similar to that of GABA; however, experimental evidence showed that GBP has, in fact, little or no action on the GABA receptor. It is highly soluble in water. It enhances the enzyme GAD, but does so weakly. It binds with the alpha$_2$ delta subunit of calcium channels in the cerebral neocortex, hippocampus, and spinal cord; this mechanism of action may be important for its efficacy in pain. At this time, the exact mechanism by which GBP increases the intracellular concentration of GABA is unknown. In vivo MR spectroscopy studies have shown that GBP increases brain levels of GABA and its metabolites homocarnosine and pyrrolidinone. It also may reduce monoamines and affect serotonin release. GBP is a competitive inhibitor of the enzyme branched chain amino acid transferase, which metabolizes the branch chain amino acids (l-leucine, l-isoleucine, and l-

valine) to glutamate. Through this mechanism, GBP may reduce brain glutamate levels (Ochoa and Richie, 2009; Suzette *et al.*, 2004).

1.4.14.2 Pregabalin

Pregabalin is an analogue of the neurotransmitter GABA and has analgesic, anticonvulsant, and anxiolytic effects. Despite being a GABA analogue, pregabalin is inactive at GABA receptors including $GABA_A$, benzodiazepine, TBPS, and $GABA_B$ radioligand binding sites (Taylor *et al.*, 2007). Neither pregabalin nor gabapentin altered GABA concentration in brain tissues (Errante and Petroff, 2003) or inhibited GABA transport in vitro. Pregabalin binds with high affinity to both the $alpha_2$ delta-1 and $alpha_2$ delta-2 subtypes. Gabapentin and pregabalin binding to the $alpha_2$ delta protein are proposed to mediate the functional effects these molecules have on calcium currents in activated neurons and on stimulated neurotransmitter release (Dooley *et al.*, 2000). The effect is a reduced release of excitatory neurotransmitters and peptide neuromodulators under membrane hyperexcitability, which is postulated to mediate the analgesic, anxiolytic, and anticonvulsant effect (Bryans *et al.*, 1998). Pregabalin is active in several animal models of seizures. In the high-intensity electroshock test, pregabalin inhibited tonic extensor seizures in rats and low-intensity electroshock seizures in mice. In the DBA/2 audiogenic mouse model and clonic seizures, pregabalin prevented tonic extensor seizures from pentylenetetrazole in mice. In a kindled rat model of partial seizures, pregabalin prevented stages 4–5 behavioral seizures. However, pregabalin was not effective in models of absence seizures (Vartanian *et al.*, 2006).

1.4.14.3 Valproate

Valproate (VPA) is the drug of choice for primary generalized epilepsies, and is also approved for the treatment of partial seizures. It was discovered by accident; first synthesized in 1882, its antiepileptic properties were recognized when it was used as a solvent for the experimental screening of new antiepileptic compounds. It was licensed in Europe in the early 1960s, where its use became extensive. It has been used in different forms, including divalproex sodium, magnesium or calcium salt, or valpromide. These forms do not differ significantly. The

mechanism of action is uncertain. VPA enhances GABA function, but this effect is observed only at high concentrations. It may increase the synthesis of GABA by stimulating GAD. It also produces selective modulation of voltage-gated sodium currents during sustained, rapid, repetitive neuronal firing (Ochoa and Richie, 2009).

1.4.15 Glutamate Blockers

Glutamate and aspartate are the most two important excitatory neurotransmitters in the brain. The glutamate system is a complex system with macromolecular receptors with different binding sites (ie, AMPA, kainate, NMDA, glycine, metabotropic site). The AMPA and the kainate sites open a channel through the receptor, allowing sodium and small amounts of calcium to enter. The NMDA site opens a channel that allows large amounts of calcium to enter along with the sodium ions. This channel is blocked by magnesium in the resting state (Ochoa and Richie, 2009).

The glycine site facilitates the opening of the NMDA receptor channel. The metabotropic site is regulated by complex reactions and its response is mediated by second messengers. NMDA antagonists have a limited use because they produce psychosis and hallucinations. In addition to these adverse effects, learning and memory may be impaired by blocking these receptors, because NMDA receptors are associated with learning processes and long-term potentiation (see figure 11) (Ochoa and Richie, 2009; Suzette *et al.*, 2004).

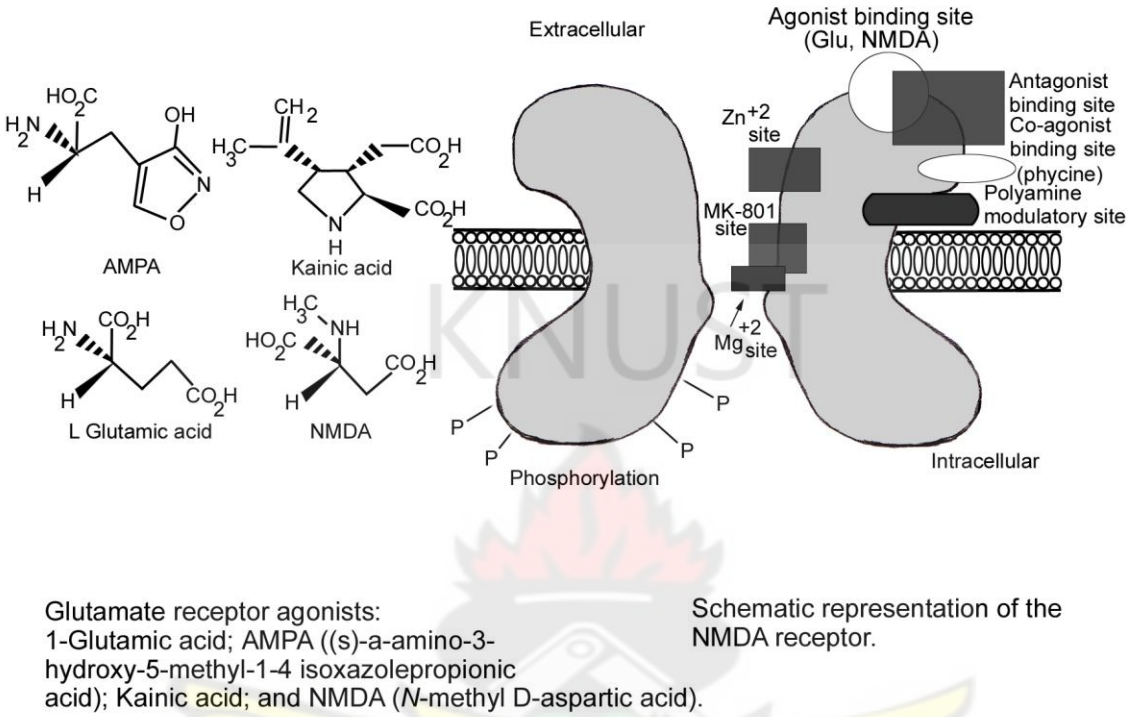


Figure 10: Schematic representation of the *N*-methyl-D-aspartate (NMDA) receptor.

Source: http://emedicine.medscape.com/article/1187334-overview#aw2aab6b3

1.4.15.1 Felbamate

Felbamate is a potent anticonvulsant, very effective against multiple seizure types. Unfortunately, after the occurrence of aplastic anemia and hepatic failure, approval for general use was withdrawn. It is now available in the United States only for a very limited use, principally by neurologists in patients for whom potential benefit outweighs the risk. It blocks the NMDA receptors and voltage-gated calcium channels and also modulates sodium-channel conductance, but has no effect on GABA receptors. In addition to its activity against seizures, felbamate has been shown to have a neuroprotective effect on models of hypoxic-ischemic injuries. Wallis and Panizzon reported neuroprotection after treatment with felbamate in the rat

hippocampal slice model following hypoxic exposure (Wallis and Panizzon, 1993). Wasterlain *et al* also demonstrated the neuroprotective effect of felbamate after bilateral carotid ligation in rat pups with a window of opportunity for neuroprotection of 1-4 hours after the ligation (Wasterlain *et al.*, 1993).

1.4.15.2 Topiramate

Topiramate is a very potent anticonvulsant. It is structurally different from other AEDs. It is derived from D-fructose and initially was developed as an antidiabetic drug. In animal models, it was found to have potent antiepileptic effects. Topiramate has multiple mechanisms of action. It exerts an inhibitory effect on sodium conductance, decreasing the duration of spontaneous bursts and the frequency of generated action potentials, enhances GABA by unknown mechanisms, inhibits the AMPA subtype glutamate receptor, and is a weak inhibitor of carbonic anhydrase (NICE, 2004; Ochoa and Richie, 2009; Suzette *et al.*, 2004).

1.4.16 AEDs with Other Mechanisms of Action

1.4.16.1 Levetiracetam

Levetiracetam (LEV) is a piracetam (S-enantiomer pyrrolidone) derivative. It was developed in the 1980s to enhance cognitive functions and for anxiolysis. It is a unique AED in that it is ineffective in classic seizure models that screen potential compounds for antiseizure efficacy such as maximal electroshock and pentylenetetrazol in rats and mice. During preclinical evaluations, it was found to be effective in several models of seizures, including tonic and clonic audiogenic seizures in mice, tonic seizures in the maximum electroshock-seizure test in mice, and tonic seizures induced in rodents by chemoconvulsants. Interestingly, LEV inhibits the development of pentylenetetrazol-induced amygdala kindling in mice, a situation in which other drugs such as PHT and CBZ are inactive. The mechanism of action is possibly related to a brain-specific stereo-selective binding site, the synaptic vesicle protein 2A (SV2A). The SV2A appear to be important for the availability of calcium-dependent neurotransmitter vesicles ready to release their content (Xu and Bajjalieh, 2001). The lack of SV2A results in decreased action potential-dependent neurotransmission, while action potential-independent

neurotransmission remains normal (Crowder *et al.*, 1999; Janz *et al.*, 1999). In addition, it reduces bicuculline-induced hyperexcitability in rat hippocampal CA3 neurons, suggesting a non-GABAergic mechanism. LEV inhibits Ca^{2+} release from the IP3-sensitive stores without reducing Ca^{2+} storage, which could explain some of LEV's antiepileptic properties (NICE, 2004; Ochoa and Richie, 2009; Suzette *et al.*, 2004).

1.5 EXPERIMENTAL MODELS OF EPILEPSY

The antiseizure or anticonvulsive pharmacology of novel test substances can be characterized using variations of two basic test methods of rodents: blockade of electroshock-induced convulsive seizures and the blockade of chemical-induced convulsive seizures. Variations of the two basic methods have lead to other classifications of the experimental models exist, namely the acute seizure model and chronic seizure models (including models of status epilepticus) (Mody and Schwartzkroin, 1997).

1.5.1 Acute seizure Models

In an effort to develop animal models for human epileptic conditions, investigators initially turned to methods of induction that were rapid and produced consistent and dramatic electrophysiological effects. Most of these approaches induced relatively brief periods of seizure activity with electrical phenomena resembling some aspects of epileptic seizure in human patients. These approaches to the understanding of seizure-like activity have been extremely important in the history of epileptic investigation, because they represent the first best method to explore basic mechanisms. In addition some of these models have become the standards against which antiepileptic drugs are evaluated for efficacy (Mody and Schwartzkroin, 1997). The potencies of most of the antiepileptics of current clinical use have been determined against two acute seizure models: the maximal electroshock (MES) and the minimal pentylenetetrazole (PTZ) seizure threshold and these tests have been used as the standard antiepileptic assay (Krali *et al.*, 1978). However, the fact that these models continue to be viewed as the standards by which antiepileptic drugs are assessed and the epilepsy-related mechanisms are explored has serious- and potentially dangerous- consequences for the

progress in the field (Mody and Schwartzkroin, 1997). Nevertheless, it is important to consider that acute experimental models of epilepsy are similar to reactive seizures in humans and can be used to study ictal events. The acute seizure models have been classified according to the type of seizure induced. The correspondence between the partial and generalized (both convulsive and non convulsive) epilepsies and their respectively agents of induction are given in Table 1 (Mody and Schwartzkroin, 1997).

Table 3: Classification of acute seizure models according to type of induced seizure

Partial
Electrical stimulation
Topical/focal convulsants
Blockers of inhibition (e.g antagonists of $GABA_A$ receptors)
Enhancement of excitation (e.g agonist of glutamate, muscarinic cholinergic receptors
Generalized
Convulsive type
Maximal electroshock (MES)
Maximal systemically administered convulsants (e.g pentyletetrazole, allyglycine, bicuculline, picrotoxine, kainic acid, convulsant barbiturates, ouabain, anticholinesterases, thiosemicarbizade)
Flurothyl seizures
Absence type
Subcortical electrical stimulation
Systemic low-dose pentylenetetrazole (PTZ)
Feline generalized penicillin
Intracerebroventricular opioids
Systemic (gamma-hydroxybutyrate (GHB))
Carbon dioxide withdrawal seizures
Hyperthermia in immature animals

1.5.1.1 Acute seizure models of Partial Epileptic seizures

These models involve the focal application or injection of several compounds or electroshocks intensities that interfere with the neurochemical balance response for normal neuronal excitability and some may lead to chronic states of epilepsy. The most commonly used compounds are the inhibitory amino acid receptor antagonists, cholinergic agonists and antagonists, excitatory amino acid receptor agonists, hormones, various metals, cryogenic injury, and electrical stimulation (Mody and Schwartzkroin, 1997).

1.5.1.2 Acute seizure model of generalized seizures

Seizures Induced by Electroshock

This acute model of convulsive activity is one of the most widely used for the anticonvulsant drug screening. Typical stimulation parameters used in antiepileptic drug screening are 50 mA,(mice) or 150 mA (rats) delivered at 60 hz for 200 ms through corneal electrodes. Two types of seizures are defined in this model: minimal (evoked by stimulus producing 5 of continuous clonus) and maximal (evoked by a stimulus producing tonic hindlimb extension/ flexion followed by clonus); various studies have focused on these seizure patterns for testing the efficacy of anticonvulsant agents (Mody and Schwartzkroin, 1997).

Seizures Induced by Systemic Application of Drugs

These models involve the systemic injection of relatively high doses of convulsants leading to reliable induction of seizures, or even status epilepticus (De Deyn *et al.*, 1992). Depending on the drug or its dose, the seizures may be *grand mal* type or absencelike. These models are easy to use and therefore convenient when large-scale drug testing is called for. However, many associated problems confound the precise interpretation of findings obtained with systemic chemoconvulsant seizure models. Chemoconvulsants injected systemically have several effects, many of them independent of the induced seizures. Some diffuse effects are often difficult to separate from the epileptogenicity,and from the direct effects of the seizures themselves (Mody and Schwartzkroin, 1997).

Convulsive seizures

The best-known systemically administered convulsantt is the pentylenetetrazole (Metrazol). Standard doses are administered intravenously or subcutaneously, usually to rats or mice. Pentylenetetrazole (PTZ) has been reported to produce seizures by inhibiting gamma-aminobutyric acid (GABA) neurotransmission (Huang *et al.*, 2002; Huang *et al.*, 2001). Given at a high doses (> 70 mg kg^{-1}) to rodents, it initially produces myoclonic jerks. These jerks then become sustained and culminate in generalized tonic-clonic seizures. In addition to the MES model, the PTZ model has become the testing ground for antiepileptic drug development (Mody and Schwartzkroin, 1997). Systemic injections of $GABA_A$ receptor antagonists e.g (Bicuculline and picrotoxin) are also a potent means of inducing convulsions. The acute phase of the seizure discharge produced by systemic administration of kainic acid (a glutamate receptor agonist) or pilocarpine (a muscarinic agonist) is very prolonged, and as such it resembles status epilepticus. The ensuing sustained and recurrent spontaneous seizures that persist throughout the life of the animal treated with kainic acid or pilocarpine are more appropriately discussed as chronic rather than acute models of epilepsy (Mody and Schwartzkroin, 1997).

Absence Seizures

In contrast to the topical application of penicillin, which produces focal seizures, systemic application of large doses of penicillin to cats produces spike- and –wave discharges and absencelike attacks. A number of other compounds, including GHB, low-dose PTZ, the $GABA_A$ receptors agonist THIP (4, 5, 6, 7-tetrahydroxyisoxazolo-[4, 5, c]-pyridine-3-ol), and AY-9944 (a drug that inhibit the reduction of 7-dehydrocholesterol to cholesterol) induces`generalized seizures in rodents. These chemical models have been useful for the understanding the thalamocortical circuits involved in the generation of spike-and –wave discharges, and in the screening of drugs effective in the treatment of absence seizures (Mody and Schwartzkroin, 1997).

1.5.2 Chronic Seizure Models

The chronic seizure models are used to investigate epilepsy mechanisms. These models lead to animals that are truly epileptic and exhibit spontaneous seizures. The chronic seizure models are also used to investigate temporal lobe epilepsy (TLE). Two most commonly used animal models of chronic seizure development are; kindling and status epilepticus. Several reviews have considered kindling and/ or status epilepticus as models of clinical epilepsy (Coulter *et al.*, 2002; Loscher, 1997; McIntyre and Poulter, 2001; McNamara, 1994; McNamara *et al.*, 1980; Morimoto *et al.*, 1998; Racine and Burnham, 1984; Sato *et al.*, 1990; Sutula, 2001).

1.5.2.1 The kindling model

Since its discovery by Goddard and colleagues (1969), the kindling phenomenon has been used extensively as a chronic animal model of TLE. With a few exceptions (Cavazos *et al.*, 1994; Sutula, 1990), kindling is still widely accepted as a functional epilepsy model in which the altered neuronal response develops in the absence of gross morphological damage, such as that seen in many other epilepsy models. In addition to its application to the investigation of epilepsy mechanisms, kindling has been used for the preclinical evaluation of antiepileptic drugs. For example, antiepileptic drugs that are clinically effective for TLE have been demonstrated to inhibit kindled seizures triggered from limbic foci, and their anticonvulsant profiles can be clearly categorized according to their mechanisms of action. Phenytoin, carbamazepine and lamotrigine, for example, act primarily on voltage-dependent Na^+ channels and elevate seizure thresholds in epileptogenic foci, whereas valproate, tiagabine and diazepam enhance GABA/benzodiazepine (BNZ) systems and block seizure propagation and generalization (Morimoto *et al.*, 1997; Otsuki *et al.*, 1998).

Kindling presents itself as an apparently simple phenomenon in which repeated induction of focal seizure discharge produces a progressive, highly reliable, permanent increase in epileptic response to the inducing agent, usually electrical stimulation (Goddard *et al.*, 1969; Racine, 1978). In limbic kindling, the initial focal seizure response is expressed mainly in the epileptiform discharge triggered at the stimulated site. The only behavioral response during the early stages of kindling is a freezing response during the evoked ictal discharge. With repeated activation, the seizure response becomes generalized to the point of driving bilateral clonic

seizures (typically referred to as "stage 5 seizures") (Racine, 1972). At this point, the initial, brief focal afterdischarge (AD) has been dramatically altered, usually increasing in duration, amplitude, spike frequency, and spike morphology (Racine, 1972; Racine, 1975). The seizure threshold is also typically reduced over the course of kindling (Racine *et al.*, 1972). Once generalized kindled seizures (stage 5 seizures) are established, these alterations persist for months or years (Goddard *et al.*, 1969).

The advantages of the kindling model for epilepsy research are clear: precise focal activation of the target brain sites is possible, development of chronic epileptogenesis is reliably observed, the pattern of seizure propagation and generalization is readily monitored, and interictal, ictal and postictal periods are easily manipulated (Morimoto *et al.*, 1998). However, there are some disadvantages, as well. Kindling experiments can be relatively labor intensive, given that electrodes are typically implanted into the brain and multiple, spaced stimulations are required. Also, spontaneous seizures do not develop unless a very large number of kindling stimulations are applied (the "over-kindling" model) (Michalakis *et al.*, 1998; Pinel and Rovner, 1978).

Several authors have claimed kindling-like phenomena in humans (Sato *et al.*, 1990; Coulter *et al.*, 2002). Although the relevance of kindling to clinical TLE is still unclear, kindling appears to be the current model of choice for investigating the functional mechanisms underlying cryptogenic TLE (without hippocampal sclerosis). A kindling-like mechanism through mesial limbic structures has also been proposed as a final common path for lesion-based focal epilepsy (Engel, 1996; McNamara, 1994).

Models of Status Epilepticus (SE)

In contrast to kindling, status epilepticus and its sequelae are easier to produce, but more variable in their expression. Most typically, high doses of a convulsant agent such as kainate or pilocarpine are injected systemically. The status epilepticus is characterized by continual, recurrent seizures.For most researchers, it is not the status epilepticus that is of interest, but the delayed appearance of spontaneous seizures. Thus, the model has been called "post-status epilepticus models of TLE" by Loscher (2002). Using these models, it is possible to create a truly epileptic rat (the most widely used animal for this research). In addition, the morphological changes that occur in the hippocampus following status epilepticus are often

quite similar to those seen in human mesial TLE, although the damage in the animal model can be more severe and widespread. The disadvantages of status models are that the status can be difficult to control (it is not unusual for a substantial proportion of the animals to die during the status phase), the expression of the spontaneous seizures can be unpredictable, and the neural damage can be extensive (Morimoto *et al.*, 2004).

The cytotoxic agents that are used to induce status epilepticus are sometimes applied to specific brain sites such as the amygdala (Tanaka, 1992) or the hippocampus (Mathern *et al.*, 1993) Also, status epilepticus can be induced by sustained electrical stimulation to specific sites such as the perforant path (Sloviter, 1987), the ventral hippocampus (Lothman *et al.*, 1990), or the anterior piriform cortex (Inoue *et al.*, 1992). All of these manipulations, however, still produce brain damage. Inoue et al. (1992) found that the most sensitive site for electrically induced status epilepticus was the deep prepiriform cortex (compared to the amygdala and dorsal hippocampus). On the other hand, Mohapel and colleagues (Mohapel *et al.*, 1996) reported that 60 min of continuous electrical stimulation triggered status epilepticus more readily when applied to the basolateral amygdala than to the central and medial amygdala or to the adjacent piriform cortex (Morimoto *et al.*, 2004).

Spontaneous seizures do not typically appear for weeks or months following the induction of status epilepticus, with a mean onset latency of around 40 days (Glien *et al.*, 2001). The onset latency does, however, appear to be related to the convulsant dose, with higher dosages yielding shorter onset latencies (Hamani and Mello, 2002). It is not surprising, then, that there is a positive correlation between the duration of status epilepticus and the development of spontaneous seizures (Klitgaard *et al.*, 2002b) or the degree of pyramidal neuron loss. (Inoue *et al.*, 1992) In the pilocarpine model, for example, at least 30 min duration of initial status epilepticus is required for manifesting an epileptogenic process (Klitgaard *et al.*, 2002b).

A number of experimental models of SE have been developed, including models in intact animals based on systemic and focal administration of chemical convulsants and focal and generalized electrical stimulation of the brain. More recently, some investigators have begun to investigate recurrent or sustained seizure activity isolated neuronal circuits using slice preparations (Treiman and Heinemann, 1997).

A variety of chemical convulsants have been administered systemically to induce various forms of experimental SE. These agents do so by increasing neuronal excitation or decreasing inhibition. Table 1 lists chemical convulsants used in the induction of experimental SE and their presumed mechanisms of action. Experimental models based on the systemic administration of chemical convulsants have the advantage of simplicity-SE can be induced simply by parenteral administration of the convulsant agent. The disadvantage of such agents is their continuing presence once SE has been induced. Results may be confounded by the continuing presence of the inducing agent or by the potential drug interactions between the inducing agent and the experimental therapeutic agent (Treiman and Heinemann, 1997).

Table 4: Mechanisms of chemical convulsants used to induce or exacerbate experimental status epilepticus

Class/Mechanism	Receptor	Chemical Convulsant
Excitatory agonists	NMDA	NMDA
Glutamate agonism	Non-NMDA	Domoic acid
		Kainic acid
		Quisqualic acid
Acetylcholine agonism	Muscurinic	Pilocarpine ± lithium
		Soman
Inhibitory antagonists		
GABA antagonism	$GABA_A$	Bicuculline
		Pentylenetetrazole
		Picrotoxin
Adenosine antagonism	A_1	Methylxanthines
Other agents		
NO synthase inhibition		L-nitroarginine
Na+ channel opening		Flurothyl
Pyridoxal phosphate	$GABA_A$	4-deoxypyridoxine
	$GABA_A$	Allygylcine
Glutamic acid decarboxylase inhibition		Mercaptoproprionic acid

1.6 EPILEPSY AND PAIN

Damage to the nervous system represents a risk for the development of chronic pain, termed neuropathic pain, with a major impact on patients and society. Neuropathic pain not only causes long lasting intense pain and reduced quality of life, but also results in a diminished working capacity and an increased need for health care. It is now well known that injury to the peripheral and central nervous systems produces a cascade of neurobiological events in the spinal cord and in brain structures receiving information from the injured area. These changes involve sensitization with a variety of clinical manifestations, including exaggerated and prolonged pains, different types of evoked pains, and pain which spreads to distant tissue structures (Jensen *et al.*, 2001; Koltzenburg, 1998; Woolf and Decosterd, 1999). Despite the discovery of multiple mechanisms underlying chronic pain, our handling of neuropathic pain is still insufficient and thus needs rethinking. The traditional classification of neuropathic pain according to aetiology or location has been of limited help in our search for a rational treatment of patients with neuropathic pain. Another approach is needed, one in which pain is classified according to underlying mechanisms (Woolf and Decosterd, 1999).

Neuropathic pain, whether of peripheral or central origin, is characterized by a neuronal hyperexcitability in damaged areas of the nervous system. In peripheral neuropathic pain, damaged nerve endings exhibit abnormal spontaneous and increased evoked activity, partly due to an increased and novel expression of sodium channels. In central pain, although not explored in detail, the spontaneous pain and evoked allodynia are also best explained by a neuronal hyperexcitability. The peripheral hyperexcitability is due to a series of molecular changes at the level of the peripheral nociceptor, in dorsal root ganglia, in the dorsal horn of the spinal cord, and in the brain. These changes include abnormal expression of sodium channels, increased activity at glutamate receptor sites, changes in -aminobutyric acid (GABA-ergic) inhibition, and an alteration of calcium influx into cells. The neuronal hyperexcitability and corresponding molecular changes in neuropathic pain have many features in common with the

cellular changes in certain forms of epilepsy. This has led to the use of anticonvulsant drugs for the treatment of neuropathic pain (Jenson, 2002).

Carbamazepine and phenytoin were the first anticonvulsants to be used in controlled clinical trials. Studies have shown these agents to relieve painful diabetic neuropathy and paroxysmal attacks in trigeminal neuralgia. Subsequent studies have shown the anticonvulsant gabapentin to be effective in painful diabetic neuropathy, mixed neuropathies, and postherpetic neuralgia. Lamotrigine is also effective in trigeminal neuralgia, painful peripheral neuropathy, and post-stroke pain. Other anticonvulsants, both new and old, are currently undergoing controlled clinical testing. The most common adverse effects of anticonvulsants are sedation and cerebellar symptoms (nystagmus, tremor and incoordination). Less common side-effects include haematological changes and cardiac arrhythmia with phenytoin and carbamazepine. The introduction of a mechanism-based classification of neuropathic pain, together with new anticonvulsants with a more specific pharmacological action, may lead to more rational treatment for the individual patient with neuropathic pain (Jenson, 2002).

1.6.1 Animal Models of Nociception

Each species expresses pain in a manner related to its own behavioural repertoire. They include basic motor responses (withdrawal, jumping, contractures, etc.), neurovegetative reactions with an increase in sympathetic tone (tachycardia, arterial hypertension, hyperpnea, mydriasis, etc.), and vocalization. The more complex reactions include conditioned motor responses, which result from a period of learning and sometimes can be very rapid (e.g., cattle avoiding an electrical enclosure). Behavioral reactions (escape, distrust of objects responsible for painful experiences, avoidance, aggressiveness, etc.) or modifications of behaviour (social, food, sexual, sleep, etc.) are often observed. The absence of verbal communication in animals is undoubtedly an obstacle to the evaluation of pain. There are circumstances however during which there can be little doubt that an animal is feeling pain. Many models for assessing analgesic activity in animals have been devised for both behavioral and non-behavioral models of nociception/pain. Generally speaking, the most reliable signs of pain are physical ones (Le Bars *et al.*, 2001).

Currently, the commonly used behavioral tests of acute nociception/pain can be broadly considered based on the use of short-duration stimuli (in the order of seconds) and then those based on the use of longer duration stimuli (in the order of minutes) (Le Bars *et al.*, 2001).

1.6.1.1 Use of Short-Duration Stimuli

These tests are the most commonly used. In general they involve a short period of stimulation. They can be classified by the nature of the stimulus, be it thermal (Tail-Flick Test, Tail immersion Test, and Hot Plate Test), mechanical (Tail or Paw Pressure Test) or electrical (Stimulation of Paw, Tail or Dental Pulp) (Chau, 1989; Le Bars *et al.*, 2001).

1.6.1.2 *Use of Long Duration Stimuli*

Basically, these tests involve using an irritant, algogenic chemical agent as the nociceptive stimulus. They are not models for chronic pain because their duration is only in the order of some tens of minutes (Le Bars *et al.*, 2001). The main types of behavioral test based on such stimuli use intradermal ("Formalin Test") or intraperitoneal injections ("writhing test"). Tests based on the stimulation of hollow organs are also used (Le Bars *et al.*, 2001).

There are models of chronic pain in animals such as the rat with induced arthritis and rats that have had various lesions to the central or peripheral nervous systems) (Chau, 1989; Colpaert, 1987; Le Bars *et al.*, 2001; Seltzer, 1995) but due to ethical considerations and technical problems the adjuvant–induced arthritis in rats is the most widely used *in vivo* model for the evaluation of drugs in chronic pain

1.6.2 Animal models of Neuropathic pain

Most models utilised in investigating neuropathic pain are performed on rats, although mice models are currently being validated. Fang and colleagues have demonstrated that mouse models of neuropathic pain, are similar to those pioneered in rats and can be useful tools in the genetic, physiological and pharmacological investigation of neuropathic pain. The various murine models of neuropathic pain include: chronic constriction injury (CCI), partial ligation of sciatic nerve (PSL), spinal nerve ligation (SNL), spared nerve injury (SNI), and streptozocin, paclitaxel, and vincristine-induced neuropathies). Sensory modalities utilized

are: mechanical allodynia (MA), cold allodynia (CA), mechanical hyperalgesia (MH) and thermal hyperalgesia (TH) (Fang *et al.*, 2007)

1.7 EPILEPSY AND ANXIETY

1.7.1 Introduction

Psychiatric aspects of epilepsy have been extensively reviewed in the past and such studies have shown that the rate of mood disorder are higher in patients with epilepsy than in those with other chronic medicals conditions such as diabetes and asthma (Hermann *et al.*, 2000; Kanner and Palac, 2000; Marsh and Rao, 2002). Most of the attention has been focused on depression, despite the fact that anxiety may actually be more common and equally disabling. A more recent study indicates that 25 % of patients with all types of epilepsy had anxiety disorders with 19% of them with other mood disorders (Swinkels *et al.*, 2001). However in some secondary care and specialist settings, the prevalence of anxiety disorder may exceed 50% (Ettinger *et al.*, 1998; Jones *et al.*, 2005).

Neurobiologically, the amagdala seems to be a particularly important structure for the production of anxiety symptoms and epileptic discharges in temporal lobe epilepsy. The amagdala is responsible for processing and relaying emotional stimuli from multiple sources to limbic and other cortical structures, basal ganglia, hypothalamus, and brain stem. The amagdala is therefore central to the generation of affective, autonomic, cognitive, and endocrine components of the clinical symptoms of "anxiety" (Chapouthier and Venault, 2001; Charney, 2003; Kalynchuk, 2000; Sah *et al.*, 2003; Trimble and Van Elst, 2003). Gamma aminobutyric acid (GABA) is the most important inhibitory transimitter in the central nervous system. Recent evidence suggests that the abnormal functioning of $GABA_A$ receptors could be of great importance in the pathophysiology of epilepsy and anxiety disorders (Lydiard, 2003). This hyopothesis is supported by the observation that some substances, including the GABAergic antiepileptic drugs gabapentin, vigabatrin, tiagabine, valproate, and pregabalin (Ashton and Young, 2003; Blanco *et al.*, 2003; Carta *et al.*, 2003; Kinrys *et al.*, 2003; Lauria-Horner and Pohl, 2003; Pande *et al.*, 2003; Pande *et al.*, 2000; Rosenthal, 2003), as well as the barbiturate,

benzodiazepines and neuroactive steroids, have antiepileptic as well as anxiolytic properties (Beyenburg *et al.*, 2001) These pharmacological properties are either mediated through the interaction of drugs with the $GABA_A$ receptor (for instance, by allosteric modulation in the case of benzodiazepines or neuroactive steroids), through agonism at the GABA binding site, or through an increase in endogenous GABA. Vigabatrin, for instance, inhibits GABA transaminase, whereas tiagabine is a selective inhibitor of GABA reuptake, thereby increasing the effects of GABA. The exact effect of the antiepileptic drugs gabapentin, pregabalin, and valproate on GABAergic neurotransmission remains unclear, but these drugs can increase GABA concentrations in certain brain regions (Ashton and Young, 2003; Blanco *et al.*, 2003; Carta *et al.*, 2003; Kinrys *et al.*, 2003; Lauria-Horner and Pohl, 2003; Pande *et al.*, 2003; Pande *et al.*, 2000; Rosenthal, 2003).

There is currently no good evidence from randomized studies of possible anxiolytic effects of AEDs in patients with epilepsy. However, one may consider choosing an AED with anxiolytic potential in a patient with epilepsy who also has symptoms of an anxiety disorder if this AED is suitable for his/her particular type of epilepsy. GABAergic drugs, in particular, have been shown to reduce anxiety in animal experiments and this 'side-effect' can therefore be put to good therapeutic use (Ashton and Young, 2003; Blanco *et al.*, 2003; Carta *et al.*, 2003; Kinrys *et al.*, 2003; Lauria-Horner and Pohl, 2003; Pande *et al.*, 2003; Pande *et al.*, 2000; Rosenthal, 2003). The GABA analogs gabapentin and pregabalin, for instance, have proven to effects in anxiety disorders and focal epilepsies (Ashton and Young, 2003; Blanco *et al.*, 2003; Carta *et al.*, 2003; Kinrys *et al.*, 2003; Lauria-Horner and Pohl, 2003; Pande *et al.*, 2003; Pande *et al.*, 2000; Rosenthal, 2003). The precise mode of action of these two AEDs is not fully understood, although it has been shown that gabapentin and pregabalin bind selectively to the a2d subunit of voltage-gated calcium channels (Ashton and Young, 2003; Blanco *et al.*, 2003; Carta *et al.*, 2003; Kinrys *et al.*, 2003; Lauria-Horner and Pohl, 2003; Pande *et al.*, 2003; Pande *et al.*, 2000; Rosenthal, 2003). Both molecules do not interact with $GABA_A$ or $GABA_B$ receptors, but do increase the concentration in some brain regions and rate of synthesis of GABA and, thereby, decrease glutamate concentration indirectly. They also modulate other neurotransmitter systems including the noradrenalinergic, dopaminergic and serotonergic systems (Ashton and

Young, 2003; Lauria-Horner and Pohl, 2003). Vigabatrin, a GABA transaminase inhibitor, and tiagabine, a GABA reuptake inhibitor (Kinrys *et al.*, 2003; Rosenthal, 2003), as well as valproate (Kinrys *et al.*, 2003), have anxiolytic properties. The possible positive effect of other AEDs like carbamazepine and oxcarbazepine on anxiety symptoms has only been reported anecdotally (Boylan *et al.*, 2004; Scicutella and Ettinger, 2002).

1.7.2 Animal models of Anxiety

Elevated Plus Maze (EPM)

The elevated plus maze was designed to provide measures of anxiety that were relatively uncontaminated by changes in overall motor activity, and has been extensively validated by (Pellow *et al.*, 1985) using behavioural, physiological, and pharmacological measures. This test relies on the inherent conflict between exploration of a novel area and avoidance of its aversive features. The plus- maze is sensitive to the anxiolytic effects of neurotoxic lesions of serotonergic neurons and to the anxiogenic effects of drugs (Pellow and File, 1986), drug withdrawal (File and Andrews, 1991), and predator odour (Zangrossi and File, 1992). Unlike the social interaction and light/dark test, the EPM does not rely on aversion to bright light, and it has been found repeatedly that behavior in the maze is independent of light levels (Becker *et al.*, 1997).

The EPM has become the most widely used animal test of anxiety and there is considerable variation in construction and testing procedures. The EPM has been used with both handled and unhandled animals, but handling is likely to change drug sensitivity (Andrews and File, 1993). There is a decline with age in the number of open arm entries and the time spent on the open arms. In addition, older rats show a reduced anxiolytic, but an enhanced sedative response to benzodiazepines (File and Wilks, 1990)

Modified Hole Board (mHB)

The modified hole board test was designed to allow a variety of behavioural dimensions to be investigated in one test (Ohl *et al.*, 2001). In addition it was designed to minimize stress by allowing the experimental animal to maintain social contact with its group mates during the

test, thus circumventing stressful social isolation (Ahmed *et al.*, 1995; Kim and Kirkpatrick, 1996). The mHB paradigm represents a test for unconditioned behavior and essentially comprises the characteristics of a hole board and an open field test. Although the original hole board was designed to investigate exploration in rodents in rodents (File and Wardill, 1975; Lister, 1990), the mHB also allows for a variety of other behavioural dimensions to be investigated. In this test, parameters measured are assigned different behavioural categories following the results of studies done in its validation (Ohl *et al.*, 2001). These categories include: Anxiety (measured by the latency to first board entry, number and duration of board entry); Social affinity (measured by the latency to group contact); Risk assessment (stretched attends); Exploration (number of and duration at Holes explored, number of rearings on board): locomotion (number of line crossings) and Physiological arousal (self-grooming and defaecation).

Light /Dark Exploration (LD)

The light/dark exploration test uses the ethological conflict between the tendencies of mice to explore a novel environment and to avoid a brightly lit, open area. Anxiolytic drugs increase the number of transitions between the light and dark compartments and non-anxiolytics do not. Crawley has found that the number of transitions is highly correlated with other exploratory behaviours and is not correlated with locomotor activity in an undifferentiated open field (Crawley, 1981) and that this paradigm is more sensitive to the detection of anxiolytics than anxiogenics. Costall and colleagues have found that the latency to leave the white side (light area) and the time spent on this side are more reliable measures, and the procedure can detect the effects of anxiogenic treatments (Costall *et al.*, 1989b). A major problem with this test is distinguishing between specific and non-specific changes in activity; this differentiation is best accomplished by performing parallel experiments in a holeboard apparatus that provides independent measures of locomotor activity, rearing and exploratory head dipping (File and Wardill, 1975). Use of male mice (rather than female mice or rats) is recommended.

1.8 ANTICONVULSANTS AS ANTIOXIDANTS

The brain is vulnerable to oxidative stress than other tissues for several reasons. One reason lies in its high oxygen consumption (Sah *et al.*, 2002), which is around 20% of the total metabolic activity (Dal-Pizzol *et al.*, 2000), although the brain represents a small percentage of the body mass. In addition, while the brain contains large amounts of polyunsaturated fatty, which are susceptible to oxidation, and pro-oxidative metals (in the cerebrospinal fluid), it has a low antioxidant capacity (the brain contains almost no catalase and less glutathione peroxidase (GPx) and vitamin E; high amounts of ascorbic (both antioxidant and pro-oxidant) as compared to liver (Bellissimo *et al.*, 2001; Frantseva *et al.*, 2000; Freitas *et al.*, 2004). Moreover, the release of excitatory neurotransmitters, such as glutamate, induces a cascade of reactions in the post-synaptic neuron, resulting in the formation of ROS (Cui *et al.*, 2004).

Reactive oxygen species have been implicated in the development of seizures under pathological conditions and linked to seizure-induced neurodegeneration. Evidence includes the temporal correlation between free radical (FR) generation and the development of seizures in some pathological conditions, (Bruce and Baudry, 1995; Jerrett *et al.*, 1973; Singh and Pathak, 1990) and the protective efficacy of antioxidative treatments against some types of seizures,(Mori *et al.*, 1991; Ogunmekan and Hwang, 1989; Whelan *et al.*, 1998; Willmore and Rubin, 1981) against cell damage caused by kainate exposure (MacGregor *et al.*, 1996) or iron-induced epilepsy (Willmore and Rubin, 1981).

Most of this evidence, however, has been indirect. Direct evidence of free radical production during epileptiform events has come primarily from the iron- or excitotoxin-induced models of epilepsy (Bruce and Baudry, 1995; Singh and Pathak, 1990). Since iron and excitotoxins are potent initiators of oxidative stress in their own right, (Bindokas *et al.*, 1996; Borg and Schaich, 1987; Garriedo *et al.*, 1998) the increased free radical generation seen in these models may relate to the inducing agents themselves, rather than to the oxidative effects of seizures. There is ample evidence to show that free radicals are actively involved in physiological processes during oxidative stress induced by convulsants such as pilocarpine

and pentylenetetrazole induced kindling (Coyle and Puttfarcken, 1993; Tejada *et al.*, 2007; Ueda *et al.*, 1997), and that some antioxidants such as ascorbic acid have been shown to be exert anticonvulsant effect in pilocarpine induced SE through the reduction of lipid peroxidation content and increased catalase enzyme activity (Xavier *et al.*, 2007). Of all the free oxygen radical species that can occur in vivo the hydroxyl free radicals ($^{\cdot}OH$) .are considered to be the most reactive and hazardous (Halliwell, 1992).

1.8.1 Antioxidants

Antioxidants are substances capable of counteracting the damaging effects of oxidation in body tissues (Scheibmeir *et al.*, 2005). The excess of ROS is generally inactivated by endogenous or exogenous antioxidant molecules that have the ability, even at low concentrations, to delay or inhibit the oxidation of a substrate (Cui *et al.*, 2004). They may do so by removing or lowering the local concentrations of one or more of the participants in this reaction, such as oxygen, ROS, or metal ions (Fe^{3+} , Cu^{2+}, etc.), which catalyze oxidation, or by interfering with the chain reaction that spreads oxidation to neighboring molecules. They may also act by enhancing the endogenous antioxidant defenses of the cell (Cui *et al.*, 2004).

1.8.2 Classification of Antioxidants

Antioxidants may be classified according to their chemical nature and mode of function:

Enzyme antioxidants: They act on specific ROS after they are formed and degrade them to less harmful products. Examples are the SODs (superoxide dismutase), catalase (CAT), and GPx (gluthathione peroxidase) The SODs, CAT, and GPx constitute the major intracellular enzymic antioxidants, while the extracellular antioxidants are mainly of the preventive and scavenging types.

The preventive antioxidants: These act by binding to and sequestering oxidation promoters and transition metal ions, such as iron and copper, which contain unpaired electrons and strongly accelerate free radical formation. Examples are transferrin and lactoferrin (which bind ferric ions), ceruloplasmin (which binds Cu, catalyzes the oxidation of ferrous ions to ferric due to its ferroxidase activity, and increases the binding of iron to transferrin), haptoglobins (which bind

hemoglobin), hemopexin (which binds heme), and albumin (which binds copper and heme) (Cui *et al.*, 2004).

The scavenging or chain-breaking antioxidants: These act by presenting themselves for oxidation at an early stage in the free radical chain reaction and giving rise to low energy products that are unable to propagate the chain further (Scheibmeir *et al.*, 2005). Lipid-soluble and water-soluble scavengers act in cellular environments that are either hydrophobic or hydrophilic, respectively. The major lipid-soluble scavengers are vitamin E (α-tocopherol), β-carotene, and coenzyme Q (CoQ), while ascorbic acid, various thiols, uric acid, and bilirubin function in the aqueous milieu (Cui *et al.*, 2004). Melatonin (N-acetyl- 5-methoxytryptamine), an endogenous substance produced in the pineal gland, is a powerful antioxidant, which is believed to be of particular interest to the nervous system (Reiter, 1997; Shen *et al.*, 2002).

1.8.3 Plants as Source of Antioxidants

Plants produce significant amount of antioxidants to prevent the oxidative stress caused by photons and oxygen (Auddy *et al.*, 2003) during photosynthesis. The major group types being the carotenoids (that suppress ROS) ascorbate (that scavenge ROS and prevent them from doing damage) and particularly thiols, which are involved in repair processes. A great number of other compounds such as the phenolic substances (especially the flavonoids) have been shown to have strong antioxidant activities *in vitro (*Jeon *et al.*, 2006). These compounds possess recognized antioxidant, anticarcinogenic, antimutagenic, antitumoral, antiartherogenic, antiplatelet aggregation, antibacterial, antiviral and anti-inflammatory effects (Jeon *et al.*, 2006; Ozgova *et al.*, 2003).

1.9 ANTICONVULSANTS AS SKELETAL MUSCLE RELAXANTS

A muscle relaxant is a drug which affects skeletal muscle function and decreases the muscle tone. It may be used to alleviate symptoms such as muscle spasms, pain and hyperreflexia. Two major therapeutic groups of skeletal muscle relaxants exist, namely; neuromuscular blockers and spasmolytics. Neuromuscular blockers act by interfering with transmission at the neuromuscular end plate and have no CNS activity. They are often used during surgical procedures and in intensive care and emergency medicine to cause paralysis. Spasmolytics,

also known as “centrally –acting” muscle relaxants, are used to alleviate musculoskeletal pain and spasms and to reduce spasticity in a variety of neurological conditions (MedicineNet.com, 1996-2007; Miller, 1998).

The ogenerations of the neuronal signals in motor neurons that cause muscle contractions are dependent on the balance of synaptic excitation and inhibition that the motor neuron receives. Spasmolytic agents generally work by either enhancing the level of inhibition, or reducing the level of excitation. Inhibition is enhanced mimicking or enhancing the actions of endogenous inhibitory substances, such as GABA (Miller, 1998).

Because of the enhancement of inhibition in the CNS, most spasmolytic agents have the side-effects of sedation, drowsiness and may cause dependence with long term use. Several of these agents also have abuse potential, and their prescription is strictly controlled. The benzodiazepines, such as diazepam, interact with the $GABA_A$ receptor in the CNS. While it can be used in patients with muscle spasm of almost any origin, it produces sedation in most individuals at the doses required to reduce muscle tone (Charney *et al*., 2001; Rang and Dale, 1991).

Baclofen was originally designed as an antiepileptic in the 1920s. Due to its low anticonvulsant effect, it is effective in patients with muscle spasiticity. Baclofen is considered to be at least as effective as diazepam in reducing spasticity, and causes much less sedation. It acts as a GABA agonist at $GABA_B$ receptors in the brain and the spinal cord, resulting in hyperpolarization of neurons expressing this receptor, most likely due to increased potassium ion conductance. Baclofen also inhibits neural function presynaptically, by reducing calcium ion influx, and thereby reducing the release of excitatory neurotransmitters in both the brain and spinal cord. It may also reduce pain in patients by inhibiting the release of substance P in the spinal cord as well (Bertrand and Galligan, 1992; Miller, 1998).

1.10 JUSTIFICATION OF RESEARCH

The currently available anti-epileptics have severe shortcomings as none has been proved to be anti-epileptogenic thus providing only symptomatic relief and their use is often plagued by significant adverse or side effects. Moreover, a significant proportion of patients (up to 40%) do not respond to these agents and this proportion is quite high in the developing countries (Kwan and Brodie, 2000; Loscher, 2002a; Regesta and Tanganelli, 1999). Additionally, a significant number of epileptic patients in Ghana are treated with older generations of anti-epileptic drugs whiles newer and more effective ones are shunned due to their high cost. A greater proportion of these patient resorts to the use of traditional medicine. There is therefore, a universal and local need for continued research into the development of newer and cost effective agents for the management of this disorder (Brodie and Kwan, 2001; Loscher, 2002b; Loscher, 2002c; McNamara, 1999). Plant sources of drugs dominate therapy in developing countries and have often served as an effective mean of getting lead compounds, from which newer and effective drugs can be developed. Thus, a search for newer and effective antiepileptic drugs from plant sources is warranted.

In Ghana, the aqueous extract from the whole plant *of Synedrella nodiflora* is being used for the treatment of epilepsy (Mshana *et al.*, 2000). It is quite laudable and beneficial that the effects of this plant are explored and possibly an antiepileptic drug developed from it. Consequently, this study was framed to investigate into the anticonvulsant and other related neuropharmacological effects of the hydro-ethanolic extract of the whole plant of *Synedrella nodiflora*.

1.11 AIM OF STUDY

The aim of the study is to investigate the anticonvulsant and related neuropharmacological effects of the hydro-ethanolic extract of the whole plant of *Synedrella nodiflora*. These finding would provide pharmacological evidence for the traditional use of the plant in the management of epilepsy and also explore the potential benefits of the plant in conditions that share similar neurobiology with epilepsy or as a consequence of epileptic seizures.

1.12 SPECIFIC OBJECTIVES OF RESEARCH

- To experimentally investigate into the anticonvulsant activity of the hydro-ethanolic extract of the whole plant of *Synedrella nodiflora* in both acute and chronic seizure models, and the mechanism(s) by which it exerts those properties, in comparison with clinically used anticonvulsants
- By laboratory research, evaluate the analgesic effect of the extract as a means of finding out if the extract as an anticonvulsant could be used to manage neuropathic pain
- To evaluate the effect of the extract in anxiety models so as to find out if it will improve or worsen anxiety resulting from epilepsy
- To evaluate the antioxidant effects of the extract as adjunct therapeutic use to its anticonvulsant effect
- To evaluate the skeletal muscle relaxant effect of the extract as adjunct therapeutic use to its anticonvulsant effect
- To evaluate the acute toxicity and the side-effects of the extract as an anticonvulsant drug

Chapter 2

MATERIALS AND METHODS

2.1 DRUGS AND CHEMICALS

The drugs and chemicals used in this study include: Pentylenetetrazole, picrotoxin, pilocarpine, *n-butyl*- bromide hyoscine, phenobarbitone sodium, baclofen, naloxone (Sigma-Aldrich Inc., St. Louis, MO, USA); diazepam (Pharm-Inter, Brussels, Belgium); thiobarbituric acid, trichloroacetic acid, formalin, acetic acid, caffeine, Folin-Cicocalteu's phenol reagent, sodium bicarbonate (BDH, Poole, England); morphine hydrochloride (Phyto-Riker, Accra, Ghana), diclofenac sodium (Troge, Hamburg, Germany)

2.2 PLANT COLLECTION

The whole plant of *Synedrella nodiflora* was collected from the Botanical Gardens, Kwame Nkrumah University of Science and Technology (KNUST), Kumasi, Ghana in August 2006 and authenticated by the Department of Pharmacognosy, KNUST. A specimen voucher, FP/08/025, has been kept at the Faculty of Pharmacy's Herbarium.

2.3 PREPARATION OF EXTRACT

The whole plant *Synedrella nodiflora* was air-dried for seven days, powdered and cold-macerated with 70 % v/v of ethanol in water. The hydro-alcoholic extract was then evaporated to a syrupy mass under reduced pressure, air-dried and kept in a dessicator. A 7% yield was obtained. This is subsequently referred to as extract of *Synedrella nodiflora* or SNE.

2.4 ANIMALS

Sprague-Dawley rats (150-200 g) and ICR mice (20-30 g) were purchased from Noguchi Memorial Institute for Medical Research, University of Ghana, Legon, and maintained in the Animal House of the Department of Pharmacology, Kwame Nkrumah University of Science

and Technology (KNUST), Kumasi. The animals were housed in groups of six in stainless steel cages (34 × 47 × 18 cm) with soft wood shavings as bedding, fed with normal commercial pellet diet (GAFCO, Tema), given water *ad libitum* and maintained under laboratory conditions (temperature 24-28 °C, relative humidity 60-70%, and 12 hour light-dark cycle).

In other experiments conducted at the Health Science Center (HSC), Kuwait University, Balb/c and MF-1 mice of either sex (20-30 g) were obtained from the Animal Resource Center (ARC), HSC, Kuwait University, Kuwait.

All procedures and techniques used in these studies were in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals (NIH, Department of Health Services publication No. 83-23, revised 1985). The protocols for the study were approved by the Departmental Ethics Committees of the Department of Pharmacology, KNUST, Ghana and the Department of Applied Therapeutics, Health Science Center, Kuwait University, Kuwait.

2.5 PHYTOCHEMICAL ANALYSIS

The hydro-ethanolic extract of *S. nodiflora* was screened for the presence of alkaloids, reducing sugars, glycosides, saponins, and tannins as described by Trease and Evans (1989).

2.6 ANTICONVULSANT EFFECT OF SNE

2.6.1 Pentylenetetrazole (PTZ)-induced Seizures

The anticonvulsant testing method of Vellucci and Webster (1984) was used with modifications. In brief, clonic-tonic seizures were induced in drug/vehicle pre-treated male Sprague-Dawley rats (150-200 g) by a subcutaneous injection of 75 mg kg^{-1} pentylenetetrazole (PTZ) into the loose skin fold on the back of the neck of the rats. The animals were pre-treated with SNE (100-1000 mg kg^{-1}) or phenobarbitone sodium (3-30 mg kg^{-1}) thirty minutes prior to the injection of PTZ. The control animals received 0.9 % saline solution (0.01 ml kg^{-1}). After the PTZ injection, the animals were placed in a testing chamber (made of perspex of dimensions 15x15x15 cm). A mirror angled at 45˚ below the floor of the chamber allowed a complete view of the convulsive event of PTZ. The behavior of the animals was captured with a camcorder (Everio^{TM} model GZ-MG 130U, JVC, Tokyo, Japan) placed directly opposite to

the mirror. The video recordings were later analyzed by tracking parameters including latencies to myoclonic jerks and clonic-tonic seizures and the duration of clonic-tonic seizures using Behavior Tracker Version 4.0 for Windows. The ED_{50} (a measure of anticonvulsant potency) was calculated by plotting the percent seizure inhibition of the drug to the vehicle-treated group. The mortality rate was also determined for each drug treatment group. The ability of a drug/extract to prevent the seizures or delay/prolong the latency or onset of the hind-limb tonic extensions was considered as an indication of anticonvulsant activity.

2.6.2 Picrotoxin-induced Seizures

In brief, clonic-tonic seizures were induced in drug or vehicle pre-treated male ICR mice (20-30g) by an intraperitoneal injection of 10 mg kg^{-1} picrotoxin into mice. The animals were pre-treated with SNE (30-300 mg kg^{-1}), or phenobarbitone sodium (3-30 mg kg^{-1}) thirty minutes prior to the injection of PIC. The control animals received distilled water (0.01 ml kg^{-1}, p.o). After the PIC injection, the animals were placed in the testing chamber and a video recording of the event made as described for the PTZ experiment. The video recordings were also later analyzed by tracking parameters including latencies to myoclonic jerks and clonic-tonic seizures and the duration of clonic-tonic seizures using Behavior Tracker version 4.0 for Windows. The ED_{50} (a measure of anticonvulsant potency) was calculated as indicated in the PTZ test. The mortality rate was also determined for each drug treatment group. The ability of a drug/extract to prevent the seizures or delay/prolong the latency or onset of the hind-limb tonic extensions was considered as an indication of anticonvulsant activity

2.6.3 Pilocarpine-induced Status Epilepticus

In this experiment, seizures were induced by an intraperitoneal injection of pilocarpine (350 mg kg^{-1}) of drug or vehicle –treated male rats. The rats were pre-treated with SNE (100-1000 mg kg^{-1}) or diazepam (1-10 mg kg^{-1}) thirty or fifteen minutes respectively before pilocarpine injection. To reduce peripheral autonomic effects produced by pilocarpine, the animals were pretreated with *n-butyl*- bromide hyoscine (1 mg kg^{-1}, 30 min before pilocarpine administration). After the injection of the pilocarpine, the animals were placed separately into the transparent plexiglass testing chamber and video recordings made as described in the PTZ- and PIC-induced seizure experiments. The latency to and duration of clonic-tonic seizures were

tracked using Behavior Tracker Version 1.5. The ED_{50} (a measure of anticonvulsant potency) was calculated.

2.6.4 Pentylenetetrazole-induced kindling

To induce kindling, a 35 mg kg^{-1} dose of PTZ was injected i.p. every 48 h into SNE or diazepam treated male Sprague-Dawley rats (200-300 g). After the PTZ injection, the rats were placed in a testing chamber (made of perspex of dimensions 15x15x15 cm^3). A mirror angled at 45˚ below the floor of the chamber allowed a complete view of the convulsive event of PTZ. The behavior of the animals was captured with a camcorder (JVC Hard Disk Camcorder, GZ-MG 130U) placed directly opposite to the mirror. Seizure intensities were classified according to the Racine score (Racine, 1972) as follows:

- ***Stage 0:*** no response
- ***Stage 1***: ear and facial twitching
- ***Stage 2***: convulsive waves throughout the body
- ***Stage 3:*** myoclonic jerks, rearing
- ***Stage 4***: turning over onto one side
- ***Stage 5***: turning over onto the back, generalized tonic-clonic seizures

In addition, the latency to the onset of myoclonic jerks was measured and analyzed. Each rat was considered fully kindled after showing stage 4 or 5 after two consecutive PTZ administrations. On day seven after kindling had been achieved, the rats were challenged with 35 mg kg^{-1} of PTZ and the entire event was also recorded. The ED_{50} (a measure of anticonvulsant potency) was calculated.

2.6.5 Lipid peroxidative assay in PTZ-Kindled rat brain homogenates

This test was done to assess the role of lipid peroxidation in PTZ- induced kindling and the beneficial role of SNE which has demonstrated in vitro antioxidant properties. On the following day after the 35 mg kg^{-1} PTZ challenge, the kindled rats were sacrificed by decapitation and forebrain was dissected out and homogenized (100 mg ml^{-1}) in ice-cold 0.1 M phosphate buffer (pH 7.4) using Ultra-Turrax T 25 homogenizer (IKA Labortehnic, Staufen,

Germany). Brain homogenate (2.5 ml) was mixed with 1 ml phosphate buffer and mixture was then incubated in an orbital shaker incubator (BoroLabs, Aldermaston Berkshire, EC) at 37 ˚C for 1 h.

To assay for thiobarbituric acid reactive substances (TBARS), 0.1ml of the incubated reaction mixture was taken into a test tube containing 1.5 ml of 10% trichloroacetic acid (TCA), and allowed to stand for 10 min. Then the tubes were centrifuged at 4000 rpm for 10 min. The supernatant was separated and mixed in a tube containing 1.5 ml of 0.67% thiobarbituric acid (TBA) in 20% acetic acid. The mixture was heated in a hot water bath at 85˚C for 1 h to complete the reaction and allowed to cool. The intensity of the pink-colored complex formed was measured at 535 nm in a spectrophotometer (Cecil CE 7200 spectrophotometer, Cecil Instrument Limited, Milton Technical Centre, England). The Absorbance decreases with increasing ability to inhibit lipid peroxidation. Phosphate buffer was used as blank throughout the experiment.

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

$$\% \text{ Inhibition} = \frac{(C_{PTZ} - C_O) - (FRM - C_O)}{(C_{PTZ} - C_O)} \times 100$$

Where:

1. C_{PTZ}: is the degree of lipid peroxidation in the kindled but untreated rats
2. **Full Reaction Mixture** (**FRM**): is the underlying lipid peroxidation of the treated rats
3. C_O:: is the absorbance of the unkindled and untreated rats

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

2.7 SKELETAL MUSCLE EFFECTS OF SNE

2.7.1 Rota-rod

The method used was adapted from the one described by Dunham and Miya (1957). The rotarod apparatus Rotamex-4/8 (Columbus Instrument, USA) consisted of a rotating bar suitably machined to provide grip. The equipment is micro-controller operated and connects to a computer via RS-232. It detects the fall of the animal tested using interruption in photobeam (IR beam). Its microstepping motor assures perfect speed stability. The standard Rotamex contains four lanes which can be expandable to eight lanes.

During the test, mice were selected and screened primarily at 12 rpm for four consecutive times (an hour interval) for a day. On day two, the speed was increased to 25 rpm and mice that could rotate on the rod for 600 sec were selected and grouped into six (three groups representing SNE (10, 100 and 1000 mg kg^{-1} , p.o), two for baclofen (1 and 10 mg kg^{-1} , i.p) and one as vehicle-treated. The mice, in their various groups, were trained for four consecutive days with four training sessions (at 1 h interval) per day. On the day of the experiment, each mouse was given a drug free rotation and thirty minutes later treated with SNE, baclofen or vehicle and tested at every thirty minutes for two and a half hours. The latency to fall was recorded as the time spent on the rotating rod until the mouse falls.

2.7.2 In vitro neuromuscular effect of SNE

Dose-responses to acetylcholine (0.1-50 µg ml^{-1}) were carried out on an isolated chick biventer-cervicis preparation in Krebs' solution as previously described (Ginsborg and Warriner, 1960). Dose-responses to acetylcholine were later repeated in the presence of each concentration of 1 and 2 mg ml^{-1} of SNE. Experiments continued until maximum or reducing responses were obtained for acetylcholine concentrations.

2.8 ANALGESIC EFFECT OF SNE

The neurobiology of neuropathic pain is similar to that epilepsy, thus certain antiepileptic drugs like carbamazepine and gabapentin are used currently in the management of neuropathic pain

(Jenson, 2002). The analgesic effect of SNE was done, using the formalin induced nociceptive assay and acetic acid induced writhing, to substantiate its traditional use as a painkiller and establish a beneficial use as anticonvulsant.

2.8.1 Formalin-induced nociception

The formalin test first described by Dubuisson and Dennis (Dubuisson and Dennis, 1977) was carried out as described by (Malmberg and Yaksh, 1992), with a few modifications. Each animal was assigned and acclimatized to test chambers (a perspex chamber 15 × 15 × 15 cm) for thirty minutes before formalin injection (Bardin and Colpaert, 2004). The mice were then pre-treated with the test drugs (30 min for i p. route and 1 h for oral route) before intraplantar injection of 20 µl of 5 % formalin. The animals were immediately returned individually into the testing chamber. A mirror angled at 45˚ below the floor of the chamber allowed a complete view of the paws. The behavior of the animals over one hour was captured with a camcorder (Everio^{TM} model GZ-MG 130U, JVC, Tokyo, Japan) placed directly opposite to the mirror and attached to a computer. The total time the animal spent licking or biting the injected paw was scored for 60 min, starting immediately after formalin injection.

In another experiment, naloxone (5 mg kg^{-1}) and caffeine (3 mg kg^{-1}) were administered intraperitoneally 15 min before the extract and morphine, and the injection of the formalin followed 30 min later for the morphine and 1 h for the extract. However, it must be noted that the intraperitoneal injections of these drugs may produce counter-irritant effects.

2.8.2 Writhing Assay

The acetic acid-induced abdominal writhing test was performed as described previously by Koster *et al.,* (1959) with slight modifications. The total number of writhings following intraperitoneal (i.p.) administration of 0.6% acetic acid (10 ml kg^{-1}) was recorded for 30 min, starting 5 min after the injection. The animals were pretreated with SNE (100-1000 mg kg^{-1}, p.o.) and diclofenac sodium (10-100 mg kg^{-1}, i.p) 30 min and 15 min respectively before acetic acid administration. Control animals were treated with normal saline solution (0.01 ml kg^{-1}). The animals were placed in a test chamber and the procedure recorded as was done for the formalin test. The total amount of time and frequency of writhe was scored for a period of 30 min following the injection of the acetic acid.

In another experiment, caffeine (3 mg kg^{-1}) was administered intraperitoneally 15 min before the extract (300 mg kg^{-1}) and diclofenac (30 mg kg^{-1}), and the injection of acetic acid followed 30 min and 1 h later for the diclofenac and extract respectively.

2.9 EFFECTS OF SNE IN MURINE ANXIETY MODELS

2.9.1 Elevated Plus Maze (EPM)

The method used was as described for rats Pellow *et al.,* (1985) with some modifications. The elevated plus maze was made from opaque Plexiglas. It consisted of two opposite open arms (15x5 cm) without side walls and two enclosed arms (15x5x30 cm), extending from a central square platform (5x5 cm). A rim of Plexiglas (0.5 cm in height) surrounded the perimeter of the open arms to provide additional grip and thus prevent the mice falling off (Rodgers and Johnson, 1995). The maze was elevated to the height of 80 cm from the floor and placed in a lit room (~750 lux). The animals were divided into ten groups of six animals each and received the extracts (100, 300 or 1000 mg kg^{-1}, p.o.), the vehicle or the standard reference drugs diazepam (0.1, 0.3 or 1 mg kg^{-1}, i.p.) or caffeine (Sigma, St. Louis, MO, USA) (10, 30 or 100 mg kg^{-1}, p.o.). Animals were placed individually in the central platform of the EPM for 5 min and their behavior recorded on a videotape with a digital camera placed 100 cm above the maze. The behaviour of the animals over the five minute period was captured with a camcorder (Everio^{TM} model GZ-MG 130U, JVC, Tokyo, Japan) placed 100 cm above the maze. Behavioural parameters were scored from the videotapes as follows: 1) number of closed and open arm entries (absolute value and percentage of the total number); 2) time spent in exploring the open and closed arms of the maze (absolute time and percentage of the total time of testing); 3) number of head-dips (absolute value and percentage of the total number) protruding the head over the edge of either an open (unprotected) or closed (protected) arm and down toward the floor; 4) number of stretch-attend postures (absolute value and percentage of the total number) the mouse stretches forward and retracts to original position from a closed (protected) or an open (unprotected) arm. An arm entry was counted only when all four limbs of the mouse were within a given arm.

2.9.2 Light Dark Box (LD)

The light-dark exploration test is typically used to more directly assess anxiety-related responses. This apparatus is based on the initial model described by Crawley (1981) and as modified by other workers (Belzung *et al.,* 1987). It consists of wooden boxes (45 cm longx30 cm wide x 30 cm deep), which are divided into two equal compartments by a wooden board with a 7x7 cm opening located centrally at the floor level, connecting the compartments. One compartment was painted black and covered with a wooden lid. The other box (not covered) was painted white and lit by a 60 W light bulb set 30 cm above the box. Mice were grouped and treated with drugs as described for the other behavioral tests described above. At the beginning of the experiment, mice were placed individually in the center of the illuminated box, facing the opening away from the dark compartment. Behaviors of the animals were recorded for 5 minutes with a digital camera placed 1 m above the box. Videotapes were scored as mentioned above for the following parameters: 1) frequency of compartment entries; 2) total time spent by mice in each compartment.

2.9.3 VersaMax animal activity monitoring system (VAMS).

The VersaMax Animal Activity Monitoring System (AccuScan Instrument Inc, USA) is equipment comprising of four animal monitor chambers (16 in x 16 in x 12 in) covered by transparent lids with perforations, an analyser and a computer. The base of each monitor chamber is lined with vertical and horizontal sensors. The behaviours to be measured are configured into the system. During the test period, any behaviour exhibited by the test animals through beam interruptions are transmitted to the analyser recorded on a computer. The data is later generated by a software and exported into an excel version. During an experiment, the system records the entire procedure as primary, secondary and auxillary in succession thus providing the researcher the opportunity to test the same animal under three different experimental conditions.

In the experiment conducted, a primary followed by a secondary session was done for 60 and 120 minutes respectively. The test animals (Balb/c male mice (20-30 g), were made to acclimatize by going through a two day procedure in the system without any drug administration. On the third day, after an initial 60 min primary session, the mice were

treated with SNE (10-1000 mg kg^{-1}, i.p) or distilled water, i.p (as a control) and tested for a 120 min secondary session.

The data obtained provided forty parameters out of which the following were analysed.

Behavioural parameter	**Definition of Parameter**
Horizontal Activity (HACTV)	The total number of beam interruptions that occurred in the horizontal sensor during a given sample period
Total Distance (TOTDIST)	Total distance traveled is the distance in centimeters traveled by the animal in a given sample period. It is a more accurate indicator of ambulatory activity.
Vertical Activity (VACTV)	The total number of beam interruptions that occurred in the vertical sensor during a given sample period
Stereotype number and duration	If the animal breaks the same beam (or set of beams) repeatedly then the monitor considers that the animal is exhibiting stereotypy. This typically happens during grooming, head bobbing, etc. This parameter corresponds to the number of times and the total amount of time the monitor observed stereotypic behavior in the animal.
Margin distance and duration(Thigmotaxis)	This parameter provides both the distance (in centimetres) travelled and the time (in seconds) spent by the animal in close proximity (within 1 cm) to the walls of the cage.
Center distance and duration	This provides the distance travelled and time spent by the animal while in the center of the cage.
LF Time	The time spent by the animal in the left-front corner of the cage
RF Time	Time spent by the animal in the right-front corner of the cage.
LR Time	Time spent by the animal in the left-rear corner of the cage.
RR Time	Time spent by the animal in the right-rear corner of the cage

2.10 ANTIOXIDANT AND FREE RADICAL SCAVENGING EFFECT OF S. NODIFLORA

2.10.1 Total Phenol Assay

The total soluble phenols present in the extracts (0.1-3 mg ml^{-1}) were quantitatively determined by colorimetric assay using the Folin-Cicocalteu's phenol reagent (Singleton and Slinkard, 1977) with some modifications. Tannic acid (0.01-0.3 mg ml^{-1}) was used as the reference drug.

Principle

Phosphomolybdate- phosphotungstate salts of the Folin-Cicocalteu's phenol reagent are reduced by the phenolic compounds in the alkaline medium giving a blue coloration, the intensity of which can be quantified spectrophotometrically at 760nm. Absorbance increases with increasing phenol content.

Experimental Procedure

The test drug (1 ml) was added to 1 ml of Folin-Cicocalteu's phenol reagent (Sigma, St. Louis, MO, USA) (diluted five folds in distilled water). The content of the test tube was mixed and allowed to stand for five minutes at 25 ^{o} C in the incubator. One millilitter of 2 % sodium bicarbonate solution was added to the mixture. The reaction mixture was then incubated at 25 ^{o} C. It was then centrifuged at 3.996 g-force for 10 min to obtain a clear supernatant. The absorbance of the supernatant was then determined at 760 nm using a Cecil CE 2040 spectrophotometer (Cecil Instrument Limited, Milton Technical Centre, England). Distilled water (1 ml) was added 1 ml Folin-Cicocalteu's phenol reagent and the mixture is processed similarly as the test drugs and used as the blank. All measurements were done in triplicates. Data obtained for the tannic acid was analyzed as linear regression of the absorbance against concentration from which tannic equivalents were obtained and the data obtained expressed graphically as graphs of concentration against the tannic acid equivalent (Total phenol content) using the GraphPad Prism for Windows version 4.02 (GraphPad Software, San Diego, CA, USA)

2.10.2 Total Antioxidant Capacity Assay

The antioxidant activity of the extracts (0.1-3 mg ml^{-1}) was evaluated by the phosphomolybdenum method (Prieto *et al.*, 1999). Ascorbic acid (0.01-0.3 mg ml^{-1}) was used as the reference drug.

Principle

The assay is based on the reduction of Mo (VI) to Mo (V) by the antioxidants and subsequent formation of a blue phosphate/Mo (V) complex in an acidic medium, the intensity of which is spectrophotometrically quantified at 695 nm. Absorbances increased with increasing antioxidant capacity.

Experimental Procedure

The test drug/ extract (0.3 ml) was combined with 3 ml of reagent solution (0.6M sulfuric acid, 28 mM sodium phosphate and 4mM ammonium molybdate) (BDH, Poole, England). The tubes containing the reaction solution were incubated at 95 °C for 90 min. The absorbance of the supernatant was then determined at 695 nm in a Cecil CE 2040 spectrophotometer (Cecil Instrument Limited, Milton Technical Centre, England).against blank after cooling to room temperature. Distilled water (0.3 ml) is processed as test/extract and used as the blank. All measurements were done in triplicates. Data obtained for the ascorbic acid was analyzed as linear regression of the absorbance against concentration from which ascorbic acid equivalents were obtained and the data obtained expressed graphically as graphs of concentration against the ascorbic acid equivalents (total antioxidant capacity) using the GraphPad Prism for Windows version 4.02 (GraphPad Software, San Diego, CA, USA)

2.10.3 Reducing Power

The reducing capacity of the extracts (0.1-3 mg ml^{-1}) in methanol was determined using the method of Fe^{3+}-reduction to Fe^{2+} (Oyaizu, 1986) as previously described (Amarowicz et al., 2005), with modifications using *n-propyl* gallate (0.001-0.03 mg/ml) as a reference antioxidant.

Principle

The method depends upon the ability of the test compound to reduce Fe^{3+} to Fe^{2+} . The resultant Fe^{2+} then reacts with ferricyanide ion to form a Prussian blue complex with maximum absorbance at 760 nm. The equations of the reducing power test are shown as follows;

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

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

The higher the reducing power, the greater the intensity of the blue complex and the higher the absorbance. Three replicates were used for each concentration.

Experimental Procedure

The drug/extract (1 ml) was mixed with 2.5 ml of 0.2 M sodium phosphate buffer (pH 6.6) and the 2.5 ml of 1 % potassium ferricyanide (BDH, Poole, England) solution in a test tube. The mixture was incubated at 50 ^{o} C for 20 min. Following this, 1.5 ml of 10 % trichloroacetic acid solution (TCA) was added to the incubated mixture, and centrifuged at 3.996 g-force for 10 min. The supernatant (2.5 ml) was then mixed with 2.5 ml distilled water and 0.5 ml of the 0.1 % ferric chloride solution ($FeCl_{3\,(aq)}$) in a test tube. The absorbance was then measured at 700 nm using Cecil CE 2040 spectrophotometer (Cecil Instrument Limited, Milton Technical Centre, England). The determination was repeated replacing the drug/extract with 1 ml distilled water. This was used as the blank. Three replicates were used. Data was presented as concentration- absorbance curves and the EC_{50} (concentration that gives 50% of maximal response) computed using GraphPad Prism for Windows version 4.02 (GraphPad Software, San Diego, CA, USA)

2.10.4 Free Radical Scavenging Assay

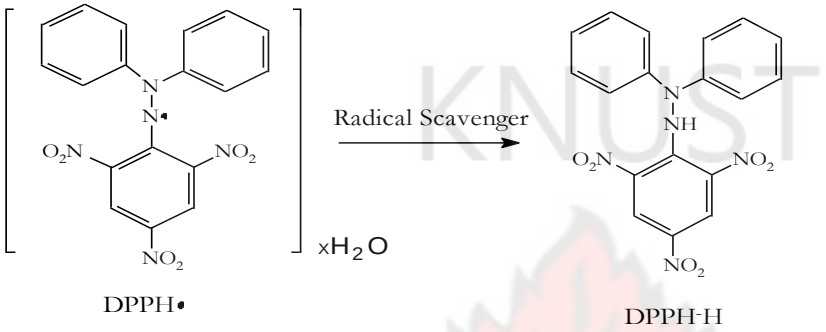
The experiment was carried out as previously described by Govindarajan (2003) with a few modifications.

Principle

The 1, 1-diphenyl -1-picrylhydrazyl hydrate (DPPH) a stable radical with characteristic violet colour (and maximum absorption at 517 nm) accepts an electron or hydrogen in the presence of a suitable free scavenger (reducing agent) to form reduced 2, 2-diphenyl -1-picrylhydrazyl,

which is yellow in colour. The residual DPPH is then determined at 517 nm in a spectrophotometer. The absorbance decreases with increasing free radical scavenging ability. The % DPPH scavenging effect of the antioxidant (% of control) is calculated as follows:

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



The absorbance decreases with increasing free radical scavenging ability.

Experimental procedure

The extracts (0.1-3 mg/ml in methanol) were compared with *n-propyl* gallate (0.001-0.03 mg/ml in methanol) as a reference free radical scavenger. The test drug/extract was centrifuged at 3000 rpm for 10 min and the supernatant collected. The supernatant (1 ml) was added to3 ml of methanolic solution of DPPH (20 mg l^{-1}) in a test tube. The reaction mixture was kept at 25 °C for 1 h in an orbital shaker (BoroLabs, Aldermaston Berkshire, EC). The absorbance of the residual DPPH was determined at 517 nm in a Cecil CE 2040 spectrophotometer (Cecil Instrument Limited, Milton Technical Centre, England). Methanol (99.8%) (1 ml) was added to 3 ml DPPH solution, incubated at 25 °C for 1 h and used as control. Methanol was used as the blank. Three replicates were used. The results were expressed as % DPPH scavenging effect against concentration and the EC_{50} determined. All data was computed using the GraphPad Prism for Windows version 4.02 (GraphPad Software, San Diego, CA, USA). Levels of significance were determined by analysis of variance (ANOVA).

2.10.5 Lipid Peroxidation

The method of Mitsuda (1967) was used with slight modification. The extract (0.1-3 mg ml^{-1} in 96% ethanol) was compared with *n*-propyl gallate (0.001-0.03 mg ml^{-1} in 96% ethanol) as a reference antioxidant. Briefly, a mixture of the sample (2 ml), 2.052 ml of 2.51% linoleic acid(BDH, Poole, England) (in 96% ethanol), 4 ml of 0.05M phosphate buffer (pH 7.0) and 1.890 ml of distilled water was put into a test tube with a screw cap and placed in an oven at 40° C in the dark for seven (7) days. To 2ml of the sample solution, which was prepared and incubated as described above, was added 2 ml of 20% trichloroacetic acid (aq) solution and 1 ml of 0.6 % thiobarbituric acid (aq) solution. This mixture was placed in boiling water bath for 10 min and after cooling, was centrifuged at 3000 rpm for 10 min. The absorbance of the supernatant was measured.

The % inhibition of linoleic acid autoxidation was calculated as follows;

$$\% \text{ Inhibition} = \frac{(C_0 - C_1) - (D - D_0 - C_0)}{(C_0 - C_1)} \times 100$$

Where

$\mathbf{C_0}$ (Full Reaction Mixture): is the degree of lipid peroxidation in the absence of antioxidant.

$\mathbf{C_1}$: is the underlying lipid peroxidation before the initiation of lipid peroxidation.

D: is any absorbance produced by the extract or the drug being tested.

$\mathbf{D_0}$ is the absorbance of the extract/drug alone

2.11 TOXICITY AND SIDE-EFFECTS OF SNE

2.11.1 Acute Toxicity

Mice were treated with SNE (100-10,000 mg kg^{-1}, p.o) and then placed inside the monitoring cage of an automated activity cage system connected to a computer. Each dose-level group consisted of five mice. The vertical, horizontal activity and the total distance covered were recorded for three hours and lethality observed 24 hr after the treatment. Mortality in mice was assessed 24 h post drug treatment. The LD_{50} was then calculated.

2.11.2 Pentobarbitone-induced sleeping time

Pentobarbitone (40 mg kg^{-1}) was injected intraperitoneally into extract/drug/vehicle- treated mice 30 min after the administration of SNE (30-300 mg kg^{-1}) orally, or diazepam 1 mg kg^{-1}, intraperitoneally. The onset time and duration of sleep were recorded.

2.12 STATISTICS

The ED_{50} (dose responsible for 50 % of the maximal effect) and inhibitory effects of drugs were analyzed by using an iterative computer least squares method, GraphPad Prism for Windows version 5.0 (GraphPad Software, San Diego, CA, USA) with the following nonlinear regression (four-parameter logistic equation).

$$Y = \frac{a + (a - b)}{1 + 10^{((Log\,ED_{50} - X) \times Hill\ Slope)}}$$

Where, X is the logarithm of concentration. Y is the response and starts at ***a*** and goes to ***b*** with a sigmoid shape.

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

Levels of significance were determined by analysis of variance (ANOVA) and Student-Newman-Keuls or Bonferroni's post test using GraphPad Prism (Version 5). All values were expressed as mean ± s.e.m. $P < 0.05$ were considered significant.

Chapter 3

RESULTS

3.1 PHYTOCHEMICAL SCREENING

Phytochemical analysis of the extract revealed the presence of glygosides, saponins, anthracene glycosides, sterols, alkaloids, tannins and pseudotannins, whereas cyanogenetic and cardiac glycosides were absent.

Table 5: Phytochemical screening of the hydro-ethanolic extract of *S. nodiflora*

Test	Observation	Inferences
Glycoside		
General Test	Brick red precipitate	Glycoside may be present
Saponin Test	Formation of stable froth after vigorous shaking	Saponin may be present
Cyanogenetic test	Discoloration of the sodium picrate paper after warming for 30 minutes	Absence of Cyanogenetic glycosides
Anthracene Test (modified BornTrager's test)	Heavy precipitate formed with brown colour	Anthracene glycoside may be present
Cardiac test		
1. Kedde's test for cardenolides	Colourless solution formed	Cardiac glycoside may be absent
2. Keller- Kiliani for digitose	Colourless solution formed in the acetic layer.	Cardiac glycoside may be absent(2-deoxysugar)
3. Liebermans test for sterols	Violet to blue colour produced	Sterols may be present

Alkaloids		
Dragendorff's Reagent Test	Orange-red precipitate produced	Alkaloids are present
Mayer's Reagent Test	White buff precipitate formed	Alkaloid are present
10% Tannic Acid Test	Brownish precipitate formed	Alkaloids are present
N/10 Iodine Test	No precipitate formed	Alkaloids are may be absent
Tannins		
1% Ferric Chloride Test	Green coloration	Condensed tannins present
1% Gelatin Test	White buff /creamy precipitate formed	Tannins present
1% Lead Acetate Test	White/cloudy precipitate	Tannins present
Pseudotannins		
Catechin Test	White match stick blackened upon gentle warming	Pseudotannins present
Chlorogenic Acid Test	No development of green colour upon exposure to air	Pseudotannins present

3.2 ANTICONVULSANT EFFECT

3.2.1 Pentylenetetrazole (PTZ)-induced Seizures

PTZ induced a sequence of events starting with myoclonic jerks which was then followed by an intense clonic-tonic convulsive phase. The extract, SNE, showed significant anticonvulsant effect against seizures induced by PTZ. SNE, dose dependently, increased both the onset of the myoclonic jerks ($F_{3,20}$=1.481, *P*=0.250, Fig. 11) and latency to myoclonic seizures and this effect was significant at the 1000 $mgkg^{-1}$ ($F_{3,20}$=3.999, *P* =0.0230, Fig. 11). SNE reduced the duration of seizures at all the dose levels used, however this effect was not significant compared to the vehicle treated group and not show dose dependency ($F_{3,20}$=4.358, *P* =0.0162,

Fig. 12). The protective index (PI) of SNE against this acute model of seizure was high (Table 8). Phenobarbitone, the reference anticonvulsant used, delayed both the onset of myoclonic jerks ($F_{3,20}$=3.818, P = 0.0259, Fig. 11) and myoclonic seizures ($F_{3,20}$=25.85, P < 0.0001, Fig. 11) and also reduced the duration of the myoclonic seizures ($F_{3,20}$=27.04, P <0.000, Fig. 12). The anticonvulsant effect of SNE was less compared to phenobarbitone as provided by the EC_{50} values, with the diazepam being the highest (Table 7).

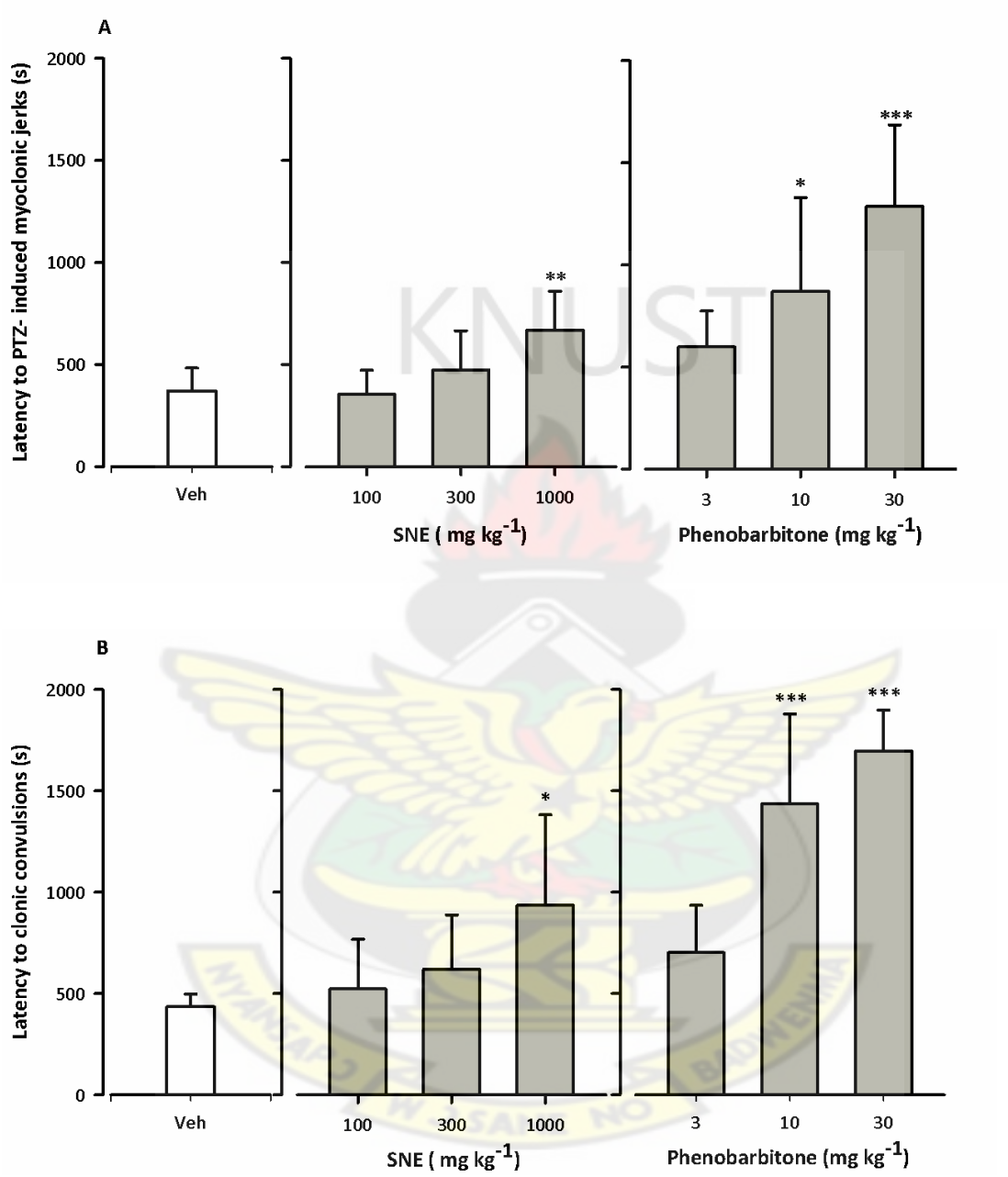


Figure 11: Effects of SNE (100-1000 mg kg^{-1}) and phenobarbitone (PHB) (3-30 mg kg^{-1}) on the latencies to myoclonic jerks and seizures induced by PTZ. Each column represents the mean ± S.E.M. n=5. *P < 0.05; ** P < 0.01; ***P < 0.001 compared to vehicle-treated group (One-way ANOVA followed by Newman-Keul's *post hoc* test)

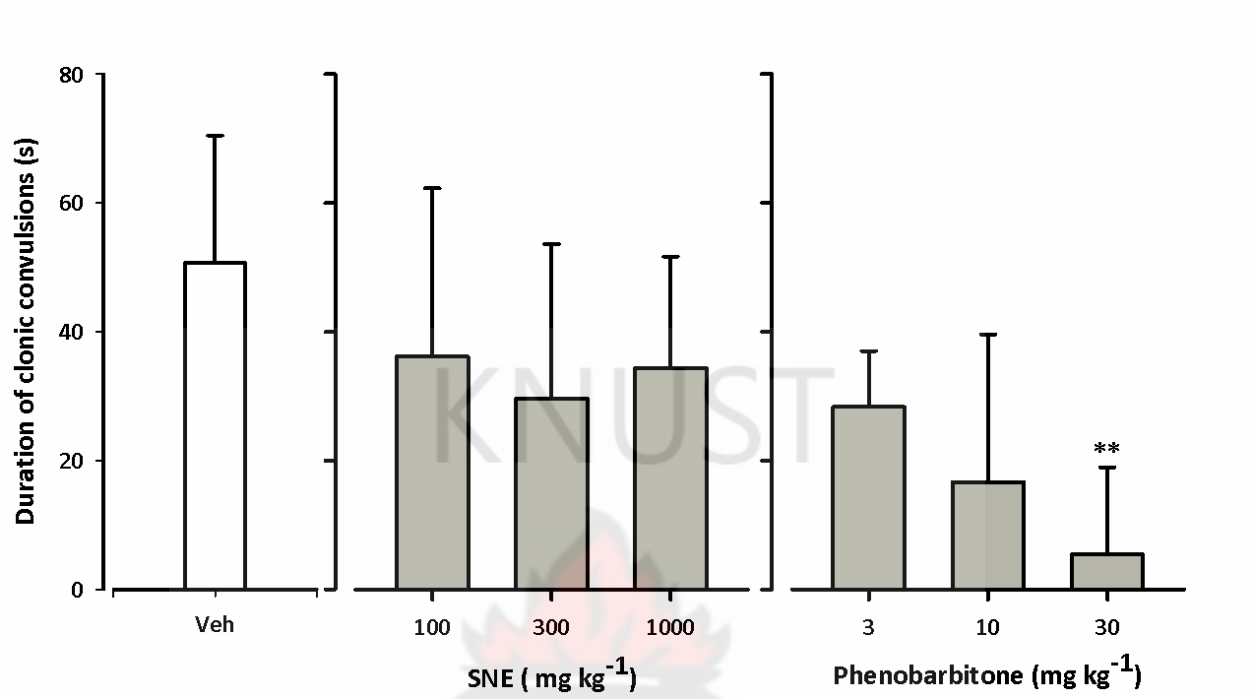


Figure 12: Effects of SNE (100-1000 mg kg^{-1}) and phenobarbitone (3-30 mg kg^{-1}) on the total duration of the seizures induced by PTZ. Each column represents the mean ± S.E.M. n=5. ** $P <$ 0.01 compared to vehicle-treated group (One-way ANOVA followed by Newman-Keul's *post hoc* test)

3.2.2 Picrotoxin - induced Seizures

Picrotoxin induced a tonic-clonic convulsive episode preceeded by myoclonic jerks in mice used. The extract showed significant anticonvulsant effect against the seizures induced by picrotoxin. SNE significantly and dose-dependently delayed the latencies to myoclonic jerks ($F_{4,20}$= 6.959, P =0.0011, Fig. 13) and tonic-clonic seizures induced by picrotoxin ($F_{4,20}$= 7.111, P =0.0010, Fig. 13). SNE also reduced the duration of seizures significantly. ($F_{4,20}$= 15.26, P <0.0001, Fig. 14). SNE provided 0% (at 30 mg kg^{-1}), 20% (at 100 mg kg^{-1}), 40% (at 300 mg kg^{-1}) and 60% (at 1000 mg kg^{-1}) protection against the picrotoxin-induced seizures. The protective index of SNE against picrotoxin- induced seizures was high (table 8). SNE significantly reduced the total frequency of seizures from 30- 300 mg kg^{-1}, with this effect, reduced, yet statistically insignificant at the 1000 mg kg^{-1}. Likewise, phenobarbitone

significantly and dose-dependently delayed the latencies to myoclonic jerks ($F_{3,16}$= 4.593, *P* =0.0167, Fig. 13) and tonic-clonic seizures induced by picrotoxin ($F_{3,16}$= 7.682, P=0.0021, Fig. 13). Phenobarbitone also reduced the duration of seizures significantly. ($F_{3,16}$= 6.208, *P* =0.0053, Fig.14). Phenobarbitone provided 0% (at 3 mg kg^{-1}), 60% (at 10 mg kg^{-1}), and 80% (at 30 mg kg^{-1}) protection against the picrotoxin induced seizures. However, the anticonvulsant effect of SNE was less potent compared to phenobarbitone as depicted by the EC_{50} values (table 7).

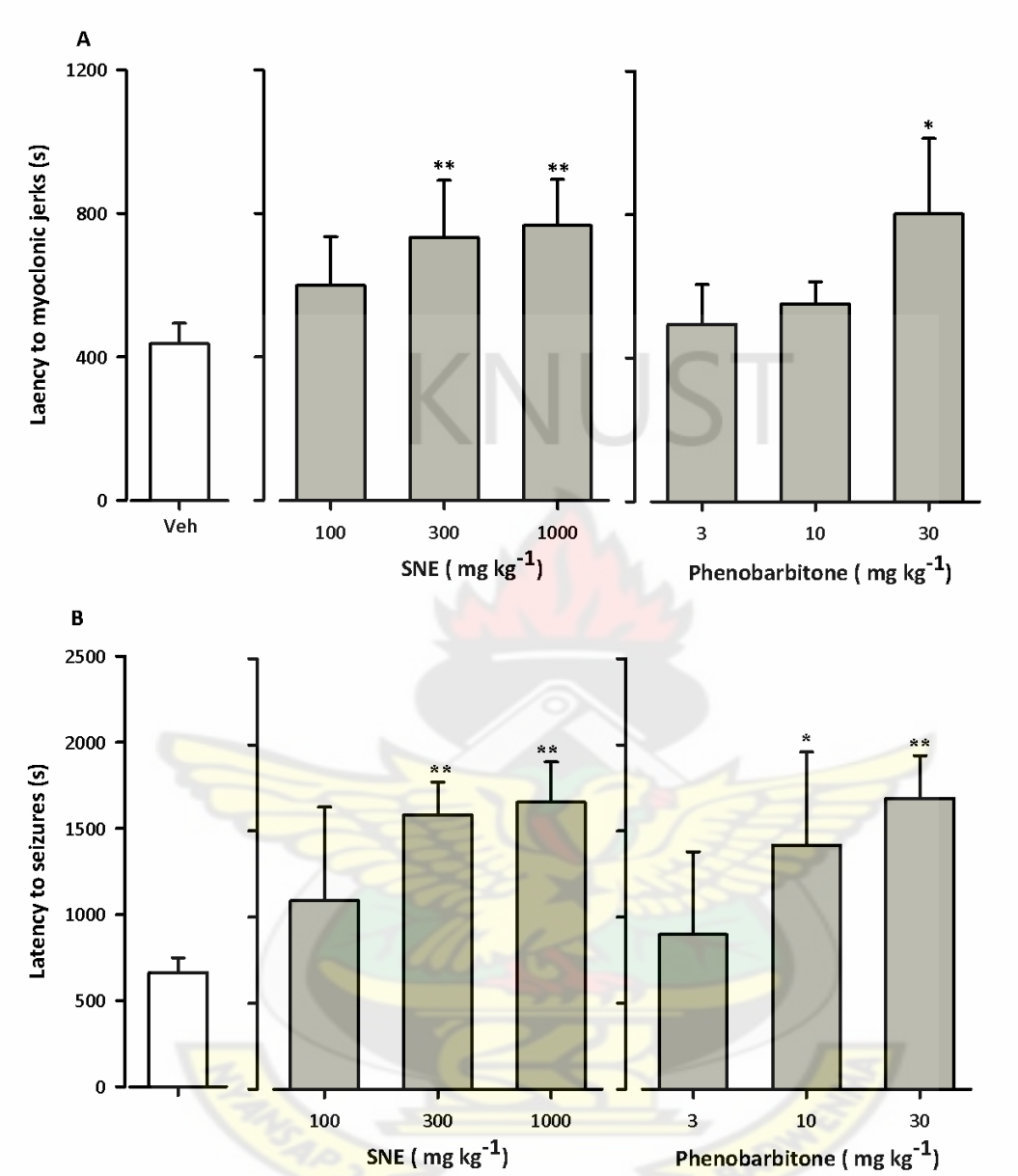


Figure 13: Effects of SNE (100-1000 mg kg^{-1}) and phenobarbitone (3-30 mg kg^{-1}), on the latency to myoclonic jerks and seizures induced by picrotoxin. Each column represents the mean ± S.E.M. n=5. *$P < 0.05$; ** $P < 0.01$; compared to vehicle-treated group (One-way ANOVA followed by Newman-Keul's *post hoc* test)

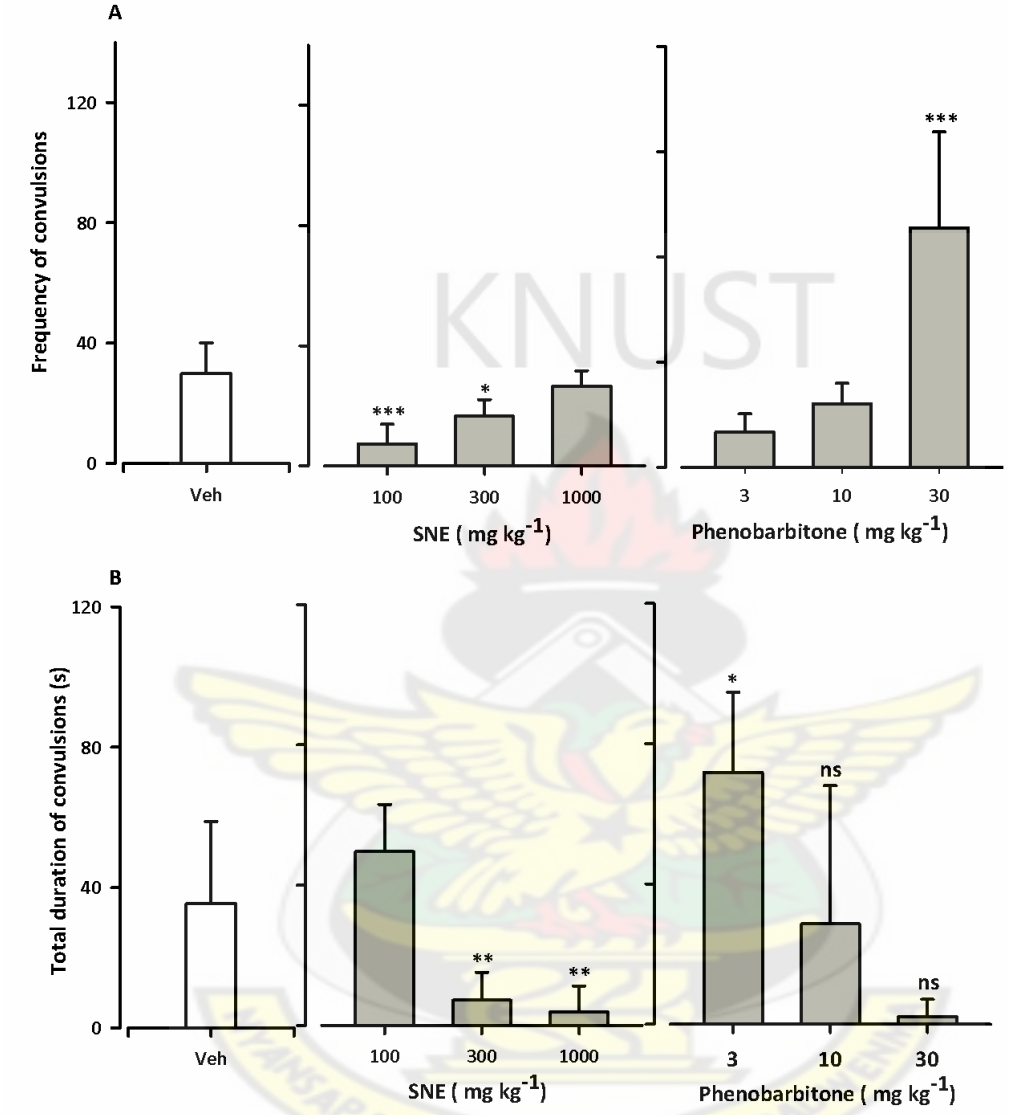


Figure 14: Effects of SNE (100-1000 mg kg^{-1}) and phenobarbitone (3-30 mg kg^{-1}) on the frequency (A) and duration (B) of the seizures induced by picrotoxin. Each column represents the mean ± S.E.M. n=5. $^{ns}P > 0.05$; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ compared to vehicle-treated group (One-way ANOVA followed by Newman-Keul's *post hoc* test)

3.2.3 Pilocarpine-Induced Status Epilepticus

Pilocarpine induced clonic convulsive episodes preceeded by myoclonic jerks in the mice. Both SNE and diazepam produced no significant effect on the latency to first myoclonic jerks compared to the vehicle treatment on the mice (SNE, [$F_{3,20}$= 0.5353, P <0.05] and phenobarbitone, ($F_{3,20}$= 0.5353, P <0.05) respectively (Fig. 15). SNE, however, dose dependently reduced the total duration of seizures ($F_{3,20}$= 7.235, P <0.001) in the mice treated. Diazepam, however, dose dependently and significantly, reduced the total duration of seizures with the 3 and 10 mg kg^{-1} dose levels completely inhibiting seizure formation ($F_{3,20}$= 26.78, P < 0.0001, Fig. 15).

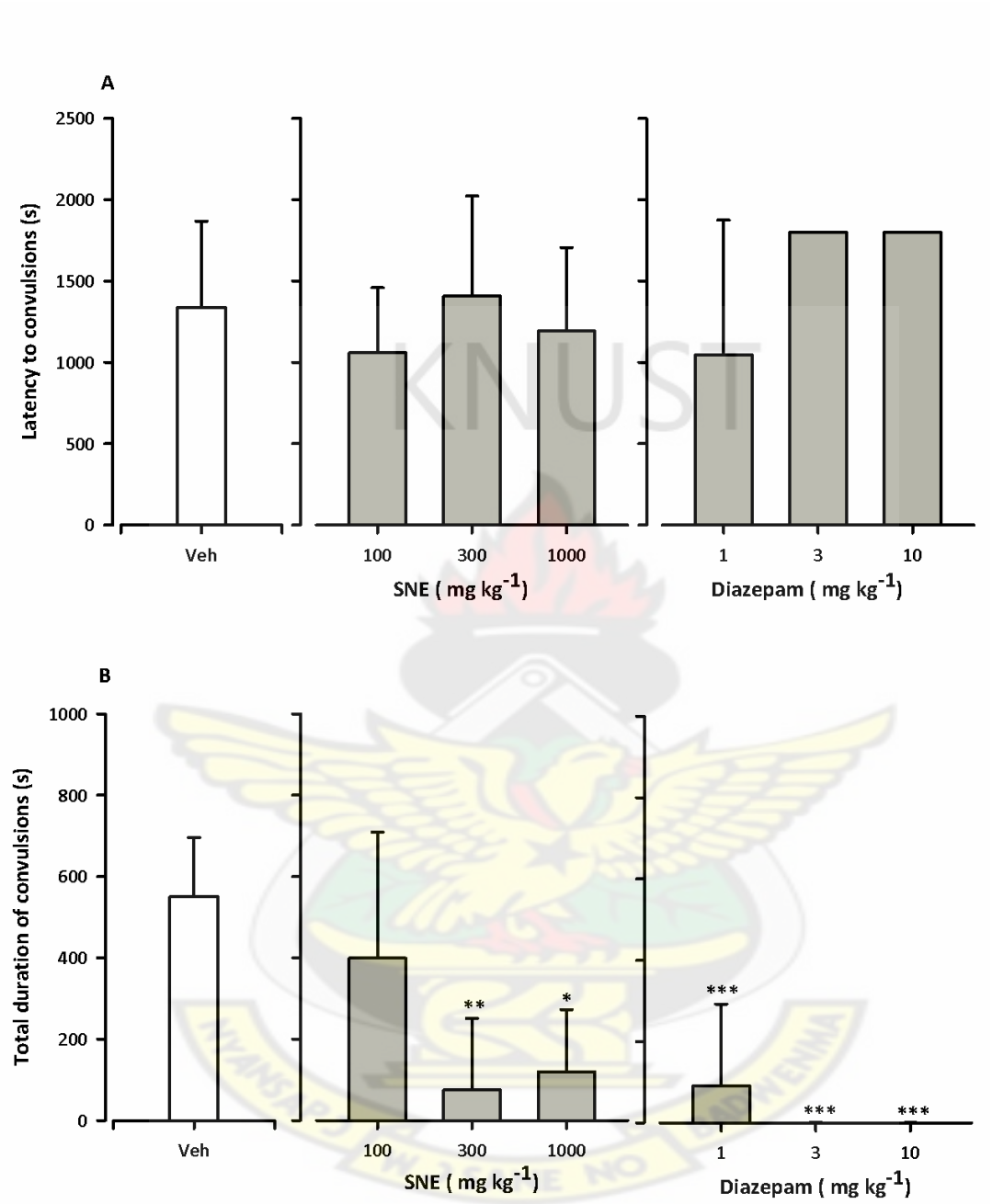


Figure 15: Effects of SNE (100-1000 mg kg^{-1}) and diazepam (1-10 mg kg^{-1}) on the latency to and total duration of seizures induced by pilocarpine. Each column represents the mean ± S.E.M. n=6. *P < 0.05; ** P < 0.01; ***P < 0.001 compared to vehicle-treated group (One-way ANOVA followed by Newman-Keul's *post hoc* test).

3.2.4 Pentylenetetrazole- Induced Kindling

In the vehicle treated group, repeated administration of 35 mg kg^{-1} of PTZ on alternate days caused a gradual increased in convulsant responses as scored by the Racine scale. By the 7^{th} day, the score had increased from 0 to 3, reaching a peak severity on the Racine score of 5 by the 15^{th} day which was maintained for the remaining duration (~2 weeks) of the study (Fig. 16). SNE significantly suppressed the kindled seizure at all the dose levels used ($F_{3,16}$ =11.48, *P*= 0.0003) (Fig. 16a), but this effect was statistically significant at both 100 and 1000 mg kg^{-1} as none of the animals in these treatment groups achieved seizure score 5 even after 16 injections of PTZ 35 mg kg^{-1} for 32 days. The % severity of seizures (as calculated from the AUC) shows that SNE attenuated PTZ kindling seizure activity by reducing the five scaled score from 50% to as low as 30% (i.e from 100-1000 mg kg^{-1}). Diazepam produced a significant dose- dependent suppression of the kindled seizure activity ($F_{3,16}$ =224.10, *P*< 0.0003, Fig.16c) and the percent severity of seizures was significant reduced from 30% (for 0.1-0.3 mg kg^{-1}), to 20% at 1 mg kg^{-1} (Fig. 16d).

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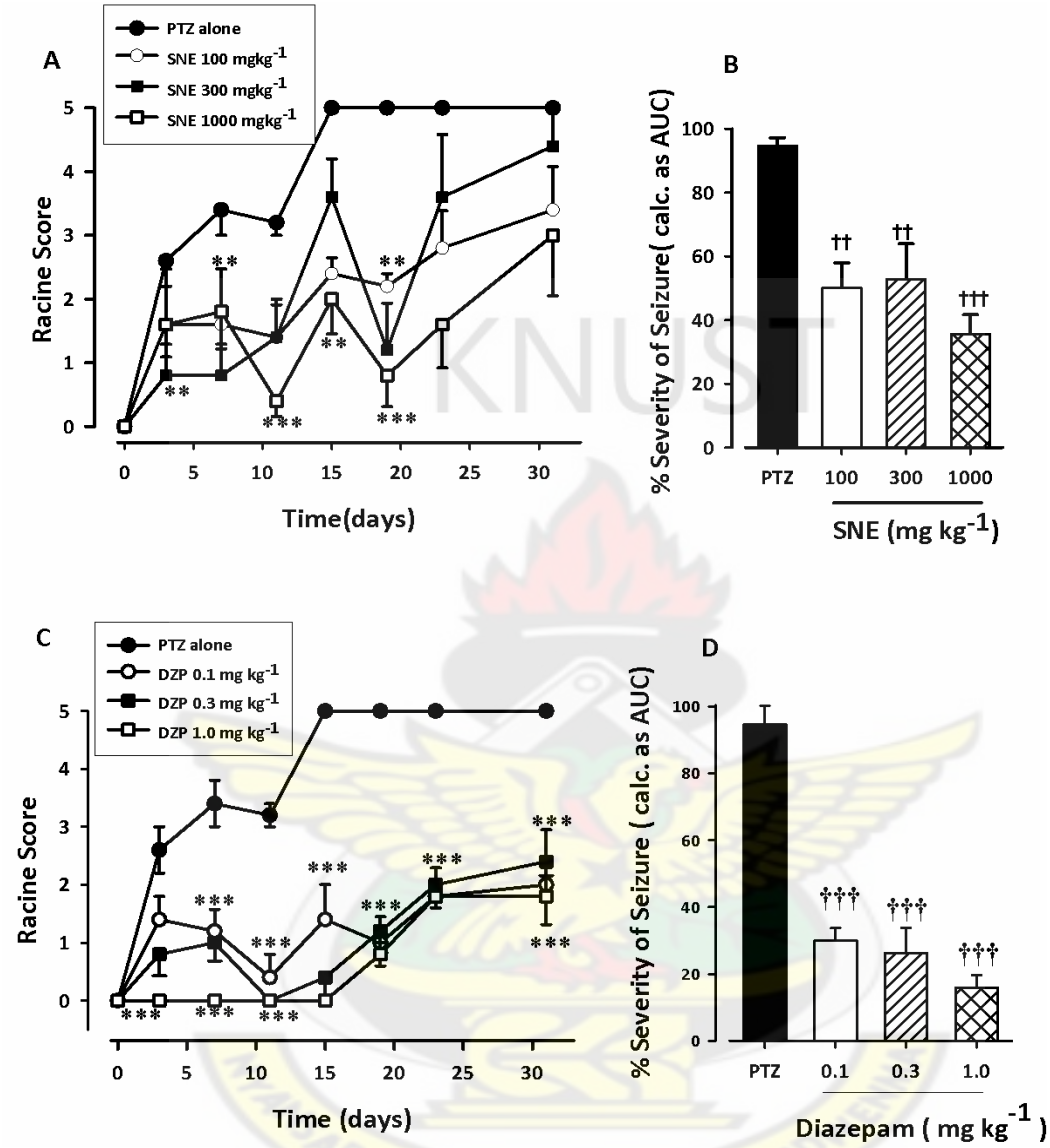

Figure 16: The dose-response effects of SNE (100-1000 mg kg^{-1}) (A and B) and DZP (0.1-1.0 mg kg^{-1}) (C and D) on the PTZ-induced kindling in rats. The left panels show the time course of effects over the 32 day period and the right panels show the percent severity of seizures calculated from the AUCs for the test duration. Values are means ± s.e.m. (n=5). ** $P < 0.01$, ***$P < 0.001$ compared to vehicle-treated group (Two-way ANOVA followed by Bonferroni's *post hoc* test). $^{\dagger\dagger}P<0.05$, $^{\dagger\dagger\dagger}P<0.001$ compared to vehicle-treated group (One-way ANOVA followed by Newman-Keul's *post hoc* test)

3.2.5 Lipid peroxidative assay in PTZ-Kindled rat brain homogenates

SNE (100-1000 mg kg^{-1}) dose-dependently inhibited lipid peroxidation in the PTZ-kindled rats whereas diazepam (0.1-1.0 mg kg^{-1}) gave an opposite effect (Fig 17)

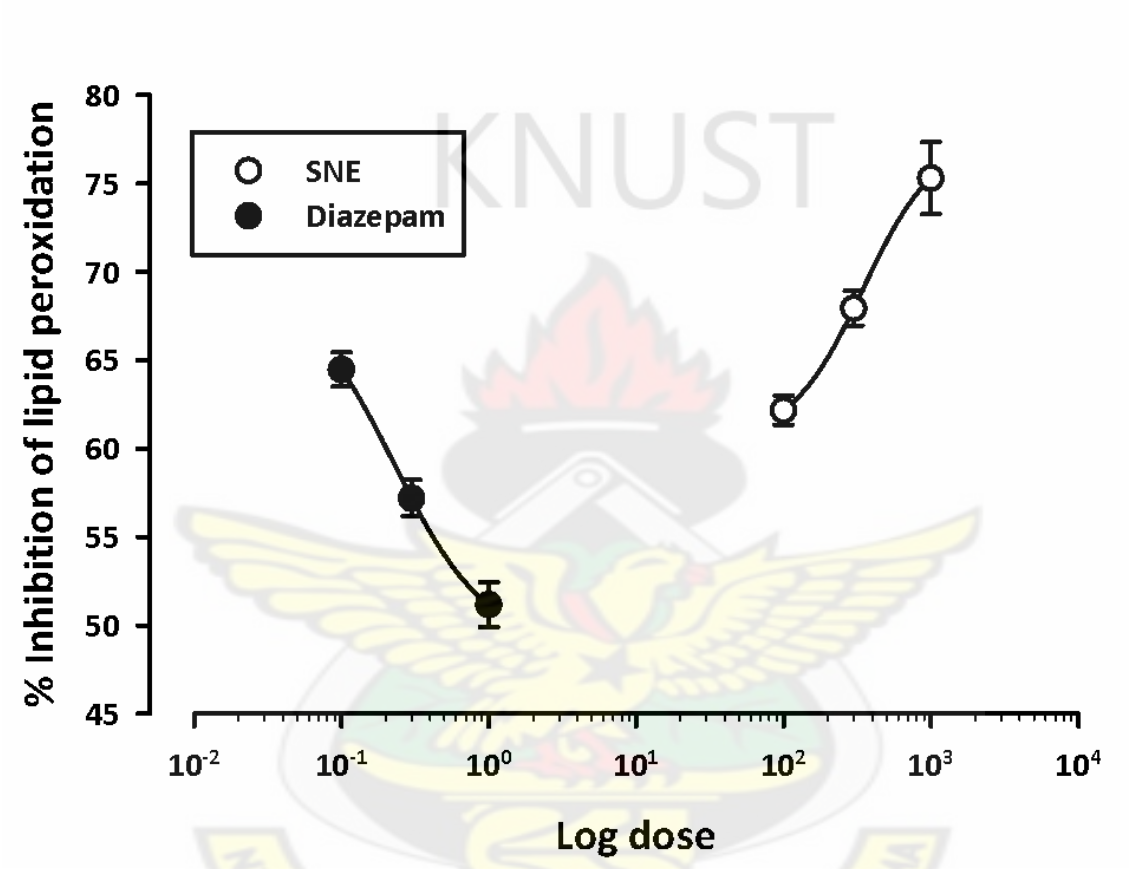

Figure 17: Percentage inhibition of PTZ induced lipid peroxidation in PTZ kindled rats by SNE (100-1000 mg kg^{-1}) and diazepam (0.1-1.0 mg kg^{-1}). Each point represents the mean ± s.e.m (n = 5).

Table 6: EC_{50} (mg kg^{-1}) of SNE, diazepam and phenobarbitone in pentylenetetrazole (PTZ), picrotoxin and pilocarpine induced seizures and PTZ-kindling in murine models of seizure

Drug	**EC_{50} (mg kg^{-1})**			
	PTZ	*Picrotoxin*	*Pilocarpine*	*PTZ kindling*
SNE	164.5	116.5	101.0	989.4
Diazepam			1.320	0.506
Phenobarbitone	5.983	9.820		

Table 7: The Protective Index (PI) of SNE against PTZ-, picrotoxin-, and pilocarpine- induced seizures and PTZ-kindling in murine models of epilepsy

Drugs	**Protective index**			
	Picrotoxin	*PTZ*	*PTZ-kindling*	*Pilocarpine SE*
SNE	6.556	4.660	0.775	7.589
Phenobarbitone	2.5*	5.61-7.7*	-	-

*(Borowicz *et al.*, 2004; White *et al.*, 2008; Yamashita *et al.*, 2004)

3.3 IN VIVO AND IN VITRO EFFECTS OF SNE ON SKELETAL MUSCLE TONE

3.3.1 Rota-Rod

Figure 18 presents the effect of treatment with SNE and baclofen on the motor coordination in the Rota-rod test. The administration of SNE, thirty minutes after the initial zero reading, produced no significant difference between the vehicle treated and the 10 and 100 mg kg^{-1} during the two–hour period of the experiment. SNE 1000 mg kg^{-1}, however, produced a significant skeletal muscle relaxant effect as the time spent on the rota-rod at 24 rpm over the two-hour period was decreased ($F_{3,16} = 34.54$, $P < 0.0001$) (Fig. 18 a). Analysis of the AUC also revealed a similar trend (Fig. 18 b). Baclofen 1 mg kg^{-1} produced no significant effect on the skeletal muscle whereas the 10 mg kg^{-1} produced a significant effect ($F_{2,12} = 5.908$, $P = 0.00164$) (Fig. 18c). An analysis of the AUC showed the same effect (Fig.18 d)

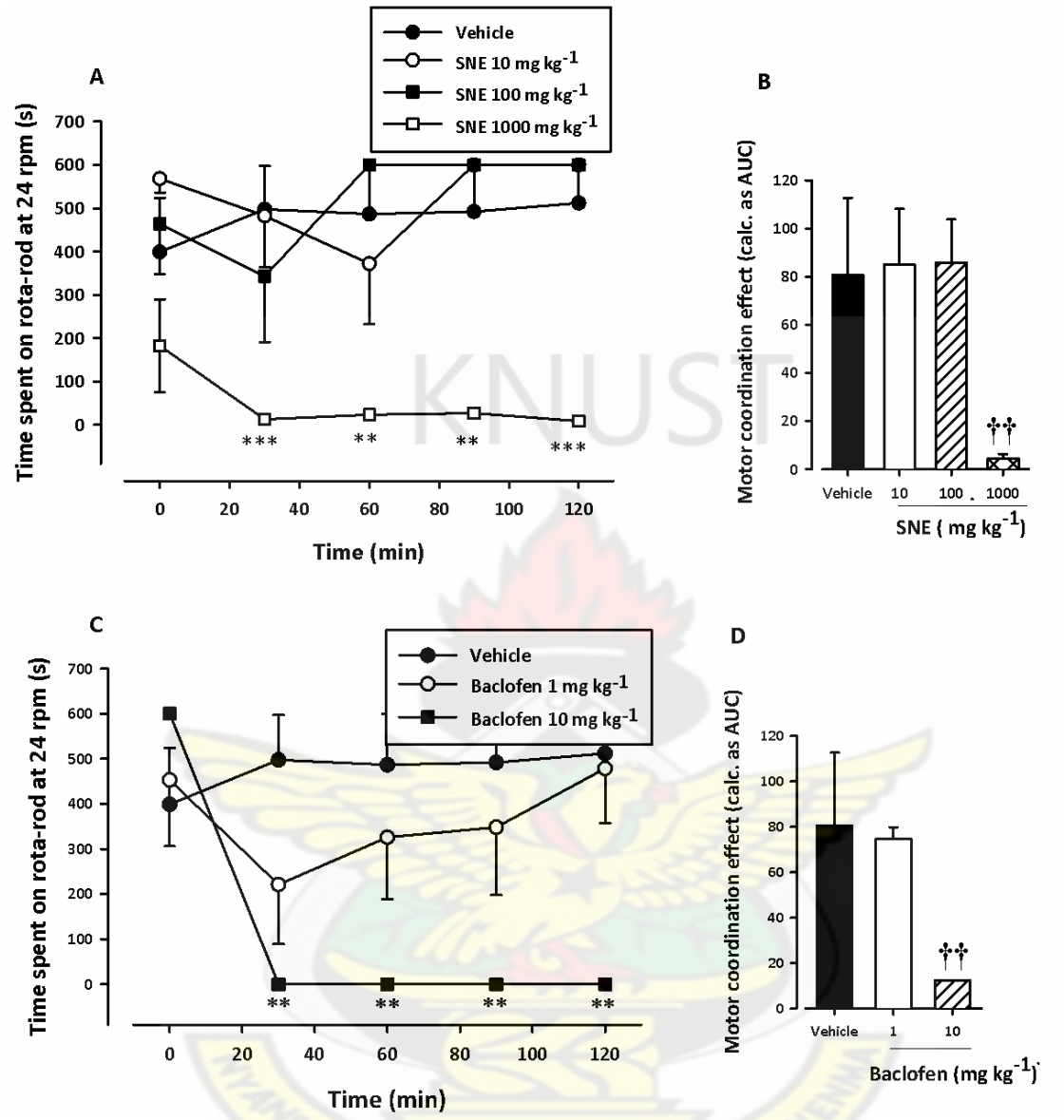

Figure 18: The effect of SNE (10-1000 mg kg^{-1}, p.o) and Baclofen (1-10 mg kg^{-1}, i.p) on the skeletal muscles of pretreated mice as measured using rotamex. Graph 'A' shows the time course of the effects over a 120 min period and 'B' the skeletal muscle effect calculated from the AUCs over the 120 min period. Data are Means ± s.e.m (n = 5). ***P < 0.001; ** P < 0.01; *P < 0.05; $^{\dagger\dagger}P$ < 0.01 compared to vehicle-treated group (one-way ANOVA followed by Neuman-Keul's post hoc test)

3.3.2 In vitro neuromuscular effect

SNE produced dose-dependent antagonism on the acetylcholine- induced contractions as shown in figure 19. The EC_{50} (mg ml^{-1}) values obtained for acetylcholine (Ach) alone and when antagonized with 1 and 2 mg ml^{-1} SNE are 1.267 x 10^{-5}, 0.023 and 0.004 respectively.

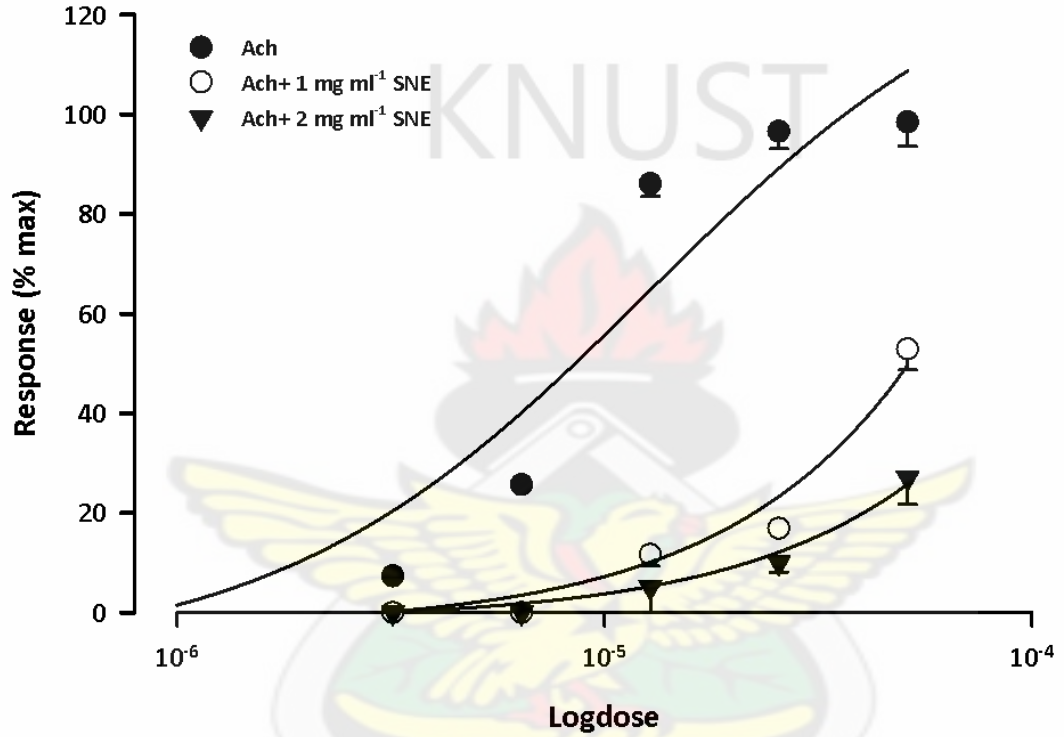

Figure 19: Effect of SNE (1-2 mg ml^{-1}) on acetylcholine (Ach)-induced contraction of chick biventer-cervicis preparations. Plots represent inhibition of Ach responses by SNE. Values are means ± s.e.m. (n=5)

3.4 ANALGESIC EFFECT

3.4.1 Formalin test

Formalin induced the characteristic nociceptive response ($F_{1,88}$ =469, P<0.0001) exhibited as biting or licking of the injected paw. The response to pain was biphasic as previously reported (Dubuisson and Dennis, 1977; Wheeler-Aceto *et al.*, 1990), consisting of an initial intense response to pain beginning immediately after formalin injection and rapidly decaying within 10 min after formalin injection (first phase). This was then followed by a slowly rising but longer-lasting response from10-60 min after formalin injection with maximum effect at approximately 20-30 min after formalin injection (second phase) (Hayashida *et al.*, 2003; Wang *et al.*, 1999).

Generally, responses to pain (defined by pain scores) were lower in the drug- treated groups than the vehicle-treated group as shown by the time course curves (Fig.20-21). Oral administration of SNE (100-1000 mg kg^{-1}; p.o.) 30 min before formalin injection significantly and dose-dependently inhibited both first and second phases of formalin-induced paw licking and biting ($F_{3,20}$ = 26.31; P<0.0001 and $F_{3,20}$ = 17.21; P <0.0001, respectively, two-way ANOVA (treatment group x time), Fig. 20a.). Analysis of the AUCs showed that SNE, at the doses used, attenuated formalin-induced pain/behaviours by 72.51-89.65 and 68.11-89.90% in the early and late phases respectively (Fig. 20b). Similarly morphine (1-10 mg kg-1; i.p.), an opioid agonist significantly attenuated the formalin-induced biting/licking in both the first and second phases ($F_{3,20}$ = 26.31; P <0.0001 and $F_{3,20}$ = 17.21; P <0.0001, respectively, two-way ANOVA (treatment group x time)) in a dose-dependent manner (Fig. 21a). One-way ANOVA followed by Bonferroni's post hoc test also revealed a significant dose-dependent decrease in total responses in the first and second phases in the presence of morphine ($F_{3,20}$ = 8.60; P = 0.0007 and $F_{3,20}$ = 8.06; P = 0.001, respectively, Fig. 21b). Also, intraperitoneal administration of diclofenac (10-100 mg kg^{-1}, i.p.) dose-dependently decreased the nocifensive behaviors induced by formalin in the first and second phases ($F_{3,20}$ = 3.22; P = 0.0446 and $F_{3,20}$ = 22.41.4; P = 0.0001, respectively, two-way ANOVA (treatment group x time),Fig. 22a). Percentage inhibitions as shown by the AUCs were 5.01-43.43% in the first phase and 79.28-93.42% in the

second phase (Fig. 22b). Comparison of ED_{50} obtained by non-linear regression (Table 9), revealed that both the extract and morphine were equipotent in both phases ($F_{1,32}$ = 0.175, P = 0.68 and $F_{1,32}$ = 0.279, P =1.212, respectively). By contrast, diclofenac was about seven fold more potent in the second phase compared to the first phase ($F_{1,32}$ = 7.982, P = 0.0081).

Figure 24 shows the effect of non-selective adenosine inhibitor, caffeine and naloxone, an opioid antagonist on the anti-nociceptive effects of SNE and morphine on formalin-induced nocifensive behaviours. Caffeine (3 mg kg^{-1}) completely reversed the effects SNE (300 mg kg^{-1}) in both phases (Fig. 24a, b; middle panels) but did not have any effects on inhibitory effects of morphine (Fig. 24a, b; outer panels). Naloxone (1 mg kg^{-1}) injected 30 min before formalin did not have any significant effects on anti-nociceptive actions of the extract (Fig. 24a, b; middle panels). By contrast, naloxone completely reversed the inhibitory effects of morphine in both phases of the formalin test (Fig. 24a, b; outer panels).

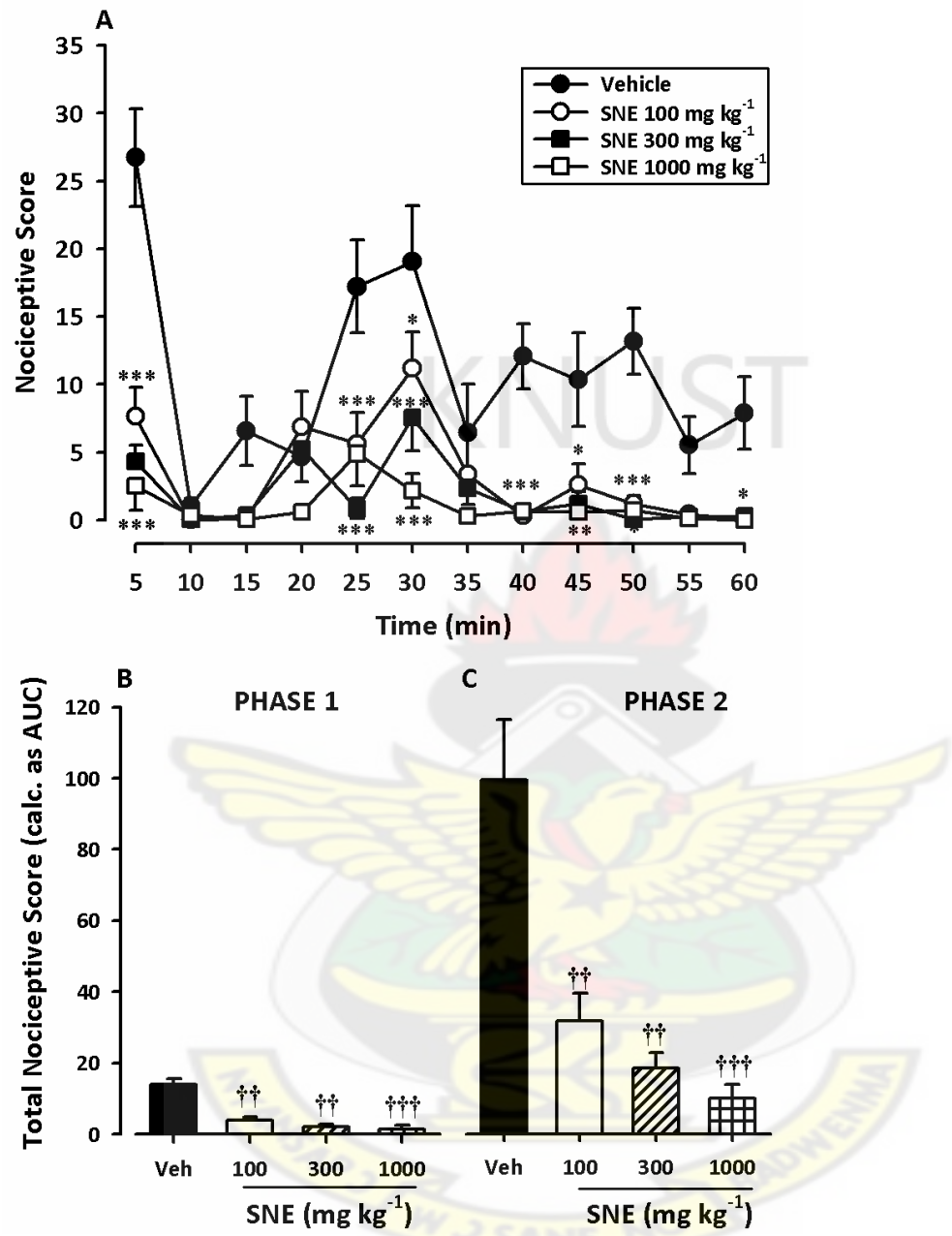

Figure 20: Effect of SNE (100-1000 mg kg^{-1} p.o.) on the time course of formalin-induced pain in mice. Nociceptive/pain scores are shown in 5 min time blocks up to 60 min post formalin injection. Each point represents mean ± s.e.m (n = 6). Values are means ± s.e.m. (n=5). *P < 0.05 ** P < 0.01; ***P < 0.001; compared to vehicle-treated group (Two-way ANOVA followed by Bonferroni's *post hoc* test). $^{\dagger\dagger}P$<0.001; $^{\dagger\dagger\dagger}P$<0.001; compared to vehicle-treated group (One-way ANOVA followed by Newman-Keul's *post hoc* test.

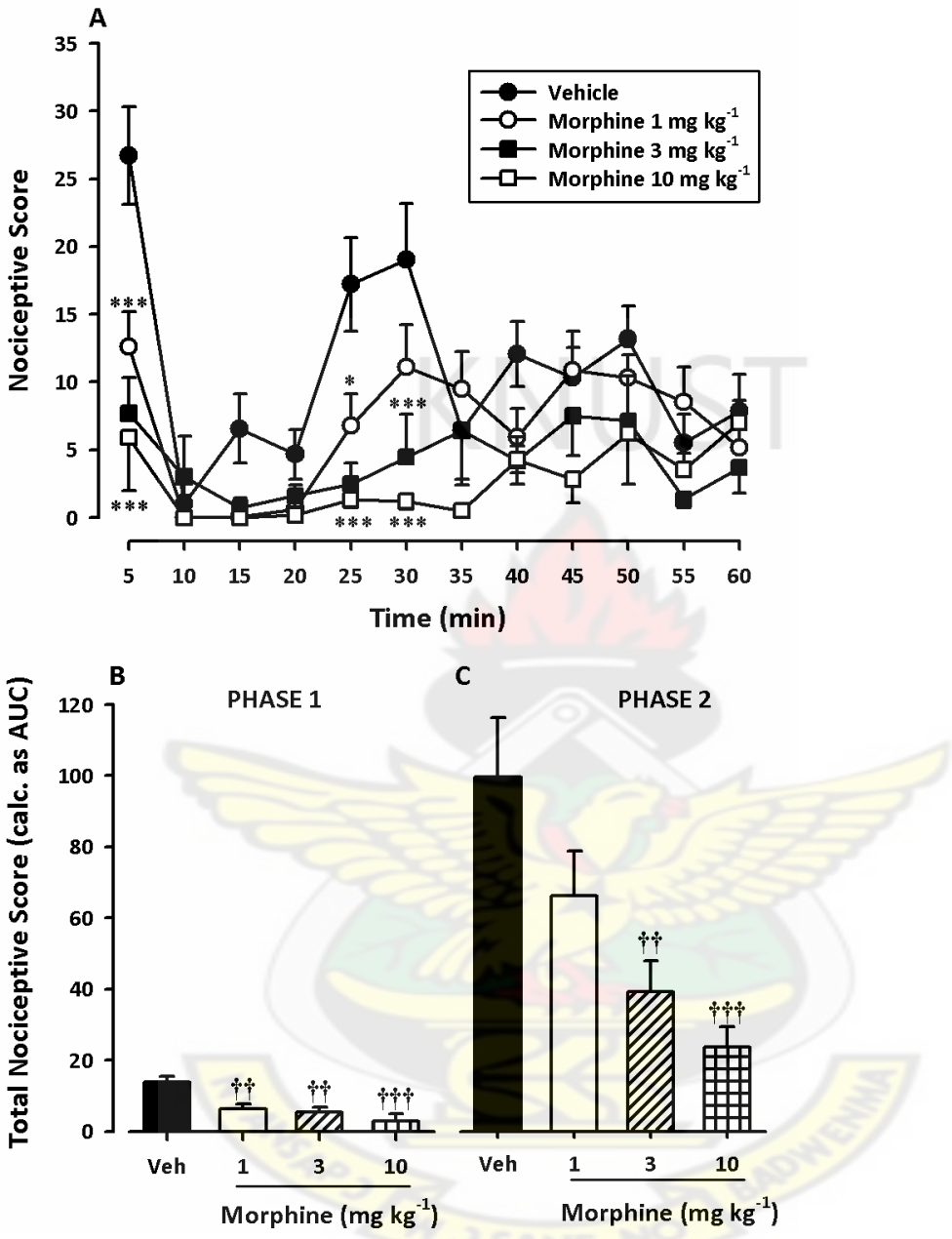

Figure 21: Effect of Morphine (1-10 mg kg^{-1} i.p.) on the time course of formalin-induced pain in mice. Nociceptive/pain scores are shown in 5 min time blocks up to 60 min post formalin injection. Each point represents mean ± s.e.m (n = 6). Values are means ± s.e.m. (n=5). **P* < 0.05; ****P* < 0.001; compared to vehicle-treated group (Two-way ANOVA followed by Bonferroni's *post hoc* test). $^{\dagger\dagger}P$<0.001; $^{\dagger\dagger\dagger}P$<0.001; compared to vehicle-treated group (One-way ANOVA followed by Newman-Keul's *post hoc* test.

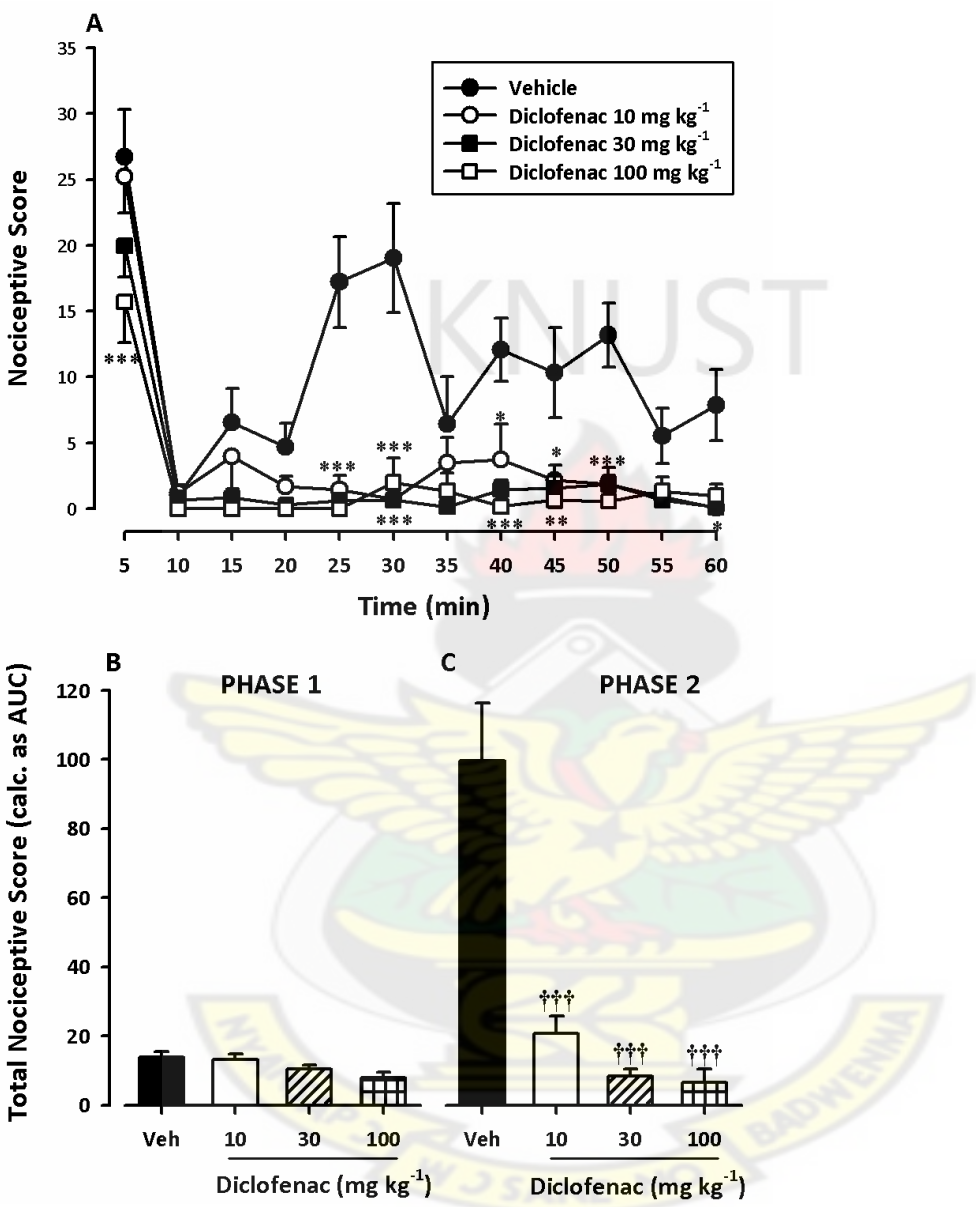

Figure 22: Effect of Diclofenac (10-100 mg kg^{-1} i.p.) on the time course of formalin-induced pain in mice. Nociceptive/pain scores are shown in 5 min time blocks up to 60 min post formalin injection. Each point represents mean ± s.e.m (n = 6). Values are means ± s.e.m. (n=5). *$P < 0.05$; ** $P < 0.01$; ***$P < 0.001$; compared to vehicle-treated group (Two-way ANOVA followed by Bonferroni's *post hoc* test). $^{\dagger\dagger\dagger}P<0.001$; compared to vehicle-treated group (One-way ANOVA followed by Newman-Keul's *post hoc* test.

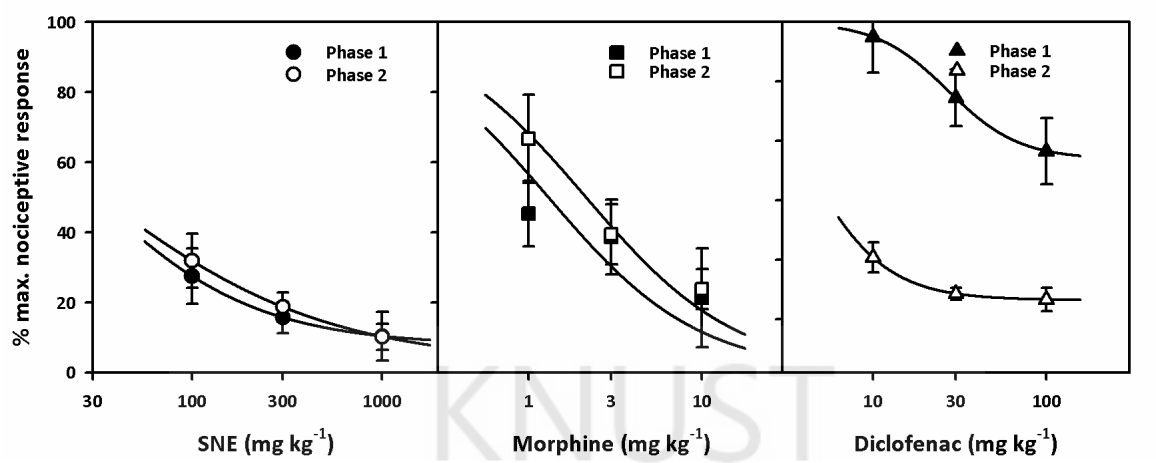

Figure 23: Dose-response curves for SNE, morphine and dicofenac in both phases 1 and 2 of formalin- induced analgesia mice. The doses of the drugs are in mg kg^{-1}.

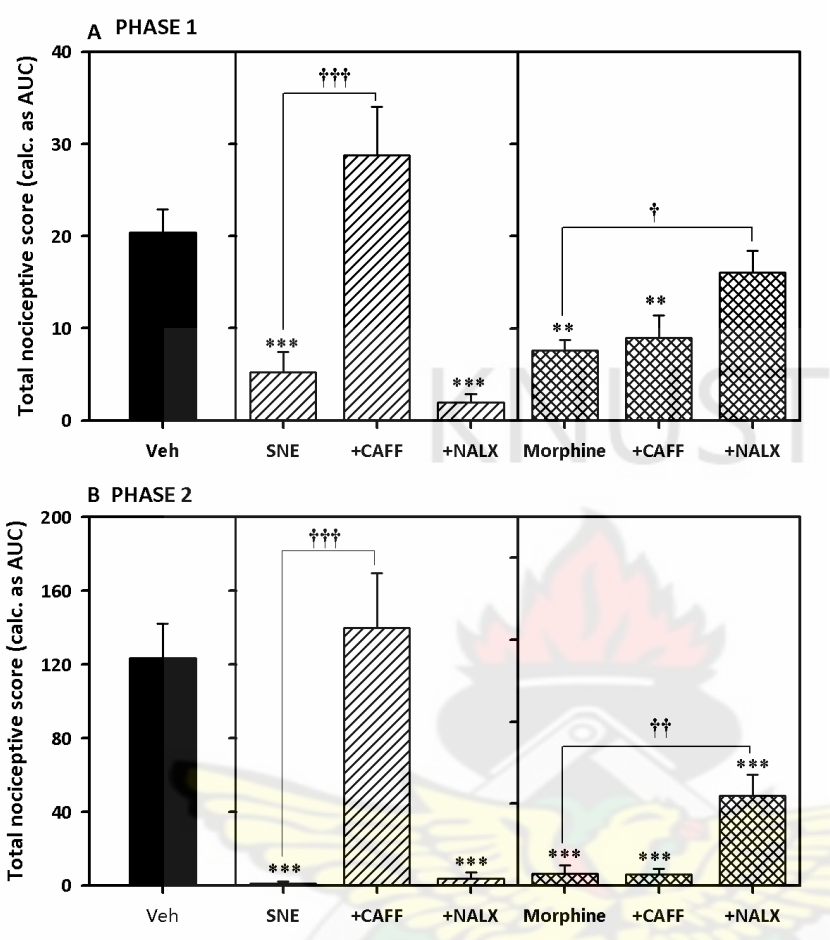

Figure 24: The antagonism of the effects of SNE (300mg kg^{-1} p.o.) and Morphine (3 mg kg^{-1} i.p.) by caffeine (3 mg kg^{-1} i.p) and naloxone (3 mg kg^{-1}i.p) on the nociceptive/pain scores of formalin-induced pain in mice. Nociceptive/pain scores are shown in 5 min time blocks up to 60 min post formalin injection. Each point represents mean ± s.e.m (n = 6). Values are means ± s.e.m. (n=5). ** $P < 0.01$; *** $P < 0.001$; compared to vehicle-treated group (Two-way ANOVA followed by Bonferroni's post hoc test). $^{\dagger}P < 0.05$; $^{\dagger\dagger}P < 0.001$; $^{\dagger\dagger\dagger}P < 0.001$compared to vehicle-treated group (One-way ANOVA followed by Newman-Keul's post hoc test.

Table 8:ED_{50} values for *S. nodiflora* extract, diclofenac and morphine in the formalin test

Drug	**ED_{50} (mg kg^{-1})**	
	Phase 1	*Phase 2*
SNE	25.98±14.59	30.24±18.08
Morphine	1.30±0.53**	2.14±0.61***
Diclofenac	27.12±12.45	3.97±9.90**††

3.4.2 Writhing Assay

In acetic acid induced writhing assay, oral administration of SNE (100-1000 mg kg^{-1}) 30 min before the injection of acetic acid significantly and dose-dependently inhibited writhes induced by the acid ($F_{3,80}$ = 9.69; P = 0.0007, two-way ANOVA (treatment group x time), Fig. 25a). Analysis of the AUCs showed that SNE attenuated acetic acid-induced writhing by 7.61-64.12% (Fig. 25b). Similarly the non-steroidal drug, diclofenac (10-100 mg kg^{-1}; i.p.) significantly reduced acetic acid-induced writhing in mice ($F_{3,80}$ = 7.11; P = 0.003, two-way ANOVA (treatment group x time), Fig. 25c). Calculated AUCs showed that diclofenac at the doses used, reduced writhing by 5.02-66.00% compared to vehicle-treated mice (Fig. 25d). Comparison of ED_{50} obtained by non-linear regression revealed that the extract (ED_{50}: 141.9 ± 37.16 mg kg^{-1}) was less potent than diclofenac (18.17 ± 6.75 mg kg^{-1}) in the writhing assay ($F_{1,26}$ = 6.78; P = 0.015).

Effects of treatment of non-selective adenosine inhibitor, caffeine is shown in Fig. 26. One-way ANOVA confirmed significant effect of caffeine (1 mg kg^{-1}) treatment on the effect on acetic acid-induced writhing ($F_{2,14}$ = 8.13, P <0.0001) post hoc between-group comparisons showed that naloxone (0.3 mg kg^{-1} and higher) antagonized the effects of SNE in this assay (Fig. 26). By contrast, caffeine pre-treatment did not have any effect on the antinociceptive effect in the writhing assay (Fig. 26) but rather seemed to potentiate the effects of diclofenac.

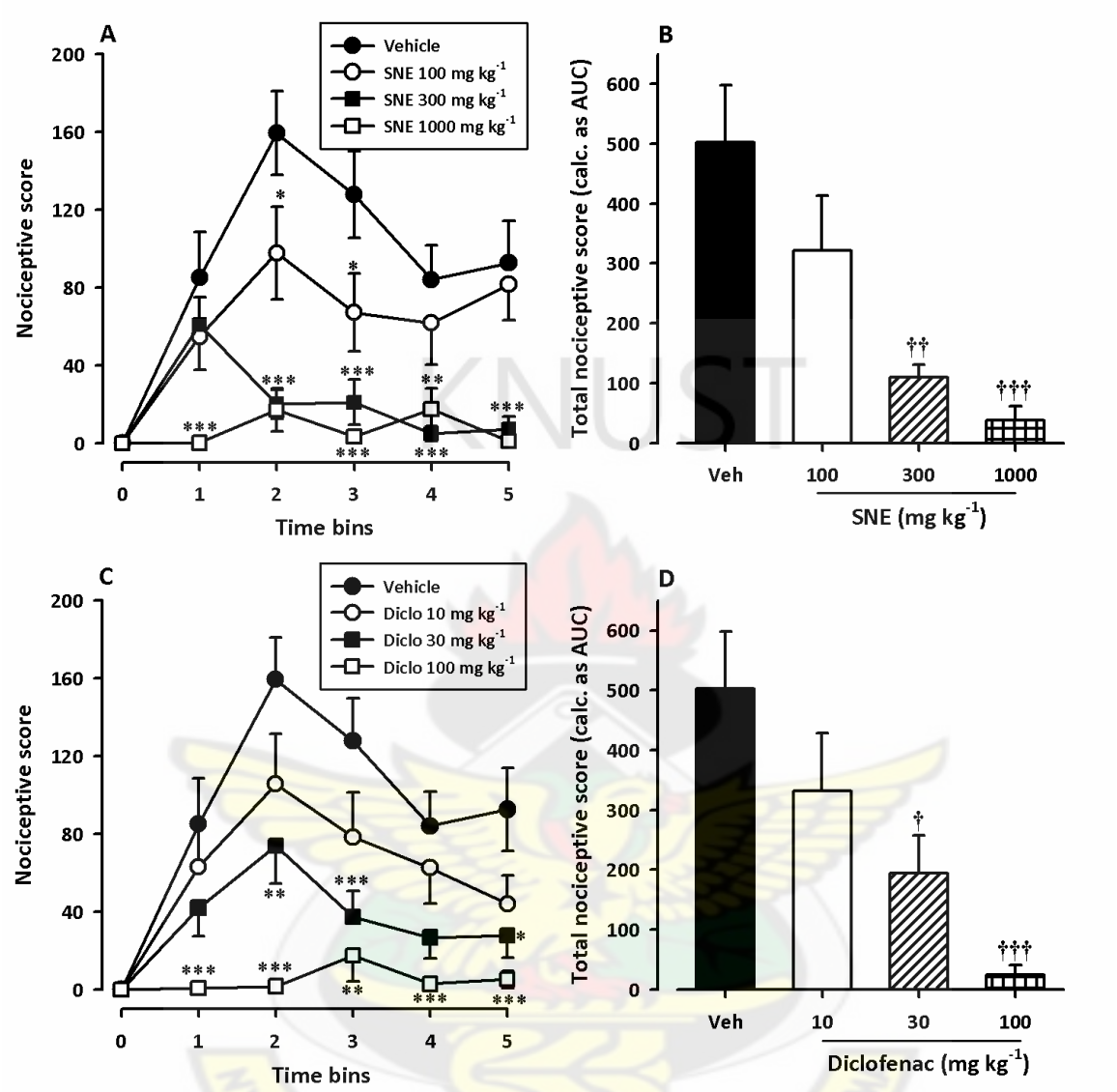

Figure 25: Effect of SNE (100-1000 mg kg^{-1} p.o.) and Diclofenac (10-100 mg kg^{-1} i.p. *)* on the time course of acetic acid-induced writhing in mice. Nociceptive/pain scores are shown in 5 min time blocks up to 30 min post acetic acid injection. Each point represents mean ± s.e.m (n = 5). Values are means ± s.e.m (n=5). *P < 0.05 ** P < 0.01; ***P < 0.001; compared to vehicle-treated group (Two-way ANOVA followed by Bonferroni's *post hoc* test). $^{\dagger}P$<0.05 $^{\dagger\dagger}P$<0.001; $^{\dagger\dagger\dagger}P$<0.001; compared to vehicle-treated group (One-way ANOVA followed by Newman-Keul's *post hoc* test).

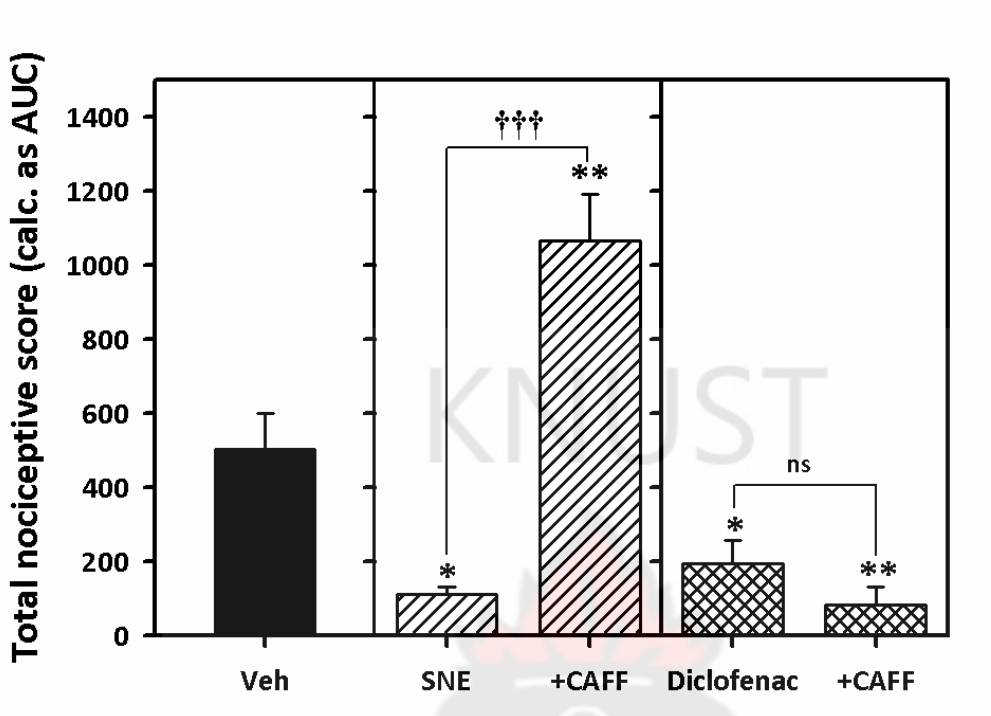

Figure 26: The antagonism of the effects of SNE (300 mg kg^{-1} p.o.) and Diclofenac (30 mg kg^{-1} i.p.) by caffeine (3 mg kg^{-1} i.p) on the nociceptive/pain scores of formalin-induced pain in mice. Nociceptive/pain scores are shown in 5 min time blocks up to 60 min post formalin injection. Each point represents mean ± s.e.m (n = 6). Values are means ± s.e.m. (n=5). *P < 0.05; ** P < 0.01; ***P < 0.001; compared to vehicle-treated group (Two-way ANOVA followed by Bonferroni's *post hoc* test). $^{\dagger}P$<0.05 $^{\dagger\dagger}P$<0.001; $^{\dagger\dagger\dagger}P$<0.001; compared to vehicle-treated group (One-way ANOVA followed by Newman-Keul's *post hoc* test).

3.5 ANTIOXIDANT AND FREE RADICAL SCAVENGING EFFECT

3.5.1 Total phenol assay

The phenol content of tannic acid (0.01-0.3 mg ml^{-1}) increased with increasing concentration (r^2 =0.9901) (Fig. 27a). The extract SNE (0.1-3 mg ml^{-1}) also showed a concentration dependent increase in phenolic content expressed as tannic acid equivalents (Fig. 27b)

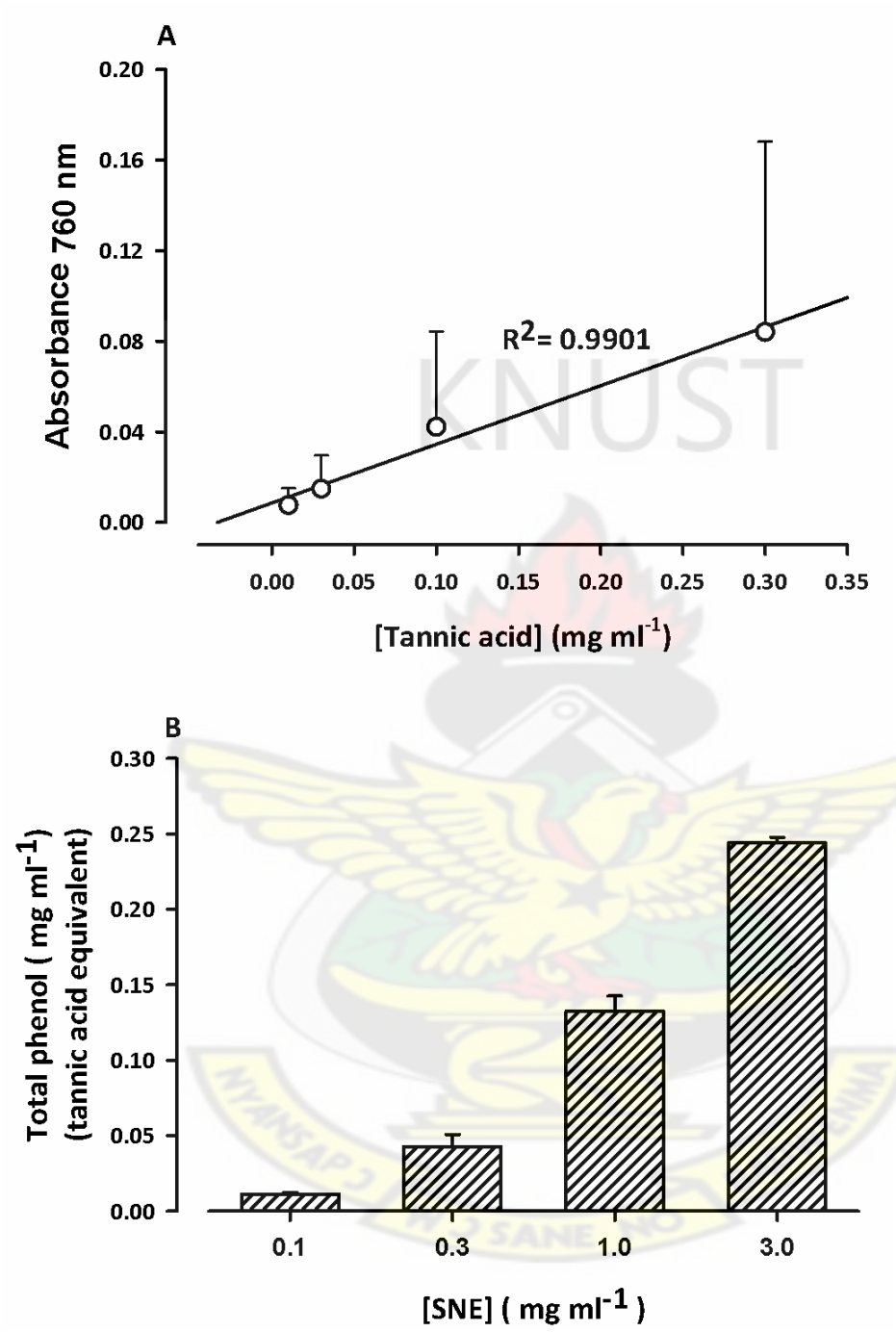

Figure 27: (A) A relationship between the absorbance and concentration of tannic acid (0.01-0.3 mg ml^{-1}) and (B) total phenolic substances present in various concentrations of SNE (0.3-3 mg ml^{-1}) expressed as tannic acid equivalent. Each point in A and columns in B represent the mean ± s.e.m (n=3).

3.5.2 Total antioxidant capacity

The total antioxidant capacity of ascorbic acid (0.01-0.3 mg ml^{-1}) increased with increasing concentration (r^2 =0.9771) (Fig. 28a). The extracts, SNE (0.3-3 mg ml^{-1}) also showed an increase in total antioxidant capacity expressed as ascorbic acid equivalent (28b).

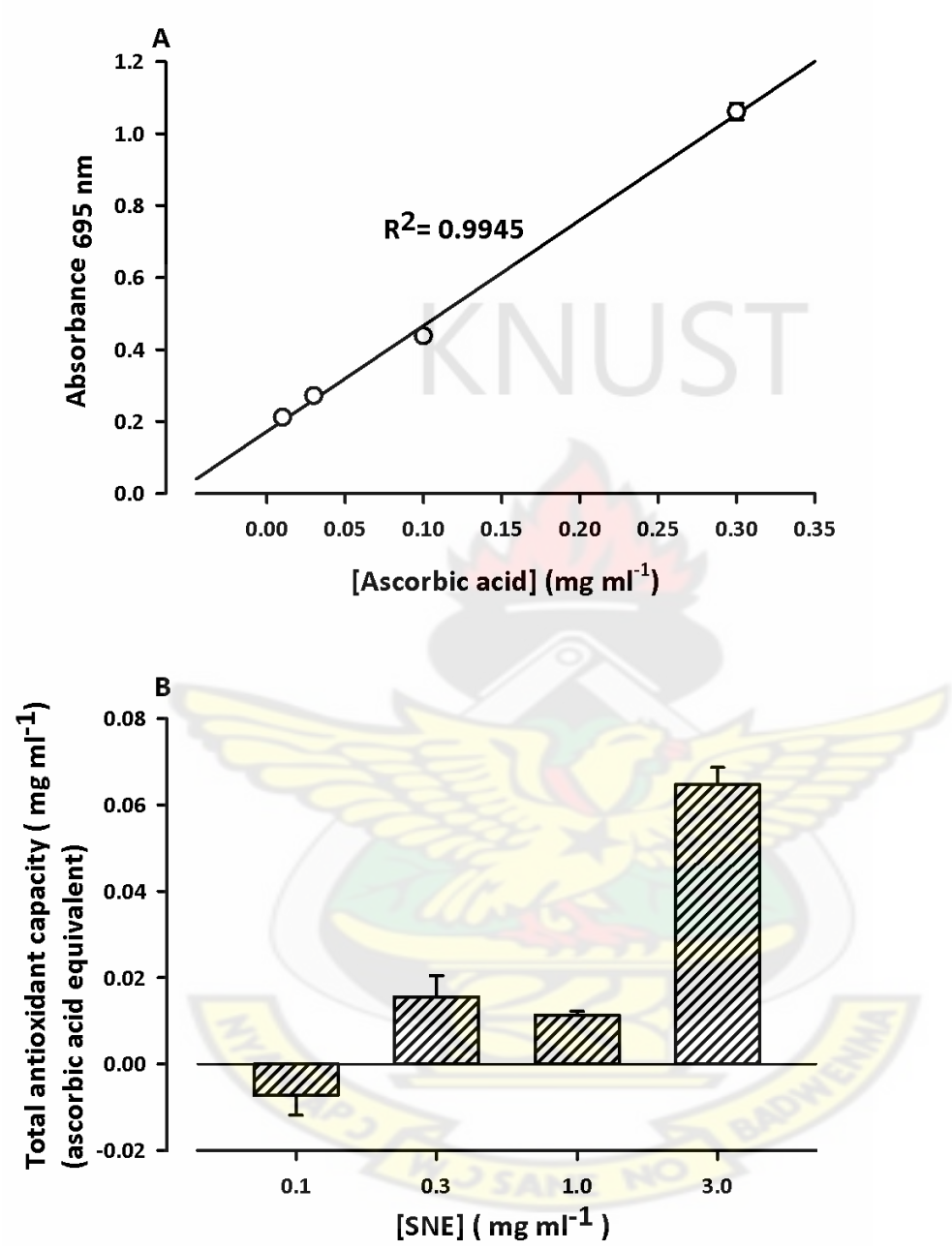

Figure 28: (A) A relationship between the absorbance and concentration of ascorbic acid (0.01-0.3 mg ml^{-1}) and (B) total phenolic substances present in various concentrations of SNE (0.3-3 mg ml^{-1}) expressed as ascorbic acid equivalent. Each point in A and columns in B represent the mean ± s.e.m (n=3)

3.5.3 Corelation between the total phenol content (TPC) and the total antioxidant capacity (TAC)

The TPC of SNE correlated with the TAC (r^2 = 0.9908) (Figure 29)

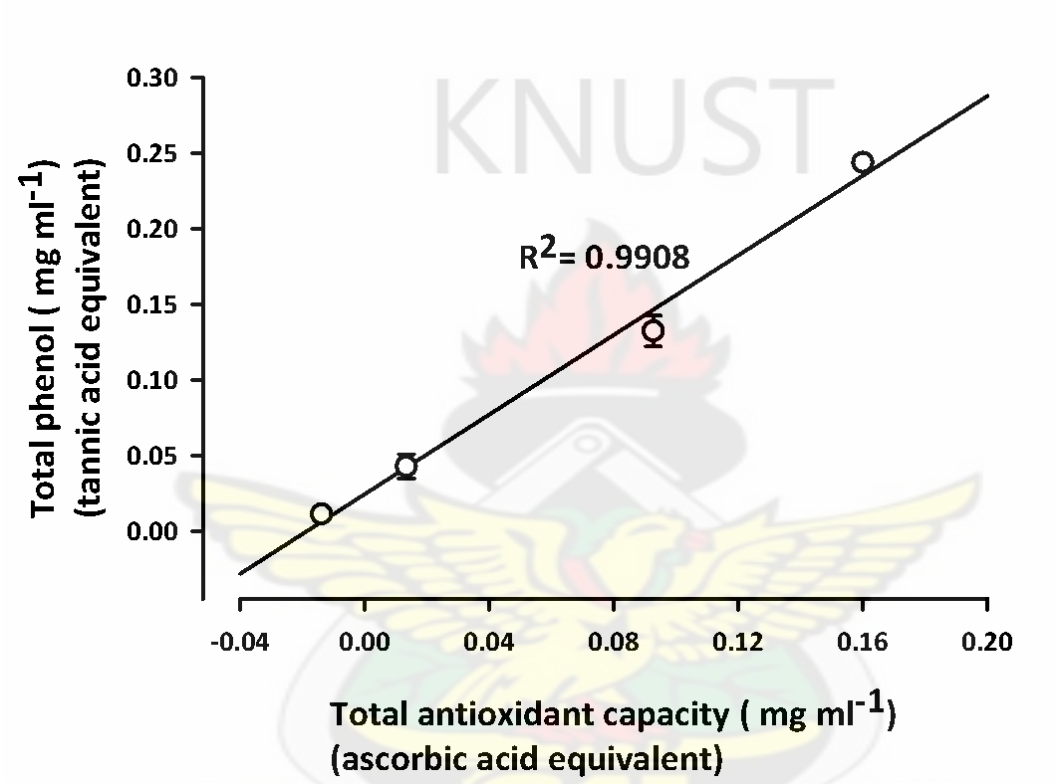

Figure 29: A relationship between the total phenol content (TPC) and total antioxidant capacity of SNE (0.3-3 mg ml^{-1})

3.5.4 Reducing Power

The extract (0.1-3 mg ml^{-1}) and the reference antioxidant *n-propyl* gallate (0.001-0.03 mg ml^{-1}) dose dependently reduced Fe^{3+} to Fe^{2+} resulting in concentration dependent increase in absorbance (Fig. 30). From EC_{50} (in mg ml^{-1} x 10^{-1}, Table 7) obtained for SNE (24.82), and *n-propyl* gallate (1.253) SNE were found to be about 20 fold less potent than *n-propyl* gallate.

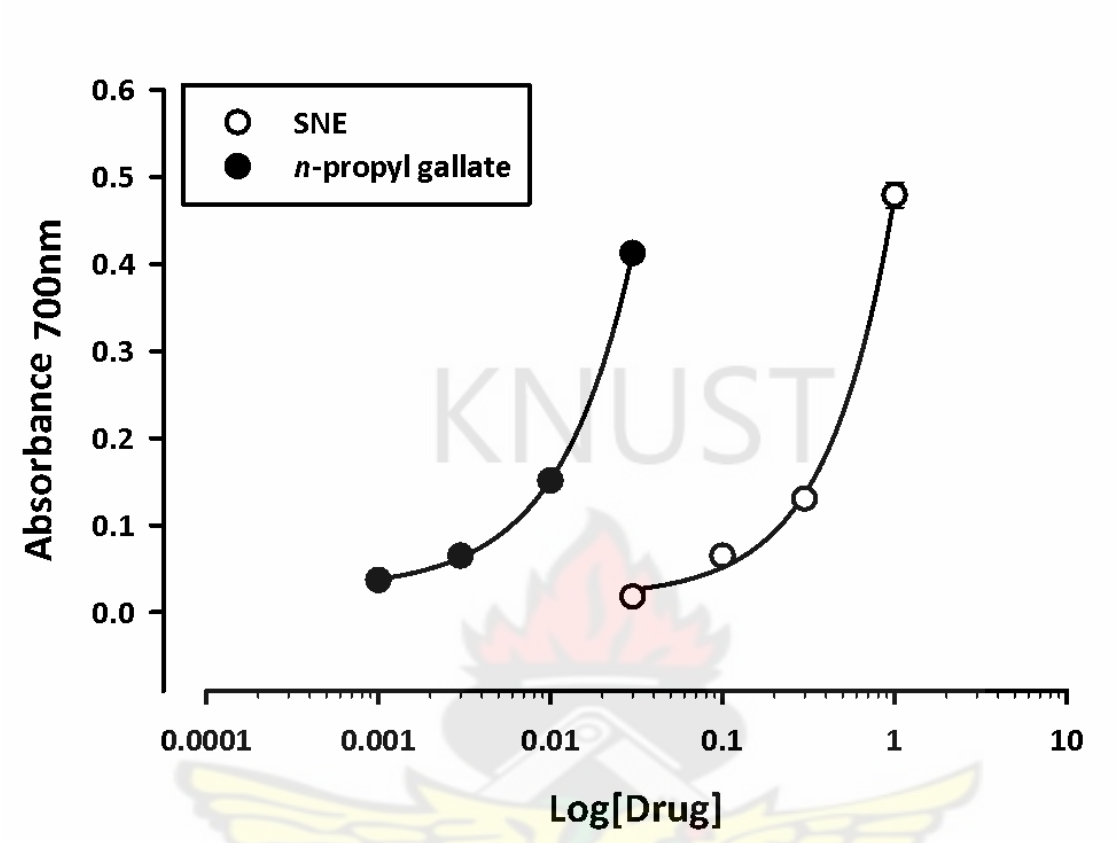

Figure 30: Reducing power of SNE (0.1-3 mg ml^{-1}) compared to *n-propyl* gallate (0.001-0.3 mg ml^{-1}). Each point represents the mean ± s.e.m.(n= 3).

3.5.5 Free radical scavenging effect

All the drugs, *n-propyl* gallate (0.001 - 0.03 mg ml^{-1}) and the extracts SNE (0.1- 3 mg ml^{-1}) exhibited dose dependent free radical scavenging activity (Fig. 31). The rank order of potency (defined by EC_{50} in mg ml^{-1} x 10^{-3}, Table 10) was found to be: *n-propyl* gallate (1.861) > SNE (331.3).

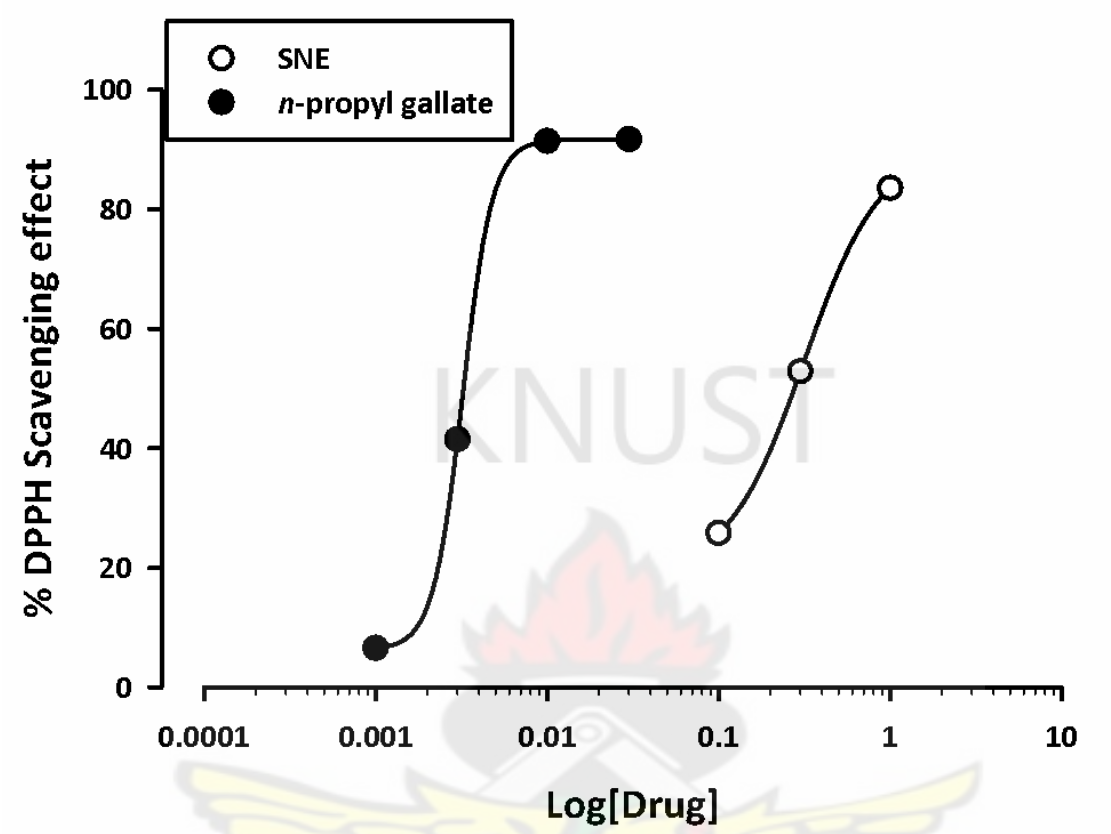

Figure 31: Free radical scavenging ability of SNE (0.1-3 mg ml^{-1}) compared to *n-propyl* gallate (0.01-0.3 mg ml^{-1}) in the DPPH radical assay. Each point represents the mean ± s.e.m (n = 3).

3.5.6 Linoleic acid autoxidation assay

All the test samples used, *n-propyl* gallate (0.001 - 0.3 mg ml^{-1}), and the extract (0.1-3 mg ml^{-1}) showed concentration dependent ability to inhibit the autoxidation of linoleic acid.lipid peroxidation (Fig. 32). The *n-propyl* gallate was found to be twice as potent as SNE (Table 10).

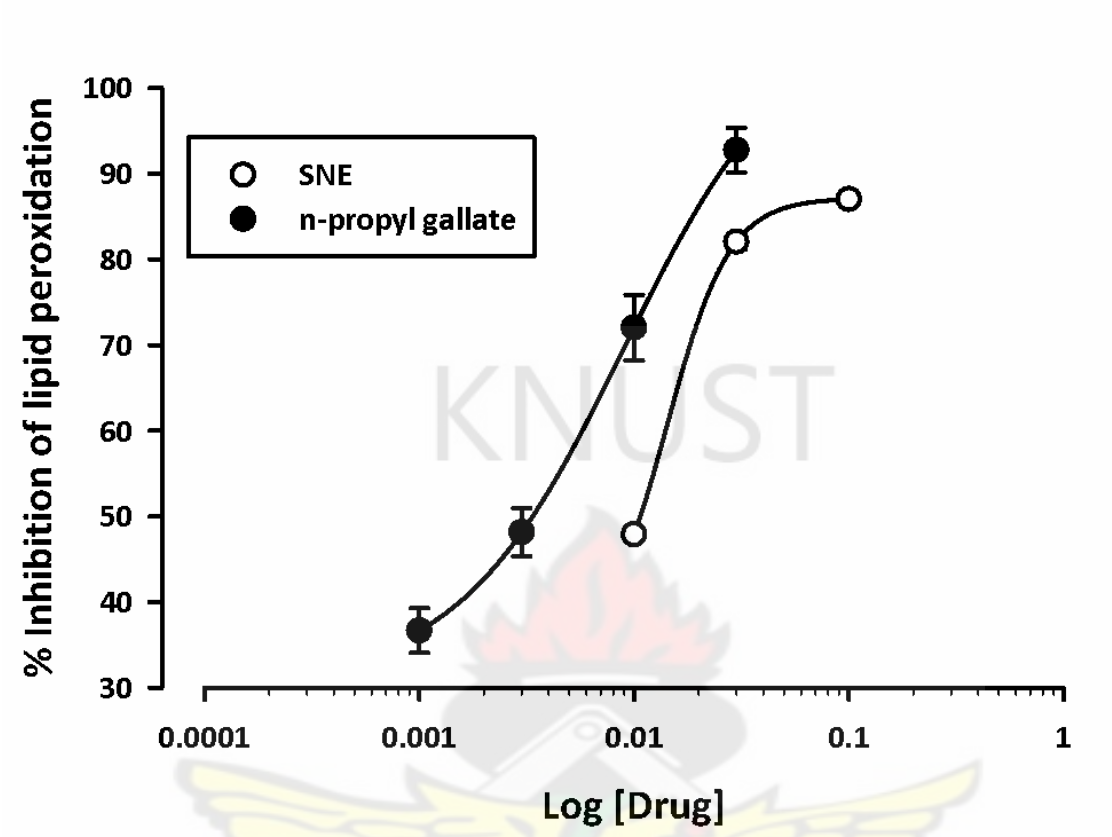

Figure 32 Percentage inhibition of linoleic acid autoxidation by SNE (0.1-3.0 mg ml^{-1}) compared to *n-propyl* gallate (0.001-0.3 mg ml^{-1}). Each point represents the mean ± s.e.m (n = 3).

Table 9: ED_{50} of antioxidant assay

	EC_{50} (mg ml^{-1})		
Drug	*Reducing Power*	*DPPH Scavenging*	*Lipid Peroxidation*
		$\times 10^{-3}$	$\times 10^{-3}$
SNE	459.7	315.9	14.35
n-propyl gallate	26.1	3.221	8.954

3.6 CENTRAL NERVOUS SYSTEM EFFECTS

3.6.1 Elevated Plus Maze (EPM)

The effects of SNE and diazepam on the various parameters measured in the EPM paradigm is as shown in figures 33-34. The administration of SNE (10-300 mg kg^{-1}) dose-dependently decreased the number of entries into both the open ($F_{4,25}$= 0.834, *P*= 0.51563, Fig. 33a) and closed ($F_{4,25}$= 2.669, *P*= 0.0556, Fig 33a) arms, no significant effect on the percent number of entry into the open arms ($F_{4,25}$= 1.478, *P*= 0.2414, Fig 33c) and a decrease in the time spent in the open arm ($F_{4,23}$= 0.3196, *P*= 0.8619, Fig 33e) in comparison to the vehicle-treated group. Similarly SNE dose-dependently, though not significant, decreased the number of head dips ($F_{4,22}$= 0.4916, *P*= 0.7398, Fig 34a) and stretch attend postures ($F_{4,25}$= 0.8171, *P*= 0.5264, Fig 34c) and significantly, decreased the duration of groomings ($F_{4,22}$= 5.0, *P*= 0.046, Fig 34e).

Diazepam (0.1-1.0 mg kg-1) dose-dependently and significantly increased the number of open arm entry ($F_{3,17}$= 5.597, *P*= 0.0074, Fig. 33b), decreased the number of entries into the closed arm ($F_{3,19}$= 3.837, *P*= 0.0265, Fig. 33b), increased the percent open arm entry ($F_{4,25}$= 4.664, *P*= 0.0140, Fig. 33d) and the percentage of the time spent in the open arm ($F_{3,20}$=4.815, *P*= 0.0141, Fig. 33f) in comparison to the vehicle-treated group. Also, diazepam dose-dependently increased the number of head dips ($F_{3,20}$=1.720, *P*= 0.1950, Fig. 34b) and significantly decreased the number of stretch attend postures ($F_{3,`18}$= 4.301, *P*= 0.0187, Fig. 34d) and not significantly, the duration of grooming ($F_{3,20}$=1.983, *P*= 0.1490, fig. 34f).

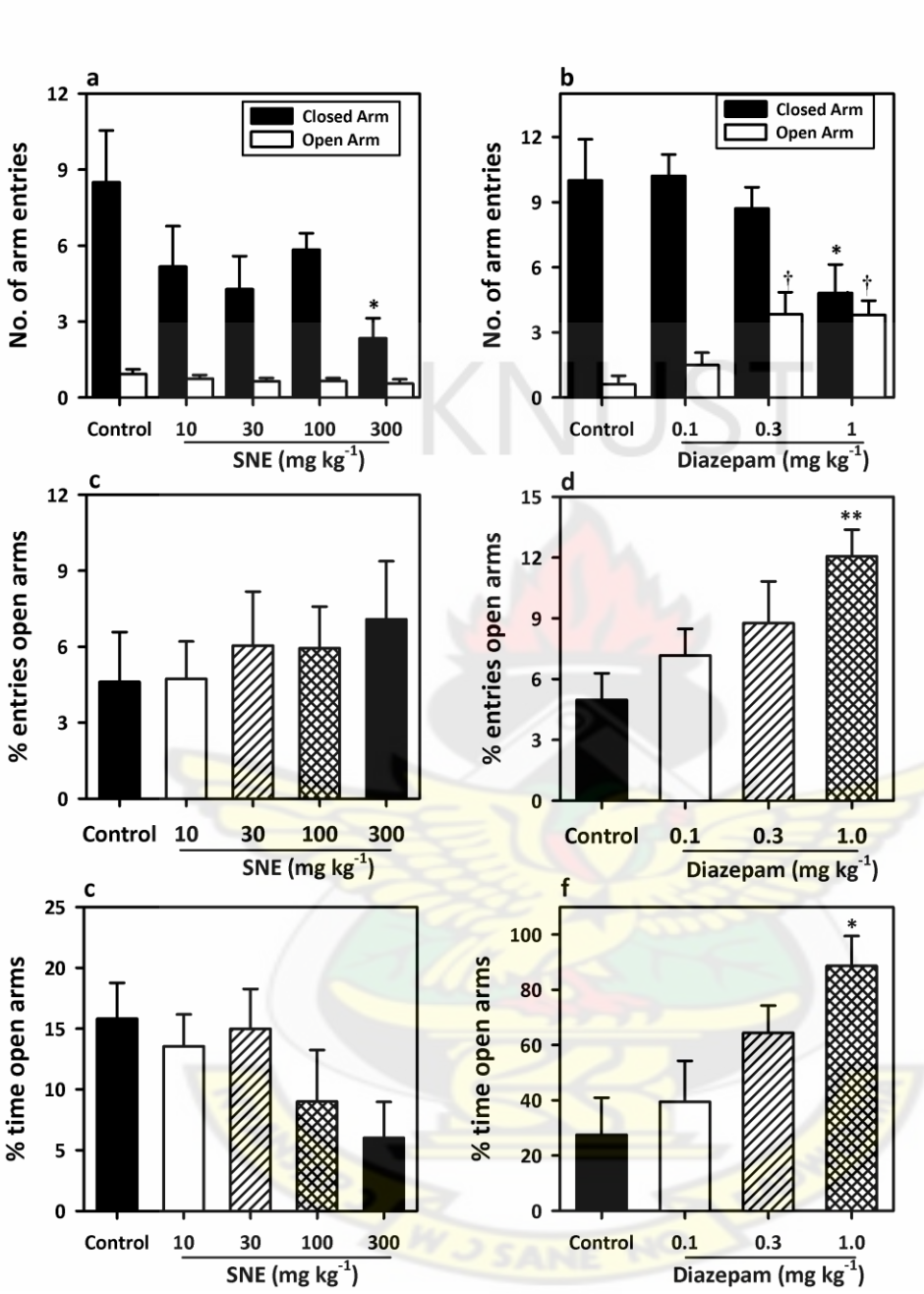

Figure 33: The effects of SNE (10-300 mg kg^{-1}) and diazepam (0.1-1.0 mg kg^{-1}) on the number entries into the open and closed arms (a and b), the percent open arm entries (c and d) and the percent time spent in the open arm (e and f). Data are presented as mean ± S.E.M. (n=5). *$P < 0.05$; ** $P < 0.01$; compared to vehicle-treated group (One-way ANOVA followed by Newman-Keul's *post hoc* test)

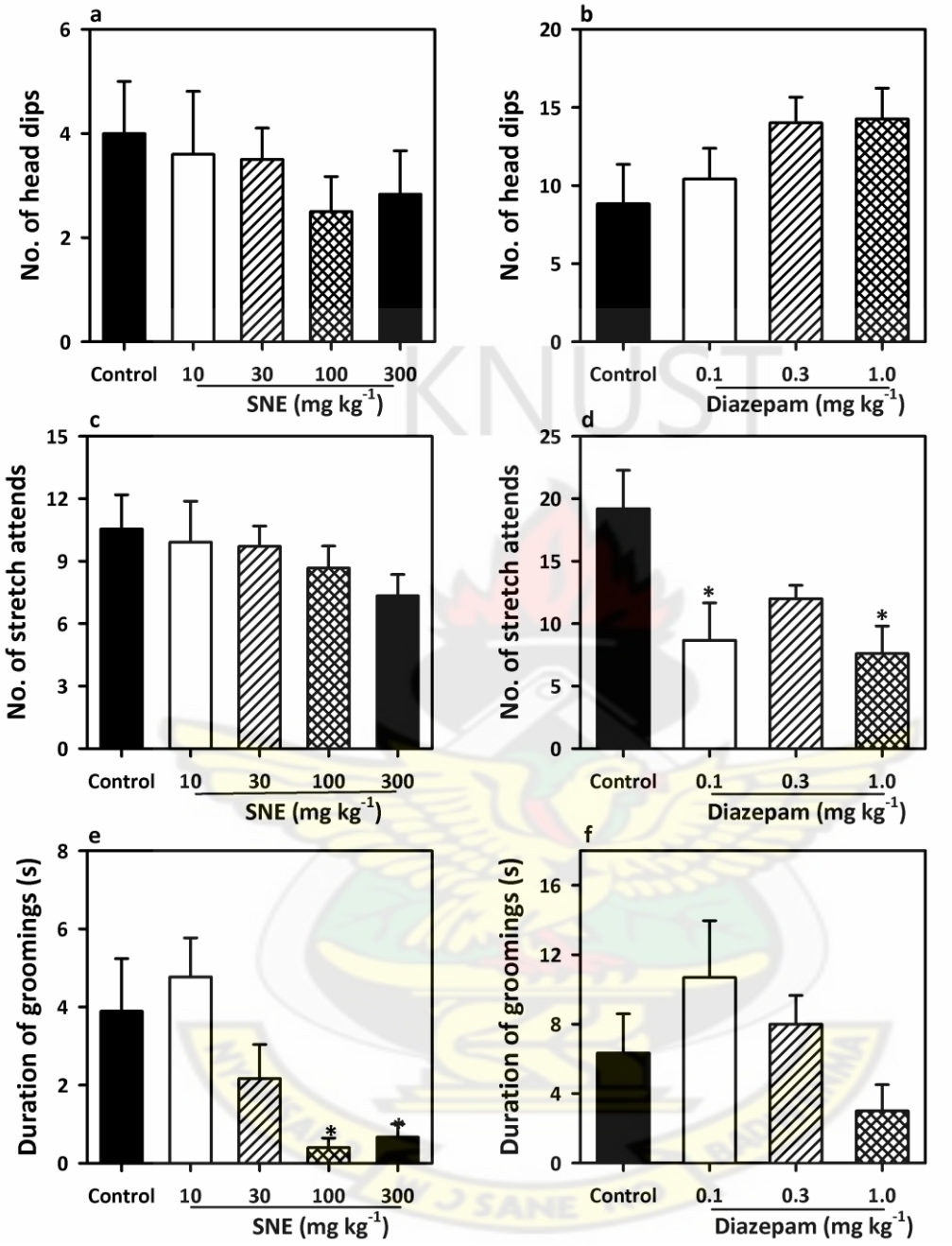

Figure 34: The effects of SNE (10-300 mg kg^{-1}) and diazepam (0.1-1.0 mg kg^{-1}) on the number entries into the open and closed arms (a and b), the percent open arm entries (c and d) and the percent time spent in the open arm (e and f) in the EPM. Data are presented as mean ± S.E.M. n=5. *$P < 0.05$ compared to vehicle-treated group (One-way ANOVA followed by Newman-Keul's *post hoc* test)

3.6.2 Light-Dark Box

The administration of SNE (10-300 mg kg^{-1}) dose-dependently and significantly reduced the number of entry in to the light compartment ($F_{4,24}$=3.429, P=0.0237, Fig 35a), the number of transition ($F_{4,24}$=5.723, P=0.022, Fig 35c) and not significantly, the time spent in the light area ($F_{4,25}$ =0.3722, P=0.8262, Fig 35a) of the Light-dark box model. Conversely, diazepam (0.1-1.0 $mgkg^{-1}$), dose-dependently and significantly increased the number of entries into the light compartment ($F_{4,24}$ =4.345, P=0.0181, Fig 35b), the number of transitions ($F_{3,18}$=3.771, P=0.0292, Fig 35d) and the total time spent in the lighted area ($F_{3,17}$ =7.470, P=0.0021, Fig 35e).

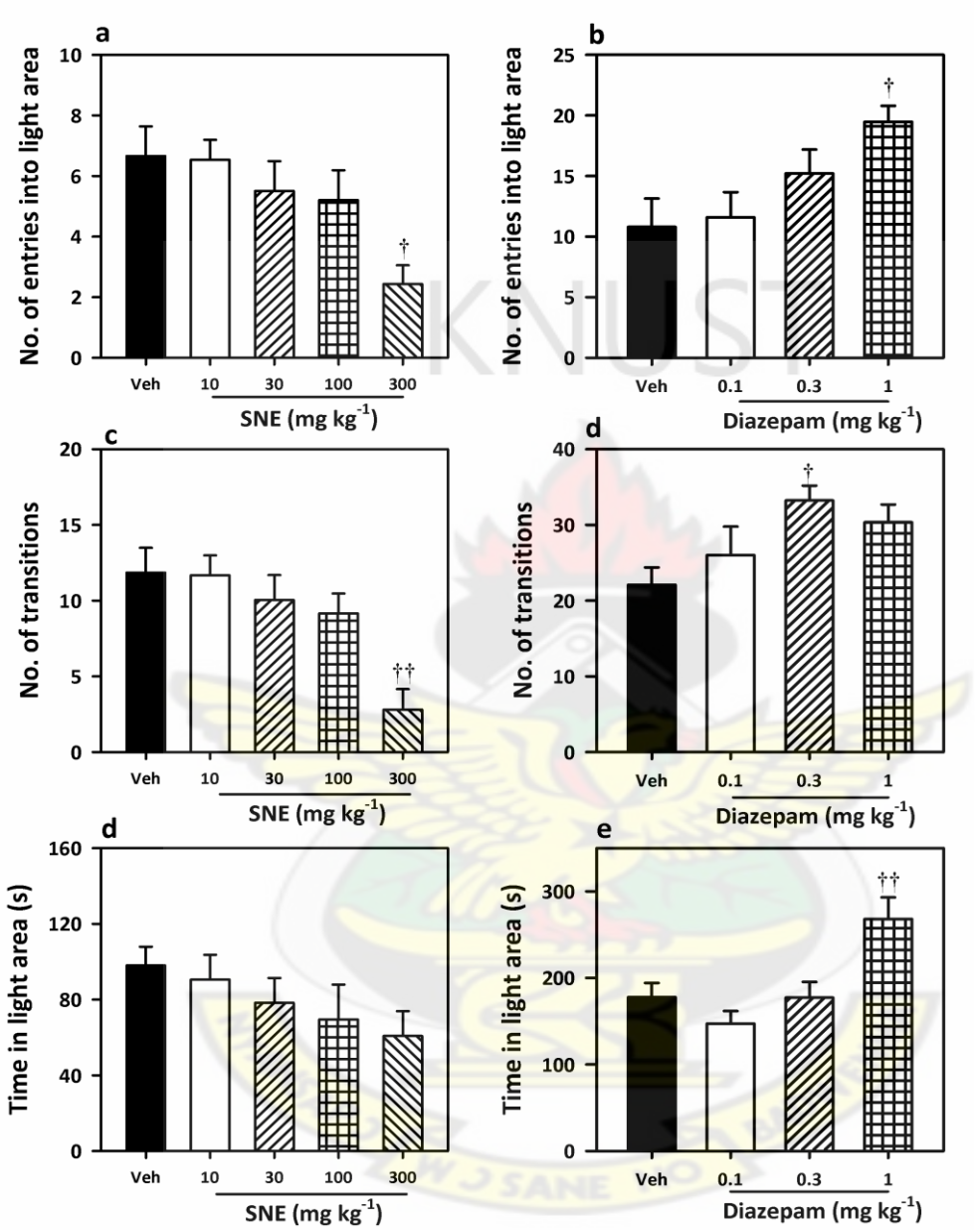

Figure 35: The effects of SNE (10-300 mg kg^{-1}) and diazepam (0.1-1.0 mg kg^{-1}) on the number entries into the open and closed arms (a and b), the percent open arm entries (c and d) and the percent time spent in the open arm (e and f). Data are presented as mean ± S.E.M. n=5. $^{\dagger}P < 0.05$; $^{\dagger\dagger}P < 0.01$ compared to vehicle-treated group (One-way ANOVA followed by Newman-Keul's *post hoc* test)

3.6.3 VersaMax Animal Activity Monitor Systems

3.6.3.1 Locomotor Activity

The locomotor activity of mice were assessed by measuring the horizontal, vertical activities and the total distance travelled by the mice pretreated with SNE (10-1000 mg kg^{-1}) or vehicle over a period of two hours. The results obtained indicated that the SNE (10- 1000 mg kg^{-1}, Fig.36) decreased dose dependently and significantly the vertical ($F_{3,68}$ =18.65, P<0.0001) and horizontal ($F_{3,68}$ =12.34, P< 0.0001, Fig.36) activities and the total distance travelled ($F_{3,68}$ =17.31, P< 0.0001, Fig. 37) with respect to the vehicle treated The percentage of the activities calculated from analysis of the AUCs revealed similar trends.

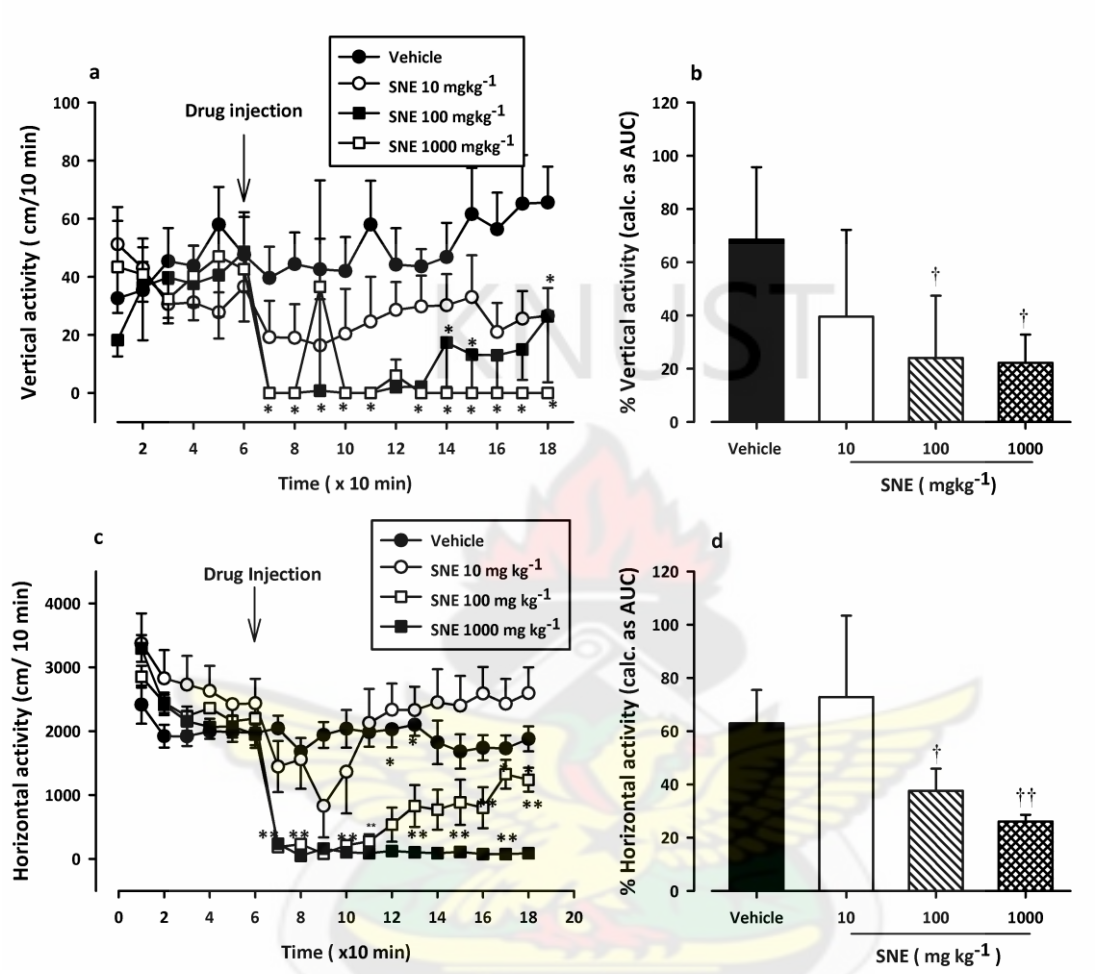

Figure 36: The effect of SNE (10-1000 mg kg^{-1}) on the vertical (**a** and **b**) and horizontal (**c** and **d**) activity of mice in the Versamax activity monitor. Panels **a** and **c** shows the time-course curve of the activity measured over a three-hour period and panels **b** and **d** represents the percent activity (calculated as AUC). Each point represents mean ± s.e.m (n = 5). Values are means ± s.e.m. (n=5). *P<0.05; ** P < 0.01; compared to vehicle-treated group (Two-way ANOVA followed by Bonferroni's *post hoc* test). $^{\dagger}P$<0.05; $^{\dagger\dagger}P$<0.01; compared to vehicle-treated group (One-way ANOVA followed by Newman-Keul's *post hoc* test

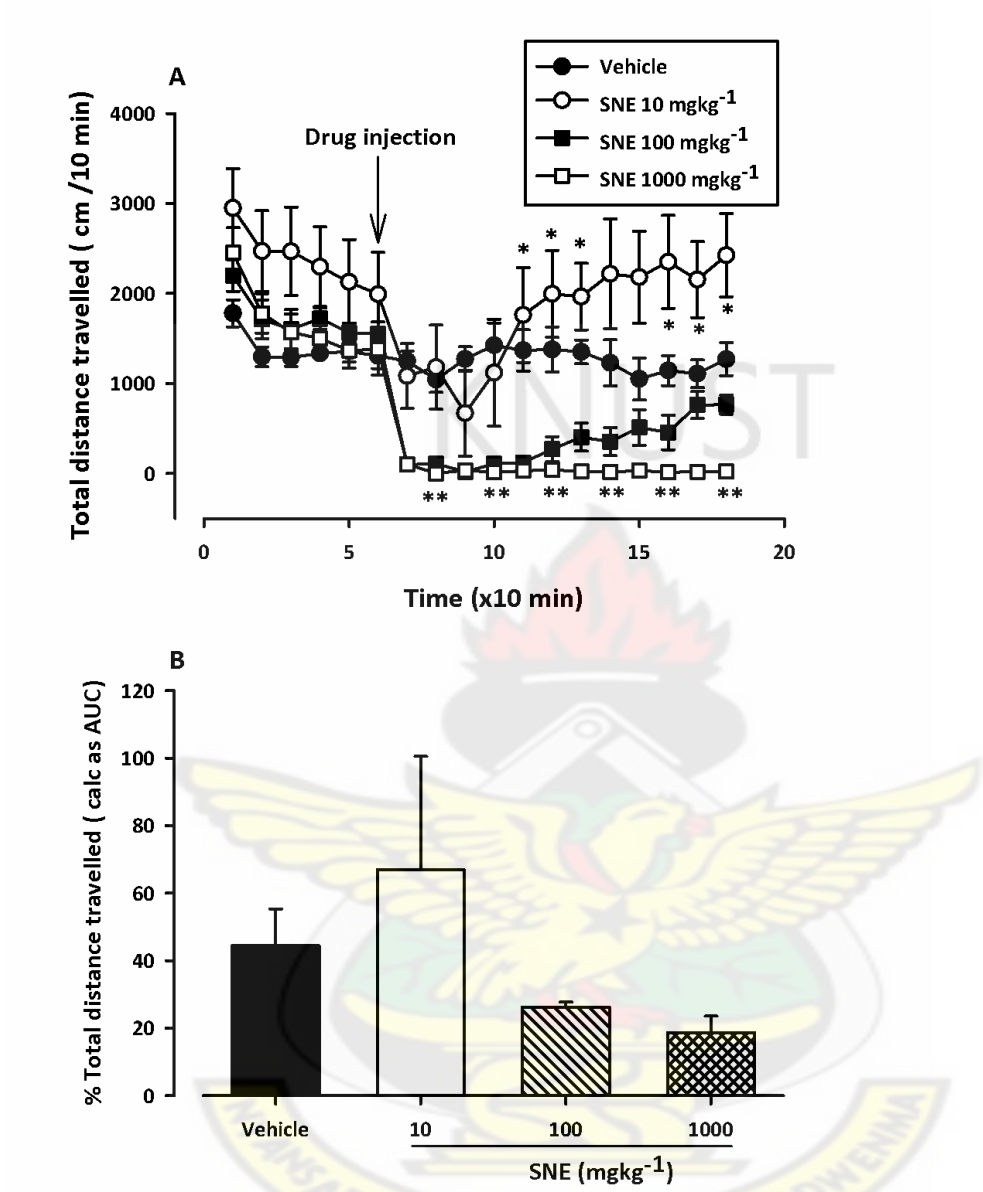

Figure 37: The effect of SNE (10-1000 mgkg^{-1}) on the total distance travelled by mice in the Versamax activity monitor. Panel **a** shows the time-course curve of the activity measured over a three-hour period and panel **b** represents the percent activity (calculated as AUC). Each point represents mean ± s.e.m (n = 5). Values are means ± s.e.m. (n=5). *P<0.05; ** P < 0.01; compared to vehicle-treated group (Two-way ANOVA followed by Bonferroni's *post hoc* test). P>0.05 not significant compared to vehicle-treated group (One-way ANOVA followed by Newman-Keul's *post hoc* test)

3.6.3.2 Anxiety Parameters

These as measured by VAMS are as follows, the margin (thigmotatic) distance covered and duration, the distance moved at the center and the time spent, and the time spent at the corners (the left front(LF), right front (RF), the left rear (LR) and the right rear (RR)).

SNE (10-1000 mg kg^{-1}) significantly decreased both the margin distance ($F_{3,68}$ = 20.82, P< 0.0001, Fig.38a) and increased the total time spent at the margin ($F_{3,68}$ = 27.56, P< 0.0001, Fig.38c) of the observation cage. The percent distance and time spent at the margin calculated from the AUCs revealed a similar trend (Fig 38b and 38d). SNE also significantly decreased the total distance travelled at the center of the observation cage ($F_{3,68}$ = 13.86, P< 0.0001, Fig.39a and 39b). Likewise, the total time spent at the center was also decreased significant and dose-dependently by SNE ($F_{3,68}$ = 27.56, P< 0.0001, Fig. 39c and 39d). There was no significant difference between the time spent in the various corners of the cage in mice pretreated with SNE and those pre-treated with the vehicle (Fig.40)

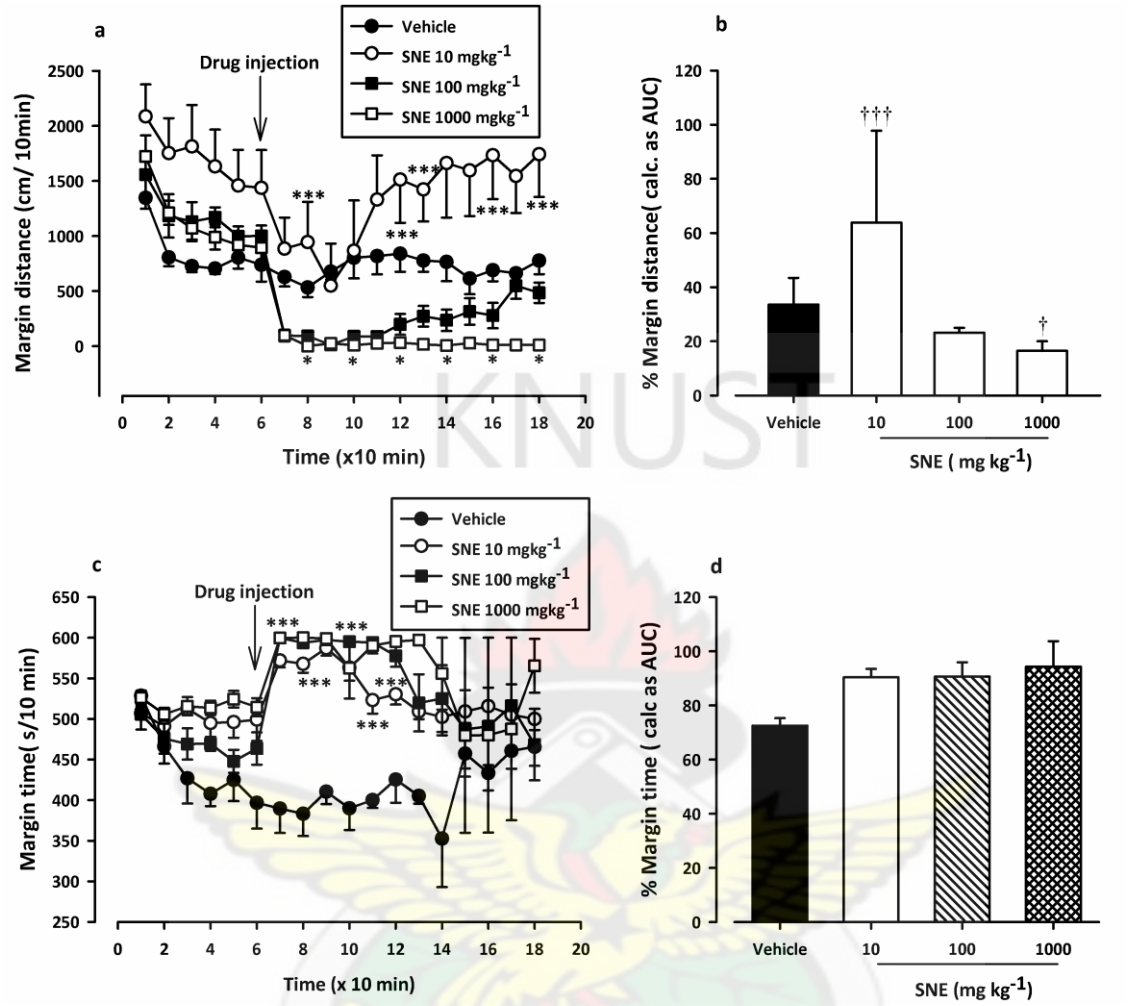

Figure 38: The effect of SNE (10-1000 mg kg^{-1}) on the margin/thigmotatic distance (**a** and **b**) and margin time (**c** and **d**) activity of mice in the Versamax activity monitor. Panels **a** and **c** shows the time-course curve of the activity measured over a three-hour period and panels **b** and **d** represents the percent activity (calculated as AUC). Each point represents mean ± s.e.m (n = 5). Values are means ± s.e.m. (n=5). $^{*}P<0.05$; $^{***}P < 0.001$; compared to vehicle-treated group (Two-way ANOVA followed by Bonferroni's *post hoc* test). $^{\dagger}P<0.05$; $^{\dagger\dagger\dagger}P<0.001$; compared to vehicle-treated group (One-way ANOVA followed by Newman-Keul's *post hoc* test)

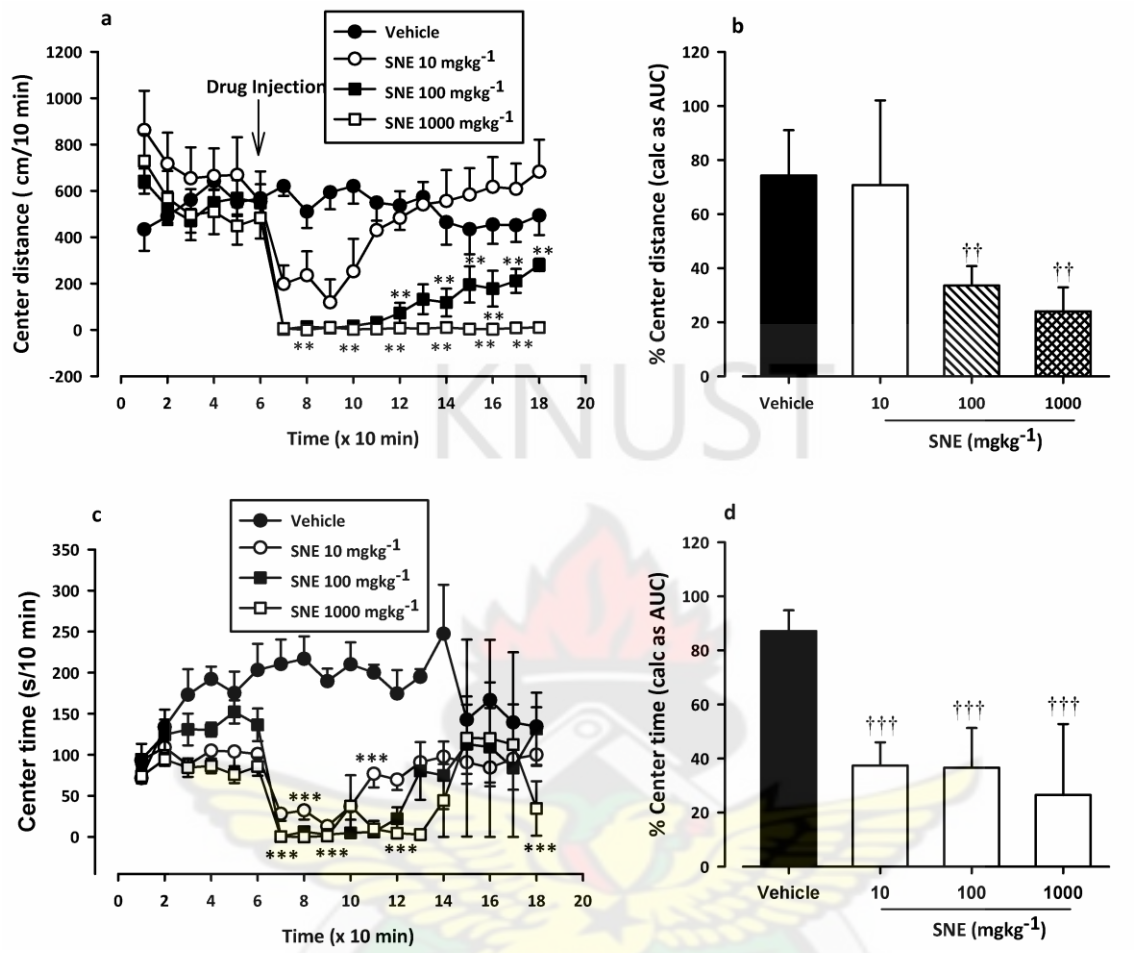

Figure 39: The effect of SNE (10-1000 mg kg^{-1}) on the center distance (**a** and **b**) and center time (**c** and **d**) activity of mice in the Versamax activity monitor. Panels **a** and **c** shows the time-course curve of the activity measured over a three-hour period and panels **b** and **d** represents the percent activity (calculated as AUC). Each point represents mean ± s.e.m (n = 5). Values are means ± s.e.m. (n=5). ** $P < 0.01$; ***$P < 0.001$; compared to vehicle-treated group (Two-way ANOVA followed by Bonferroni's *post hoc* test). $^{\dagger\dagger}P<0.01$; $^{\dagger\dagger\dagger}P<0.001$; compared to vehicle-treated group (One-way ANOVA followed by Newman-Keul's *post hoc* test)

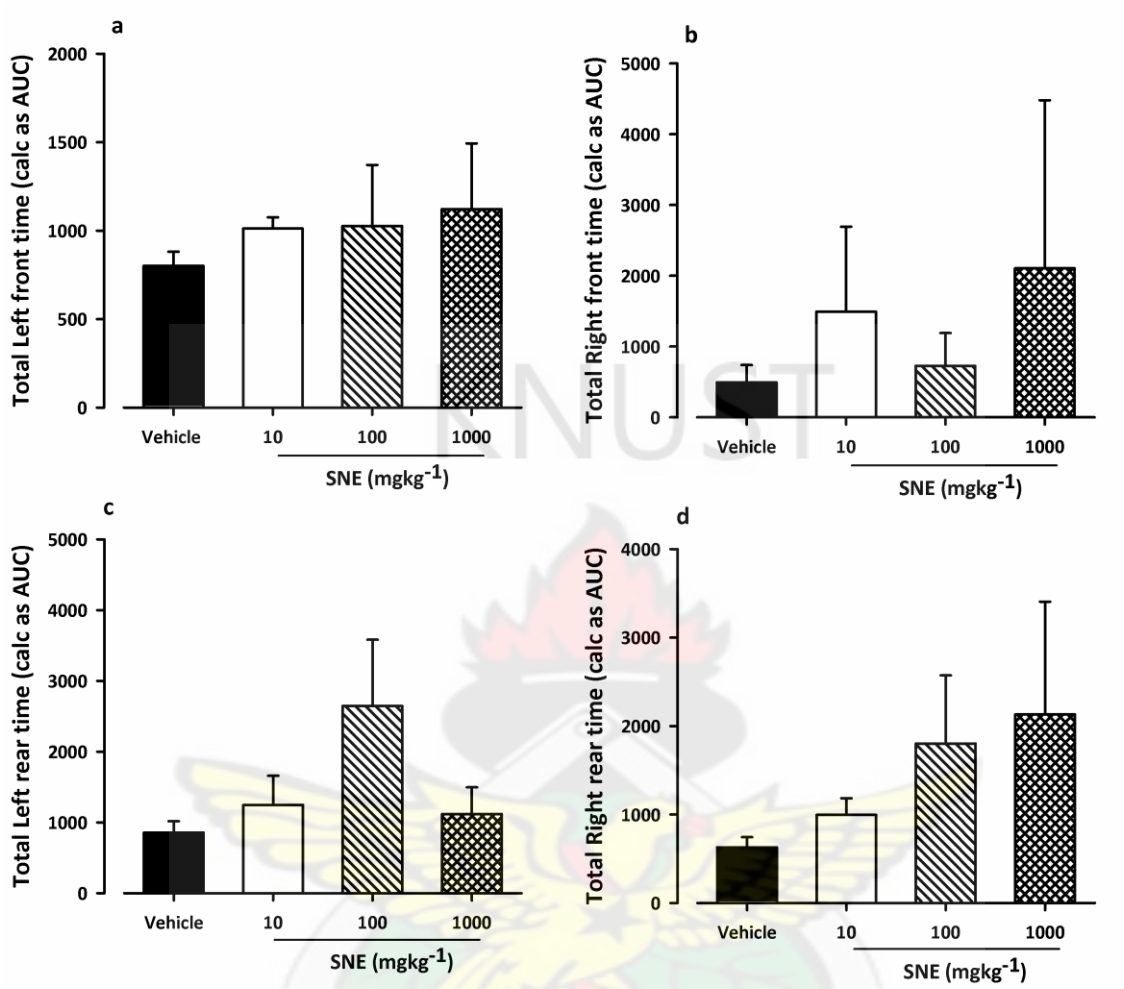

Figure 40: The effect of SNE (10-1000 mg kg^{-1}) on the time spent by mice at the left front (**a**), right front(**b**), left rear(**c**) and right rear (**d**) areas of the observation cage of VAMS measured over 180 min. Each point represents mean ± s.e.m (n = 5). Values are means ± s.e.m. (n=5). $P > 0.05$ not significant compared to vehicle-treated (One-way ANOVA followed by Newman-Keul's *post hoc* test)

3.6.3.3 Physiological Arousal Parameters

These as measured by VAMS include the stereotype count and duration. The results indicate that stereotype count and duration decreased significantly with increasing concentration of SNE (10-1000 mg kg^{-1}) ($F_{3,68}$ (count)= 11.02; (duration)= 9.559, P<0.0001, Fig 41)

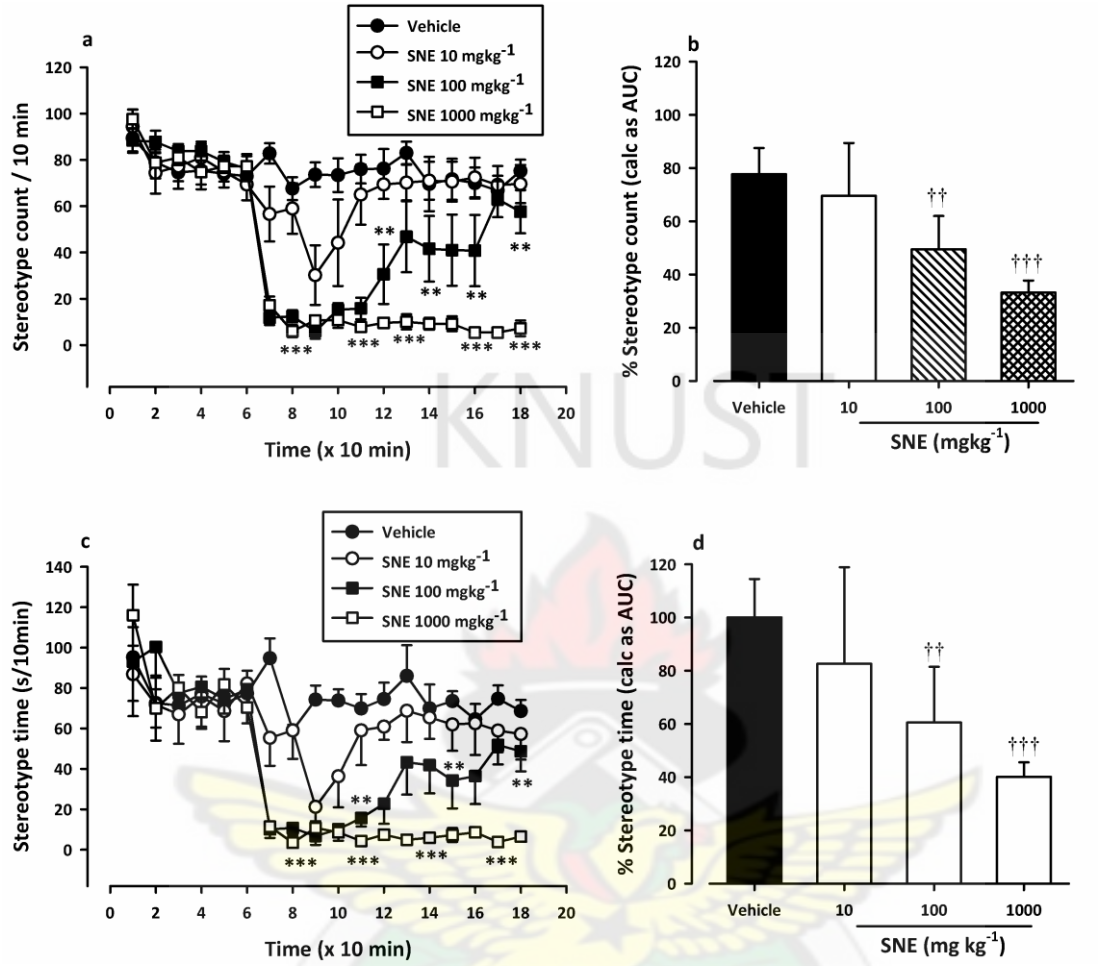

Figure 41: The effect of SNE (10-1000 mgkg^{-1}) on the stereotype count (**a** and **b**) and stereotype time (**c** and **d**) activity of mice in the Versamax activity monitor. Panels **a** and **c** shows the time-course curve of the activity measured over a three-hour period and panels **b** and **d** represents the percent activity (calculated as AUC). Each point represents mean ± s.e.m (n = 5). Values are means ± s.e.m. (n=5). ** $P < 0.01$; ***$P < 0.001$; compared to vehicle-treated group (Two-way ANOVA followed by Bonferroni's *post hoc* test). $^{\dagger\dagger}P<0.01$; $^{\dagger\dagger\dagger}P<0.001$; compared to vehicle-treated group (One-way ANOVA followed by Newman-Keul's *post hoc* test)

3.7 TOXICITY AND SIDE EFFECTS OF SNE

3.7.1 Acute toxicity

Oral administration of SNE (100-10,000 mg kg^{-1}) yielded no death after 24 hrs and the LD_{50} was approximated to exceed 10,000 mg kg^{-1}. However, an intraperitoneal administration of similar doses produced 100% mortality in mice pretreated with 10,000 mg kg^{-1}. From the spontaneous locomotor activity measured over the three hour it could be realized that movement among the SNE 10,000 mg kg^{-1}– pretreated mice (i.p) was brought to zero around the 90^{th} min (Fig.42), suggesting the occurrence of death.

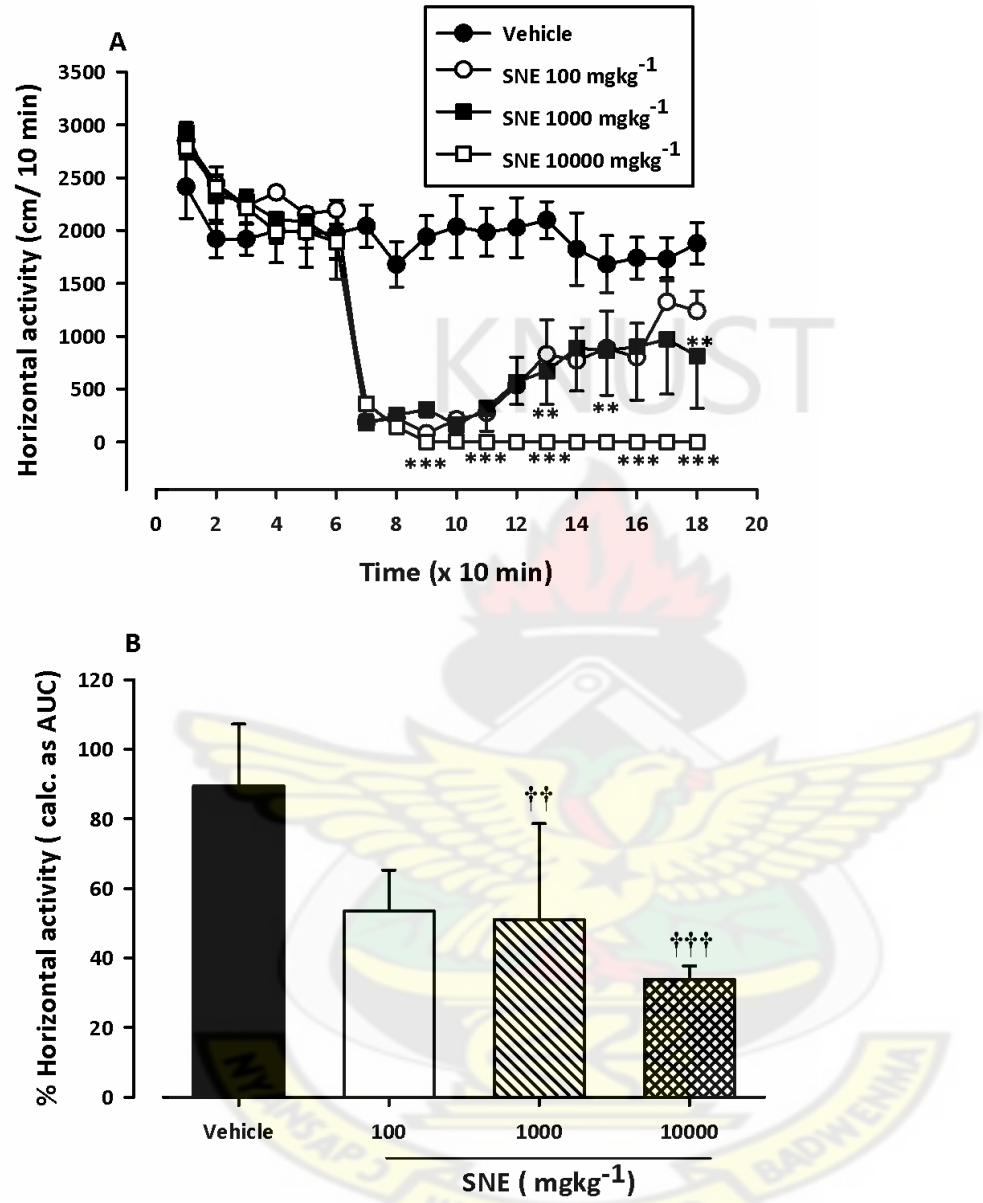

Figure 42: The effect of SNE (10-1000 mg kg^{-1}) on the horizontal activity of mice in the Versamex activity monitor. The graph labeled 'A' shows the time-course curve of the horizontal activity measured over a three-hour period and that labeled 'B' represent the AUC measured from the time-course curve. Each point represents mean ± s.e.m (n = 5). Values are means ± s.e.m. (n=5). ** $P <$ 0.01; ***$P < 0.001$; †† $P < 0.01$; $^{\dagger\dagger\dagger}P < 0.001$; compared to vehicle-treated group (one-way ANOVA followed by Newman-Keul's *post hoc* test)

3.7.2 Pentobarbitone- induced sleeping time

Pentobarbitone induced sleep in all the mice used. The sleep time was however increased in mice pre-treated with the extract (30-300 mg kg^{-1}) and this effect was dose dependent, though statistic significance was obtained for 100- 300 mg kg^{-1} ($F_{3,20}$ = 11.11, P= 0.0002 , Fig.48) . Diazepam, the reference drug, also increased significantly the sleep time of the mice (P< 0.01) (Fig.48).

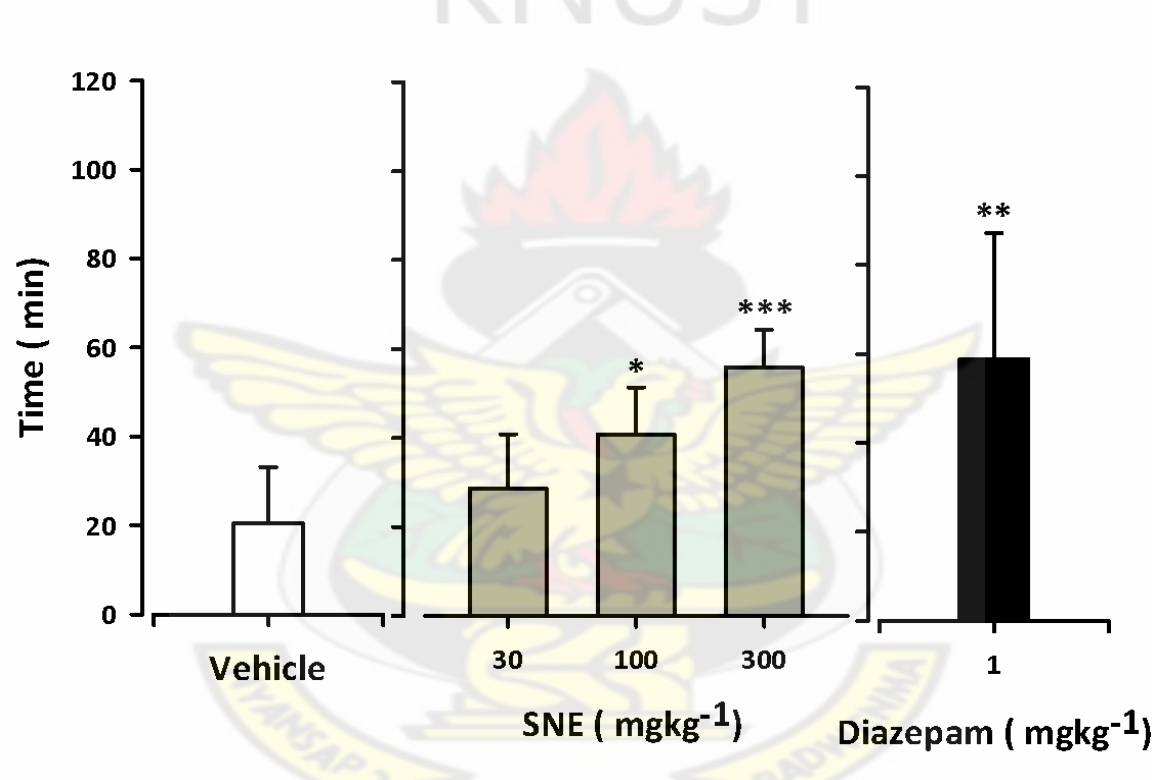

Figure 43: Pentobarbitone- induced sleeping time among mice pre-treated with SNE (30-300 mg kg^{-1}) and diazepam 1 mg kg^{-1}. *P < 0.05, ** P < 0.01, ***P < 0.001, compared to vehicle-treated group (One-way ANOVA followed by Newman-Keul's *post hoc* test)

Chapter 4

DISCUSSION

4.1 THE ANTICONVULSANT EFFECT OF S. NODIFLORA

The outcome of the study provides evidence that SNE possess anticonvulsant activity in the experimental animal models used. The effectiveness of the plant's extract in the experimental convulsion paradigm used probably suggests that the herb could be used in both *petit* and *grand mal* types of epilepsy (Mahomed and Ojewole, 2006).

The study revealed that SNE, diazepam and phenobartitone inhibited pentylenetetrazole (PTZ)-induced seizures. The pentylenetetrazole (PTZ) test represents a valid model for human generalized and absence seizures (Loscher and Schmidt, 1988). Pentylenetetrazole has been used experimentally to study seizure phenomenon and to identify pharmaceuticals that may control seizure susceptibility. The exact mechanism of the epileptogenic action of PTZ at the cellular neuronal level is still unclear but it has been generally reported to produce seizures by inhibiting gamma-aminobutyric acid (GABA) neurotransmission (De Sarro *et al.*, 2003). Enhancement of GABAergic neurotransmission has been shown to inhibit or attenuate seizures, while inhibition of GABAergic neurotransmission or activity is known to promote and facilitate seizure. Anticonvulsant agents such as diazepam and phenobarbitone inhibit PTZ-induced seizure by enhancing the action of $GABA_A$-receptors, thus facilitating the GABA-mediated opening of chloride channels (Gale, 1992; Olsen, 1981). Postsynaptic $GABA_A$-receptors are multi-unit complexes with binding sites for the endogenous ligand GABA, benzodiazepines, barbiturates and other ligands with a central chloride ion channel (Olsen and Leeb-Lundberg, 1981). Thus the inhibition of PTZ-induced seizures by SNE suggests that SNE may produce this effect by enhancing GABAergic neurotransmission although it is also possible that it could have done so by depressing glutamate-mediated excitation.

Picrotoxin, a $GABA_A$-receptor antagonist, produces seizures by blocking the chloride-ion channels linked to $GABA_A$ receptors, thus preventing the entry of chloride ions into neurons. This leads to decreased GABA transmission and activity in the brain. Thus,

convulsions arising from picrotoxin are due to the decreased $GABA_A$ receptor-mediated inhibition which tips the balance in favor of the glutamate-mediated excitatory transmission (Gale, 1992; Leidenheimer *et al.*, 1991). The ability of SNE to attenuate seizures induced by picrotoxin may possibly be due to an interaction with $GABA_A$ receptors and / or GABA neurotransmission. Phenobarbitone, a reference anticonvulsant, produced similar effects on picrotoxin-induced seizures. Phenobarbitone is known to enhance GABAergic neurotransmission by increasing chloride ion flux through the chloride channels of $GABA_A$-receptors (Davies *et al.*, 1997; Meldrum, 1975). Since SNE mimicked, to a large extent, the anticonvulsant actions of phenobarbitone, it is possible that SNE antagonizes picrotoxin-induced seizures by opening the chloride channels associated with $GABA_A$-receptors. It is possible to achieve these effects by suppressing glutamate-mediated excitation and this had to be verified by studying the effects of SNE on pure glutamate-mediated excitatory postsynaptic responses in a relevant brain region. Thus, further *in vitro* studies are needed to unequivocally determine the exact mechanisms by which SNE attenuate these seizures.

SNE also showed anticonvulsant effects against pilocarpine- induced seizures. Pilocarpine (PILO), a cholinergic agonist, is widely used in studies of epilepsy as a model of experimentally induced limbic seizures (Turski *et al.*, 1987). Pilocarpine induced seizures and status epilepticus (SE) produces many alterations in the central nervous system neurotransmission. There is evidence to demonstrate that an i.p administration of pilocarpine (300-400 mg kg^{-1}) significantly decreases not only the M_1, M_2, and GABAergic receptors densities, but also decrease acetylcholinesterase (AchE) and increases superoxide dismutase and catalase enzymatic activities in the rat straitum, frontal cortex and hippocampus (Frietas *et al.*, 2004a). Moreover, it has been observed that an acute treatment with 300-400 mg kg^{-1} pilocarpine induces long-lasting alterations in the serotonergic and glutamatergic receptor in the rat frontal cortex, hippocampus and striatum (Frietas *et al.*, 2004a). It is also known that the seizures and SE induced by pilocarpine produces alterations behaviourally and electroencephalographically similar to temporal lobe epilepsy (TLE). The results obtained suggest that SNE can exert anticonvulsive activity on seizures and SE induced by pilocarpine, as revealed by the decrease in the duration of the seizures

and mortality rate. Although the exact pathophysiological mechanism seems unknown, the whole process can be related to decreases in lipid peroxidation levels and free radical content. A variety of biochemical processes, including the activation of membrane phospholipases, proteases and nucleases which cause degradation of membrane phospholipids, proteolysis of cytoskeleton proteins and protein phosphorylation (Bostek, 1989; Erakovic *et al.*, 2000; Halliwell and Gutteridge, 1984; Savoure, 1993), can be triggered during seizures induced by pilocarpine. These seizures also lead to marked alterations in membrane phospholipid metabolism which further results in liberation of free fatty acids (FFA), particularly free arachidonic acid, diacylglycerols, eicosanoids, lipid peroxides and free radicals (Bostek, 1989; Erakovic *et al.*, 2000; Halliwell and Gutteridge, 1984; Savoure, 1993). These lipid metabolites along with abnormal ion homeostasis and lack of energy generation may contribute to cell injury and cell death (Pellegrini-Giampietro et al., 1988; Sugar et al., 1987). There are ample evidence to show that lipid peroxidation levels are increased during the acute period of pilocarpine seizures and SE induced by pilocarpine in adult rats (Frietas *et al.*, 2004b; Xavier *et al.*, 2007) and that certain antioxidants such as ascorbic acid have shown anticonvulsant activity against pilocarpine induced SE (Tejada *et al.*, 2007; Xavier *et al.*, 2007). In their finding it became clear that pilocarpine SE increases lipid peroxidation content in the hippocampus and confirmed the involvement of free radical levels in the pilocarpine induced brain damage. Unpublished research work done in our laboratory, also included in this dissertation, indicates that SNE has antioxidant and free radical scavenging effects in both *in vitro* and *in vivo* models of experimental oxidative stress. Thus it can be suggested that the effect exhibited by SNE on the pilocarpine induced SE could be partly due to its antioxidant effect.

Oral administration of SNE to rats decreased the progression of epileptogenesis induced by the subconvulsive dose of PTZ on alternate days for thirty-two days.The kindling model has become a widely employed tool for the study of seizure mechanisms and a useful experimental model of human epilepsy (Mason and Cooper, 1972). Pentylenetetrazole (PTZ) kindling is an acknowledged model for epilepsy and refers to a phenomenon in which repeated injection of subconvulsive doses of PTZ causes gradual seizure

development culminating in generalized tonic – clonic seizures associated with a cognitive deficit (Becker *et al.*, 1994), changes in emotional behavior (Duncan, 2002), and neuronal cell loss in hippocampal CA1, CA3, CA4 structures, dentate gyrus and the hilus (Franke and Kittner, 2001; Pavlova *et al.*, 2003; Pohle *et al.*, 1997). It is reported that free radical generation due to the increased activity of the glutamatergic transmitter plays a fundamental role in neuronal cell death of the PTZ kindling in rats (Becker *et al.*, 1997; Rauca *et al.*, 1999; Rocha *et al.*, 1996; Schroder *et al.*, 1993; Sechi *et al.*, 1997; Sejima *et al.*, 1997). Free radicals have been implicated in a number of seizure models including PTZ kindling (Gupta *et al.*, 2003; Sejima *et al.*, 1997) and that some antioxidants have been shown to be effective in these seizure models (Gupta *et al.*, 2003; Kabuto *et al.*, 1998; Willimore and Rubin, 1981). However, when the production of free radicals increases or the defense mechanism of the body decreases, they cause cellular dysfunction by attacking at the polyunsaturated sites of the biological membranes leading to lipid peroxidation (Gupta and Sharma, 1999). Zonisamide, a new AED, though the exact mechanism of action is unknown, has been reported to have free radical scavenging effects (Oommen and Mathews, 1999). SNE's ability to reduce the maximal effect of PTZ kindling in the rats used may be attributed to its ability to inhibit lipid peroxidation and scavenge free radicals. This possibility is supported by the *in vitro* experiments whereby SNE was observed to dose-dependently inhibit lipid peroxidation in brains from PTZ kindled rats. Additional *in vitro* experiments also show that SNE possesses anti-lipid peroxidation activity and free radical scavenging effects (Unpublished data). Thus, it is possible that SNE slowed the progression of epileptogenesis through antioxidant mechanisms. By contrast, diazepam, while slowing the progression of epileptogenesis, did not show any antioxidant properties.
The protective effect of diazepam on PTZ-induced kindling is well established and is known to occur via an interaction with the benzodiazepine –binding site of the GABA-benzodiazepine receptor ionophore complex (Schwark and Haluska, 1987).

The characterization of the anti-epilepsy potential of a test substance requires the calculation of a protective index (PI), which is the ratio of a CNS side effect to the anticonvulsant efficacy of the test substance. The PI ratio is the TD_{50} value for rota-rod failure or sedation divided by the ED_{50} value for seizure protection (Klitgaard *et al.*,

2002a). The PI is considered an index of the margin of safety and tolerability between anticonvulsant doses of anticonvulsant and the doses exerting acute adverse effects (e.g. sedation, ataxia, impairment of motor coordination or other neurotoxic manifestations) in preclinical studies. A large PI value, for example 10, indicates a potential for a clinically meaningful separation between the sedating or depressant CNS side effects commonly seen in this class of compounds, and the antiepileptic activity. The adverse effect used in this case is the motor incoordination as provided by the effect of the extract in mice using the rot-rod test. SNE produced high PI against PTZ, picrotoxin and pilocarpine induced seizures but showed a low PI against PTZ induced kindling. This shows that SNE possesses a clinically meaningful separation between its side effects and the anticonvulsant effect. The low PI for PTZ-kindling could mean than a high dose of SNE is needed to achieve the inhibition of epileptogenesis which could be accompanied by CNS side effects.

4.2 THE SKELETAL MUSCLE RELAXANT EFFECT OF SNE

The ability of the extract to significantly reduce the time spent on the rotating rod at 1000 mg kg^{-1} suggests that at higher doses, the extract impairs motor coordination. The accelerating rota-rod is a valid animal model used to assess the effects of drugs on motor coordination in rodents (Bohlen *et al.,* 2009). Time spent on the rotating rod is presumably affected by both sedation and loss of muscle tone (Green *et al.,* 2001). Regardless, some benzodiazepine such as diazepam causes a reduction in muscle tone through central mechanisms independent of sedation (Green *et al.,* 2001; Rang *et al.,* 2003). Therefore, the effect observed for SNE at 1000 mg kg^{-1} could be due to either sedation or centrally mediated mechanisms. Baclofen, the reference muscle relaxant used; at 10 mg kg^{-1} produced a significant reduction in the time spent on the rotating bar. Pharmacologically, it exerts this effect as a selective agonist at presynaptic $GABA_B$ receptor mostly abundant in the brain and spinal cord (Kuroiwa *et al.,* 2009; Waldman, 1994).

The in vitro effect of SNE indicates an antagonism on acetylcholine-induced contraction of the chick biventer cervicis preparation. The isolated chick biventer cervicis has been widely used to investigate the effects of drugs on neuromuscular transmission and is been known

to demonstrate response that would be seen in both rat diaphragm and frog/toad Rectus abdominis (Chang and Tang, 1974; Kitchen, 1984). Depolarising and non-depolarising neuromuscular agents are known to effect muscle relaxant effect on this model of isolated skeletal muscle preparation since it contains both slow and fast (twitch) muscle fibres (Kitchen, 1984). Competitive or non-depolarising neuromuscular blockers are known to block acetylcholine's response in the slow-contracting, multiply-innervated fibres of this muscle preparation (Marshall, 1971). Therefore, SNE can be said to exert muscle relaxant effect by employing similar mechanisms and that this effect is dose-dependent.

4.3 THE ANALGESIC EFFECT OF S.NODIFLORA

The present study demonstrates that the hydroalcoholic extract of *S. nodiflora* possess antinociceptive effect of in the formalin and writhing assays and attempts to investigate its mode of action. The formalin test first described by Dubuisson and Dennis (Dubuisson and Dennis, 1977) has been shown as the most predictive of acute pain (Le Bars *et al.*, 2001) and a valid model of clinical pain (Costa-Lotufo *et al.*, 2004; Vasconcelos *et al.*, 2003; Vissers *et al.*, 2003). The formalin test, is a well characterized and accepted method in pre-clinical screening of analgesics (Abbott, 1988; Vissers *et al.*, 2003) Intradermal injections of formalin into the rat paw resulted in a biphasic nociceptive response evidenced by flinching, licking or biting of the injected paw as reported earlier (Dubuisson and Dennis, 1977; Wheeler-Aceto *et al.*, 1990). An analgesic drug would tend to decrease the incidence of flinching, licking or biting of the injected paw (Courteix *et al.*, 1998). It is suggested that the first phase of the formalin response results essentially from the direct stimulation of nociceptors (Chau, 1989; Le Bars *et al.*, 2001; Szolcsanyi *et al.*, 2004) whereas the second phase involves inflammatory components with the release of different pain mediating substances that possibly activate small afferents (Le Bars *et al.*, 2001; Malmberg and Yaksh, 1992; Yashpal and Coderre, 1998). Centrally acting analgesics are known to inhibit the first phase (Le Bars *et al.*, 2001; Malmberg and Yaksh, 1992; Yashpal and Coderre, 1998), whereas NSAIDs and corticosteroids are known to inhibit the second phase (Vasconcelos *et al.*, 2003). Hence the analgesic properties exhibited by the *S. nodiflora* and morphine in both the first and second phases are characteristic of analgesics with central effects and peripheral anti-inflammatory properties (Le Bars *et al.*, 2001; Mino *et al.*, 2004)

and that exhibited by diclofenac in only the second phase is characteristic of cyclo-oxygenase inhibitors and therefore consistent with NSAIDs, (Malmberg and Yaksh, 1992; Mino *et al.*, 2004; Rosland *et al.*, 1990; Yashpal and Coderre, 1998).

Since *S. nodiflora* inhibits the second phase of formalin algesia it is likely to possess some peripheral anti-inflammatory properties. This assertion supports the findings published by Abad et al, that the plant extract possesses potent anti-inflammatory effect (Abad *et al.*, 1996).

The aqueous extract of *Synedrella nodiflora* is traditionally used in the management of epilepsy (Mshana *et al.*, 2000) and the hydro-ethanolic extract has demonstrated potent anticonvulsant activity in acute and chronic seizure models as explained earlier in this thesis dissertation. It has been discovered that anticonvulsants such as gabapentin and lamotrigine among others have exhibited analgesic effects in the formalin test (Sawynok *et al.*, 2001; Chesler *et al.*, 2003; Vissers *et al.*, 2003). It is now a common phenomenon for clinicians to use anticonvulsant such as phenytoin, carbamazepine, oxcarbazepine and gabapentin in the management of neuropathic pain (Carrazana and Mikoshiba, 2003; Jensen, 2002). Thus it is not surprising that the whole plant extract of *S. nodiflora* is used traditionally in treating epilepsy and pain (Mshana *et al.*, 2000; Burkill, 1985).

Phytochemical screening of the water and ethanolic extracts of *S. nodiflora* revealed the presence of sterols, reducing sugar, alkaloids, phenolic compounds, tannins and aromatic acid (Martin-Rathi and Gopalakrishnan, 2005). These substances were also detected during the phytochemical screening of the hydroalcoholic extract of *S. nodiflora* with the alkaloids being abundantly present. Reports suggest that alkaloids and phenolic compounds possess potent analgesic, anti-inflammatory and antioxidant effects. (Calixto *et al.*, 2000; Henriques *et al.*, 1996) Hence the potent analgesic effect exhibited by *S. nodiflora* may be related to the presence of these constituents present.

In an attempt to investigate into the mode of the analgesic activity of *S. nodiflora*, the effect of the extract was antagonized by naloxone and caffeine. Naloxone, an opioid antagonist, significantly blocked the analgesic effect of morphine in both phases of the formalin test but did not block the analgesic of *S.nodiflora* whereas caffeine, a non-specific adenosine receptor antagonist, (Fredholm *et al.*, 1994; Sawynok and Reid, 1996) significantly blocked the analgesic of *S. nodiflora* but had little effect on morphine-induced analgesia. Similarly,

caffeine inhibited the *S. nodiflora* - induced analgesia in the writhing test as well. Caffeine is a non-selective adenosine A_1 and A_2 receptor antagonist with comparable affinity at both receptors, but lacks activity at the adenosine A_3 receptor (Daly, 1993; Fredholm *et al.*, 1994). Current animal-based studies have repetitively demonstrated than caffeine enhances the anti-nociceptive activity of acetaminophen and non-steroidal anti-inflammatory drugs when in combined with them and studies have also revealed consistent intrinsic anti-nociceptive properties in the formalin test (Sawynok *et al.*, 1995). However, when administered with morphine, caffeine exhibits both inhibition (Sawynok *et al.*, 1993) and enhancement of the anti-nociceptive action of morphine (Ahlijanian and Takemori, 1985; Malec and Michalska, 1988; Misra *et al.*, 1985; Person *et al.*, 1985). Morphine has been shown to release adenosine in both in vivo and in vitro spinal preparations and this release of adenosine has been shown to contribute to its anti-nociceptive effect (Sweeney *et al.*, 1987). Hence, the ability of caffeine to inhibit the anti-nociceptive effect of morphine reflects antagonism of adenosine. Quite similarly, the ability of caffeine to inhibit the anti-nociceptive effect of SNE in the formalin and acetic acid induced writhing test indicates that adenosine probably plays a role in the antinociceptive effect of SNE. There is ample evidence to prove that adenosine and its analogs alter pain transmission by actions on both nociceptive afferent and transmission neurons, and these actions are principally mediated by adenosine A_1 receptor (Sawynok, 1998; Sawynok and Liu, 2003; Sawynok *et al.*, 1999). Thus the effect of *S. nodiflora* on nociception may probably be due to the release of adenosine or direct activation of adenosine A_1 receptor. Further experiments involving the use of specific agonists and antagonists of A_1 receptors are necessary to confirm this assertion. Furthermore, findings have shown that adenosine A_1 receptor agonists have been shown to exert anticonvulsant effects within a number of models of epilepsy and this effect is mainly due to the well known inhibitory effect of A_1 receptors upon synaptic transmission in the hippocampus (Dragunow, 1990; Dunwiddie and Worth, 1982), and also some anticonvulsants like diazepam and sodium valproate have been found to exhibit their anticonvulsant properties through the activation of adenosine receptors (Candenas *et al.*, 1991; Gupta and Malhotra, 1997). Hence, it is not startling to realize that the anti-nociceptive effect of SNE may probably be linked to adenosinergic mechanisms, which could possibly play a role in its anticonvulsant effect.

The findings of the present study lend pharmacological support to the suggested traditional uses of *S. nodiflora* plant extract in the treatment, management and/or control of epilepsy in some rural communities of Ghana.

4.4 THE EFFECTS OF SNE IN EXPERIMENTAL MODELS OF ANXIETY

To assess the effect of the extract in anxiety, various models of anxiety-like effects were employed including the elevated plus maze, the light/dark box and the versamax animal monitor systems. The extract produced anxiogenic-like effects in all the models used and also decreased the locomotor activity of the mice pretreated with it.

The elevated plus maze (EPM) is considered as one of the valid ethological animal model of anxiety since it employs natural stimuli (fear of a novel, brightly lit open space and fear of balance of a relatively narrow and raised platforms) capable of inducing anxiety in humans (Dawson and Tricklebank, 1995; Imaizumi and Onodera, 2000; Jung *et al.*, 2000) . This test has been described to produce bi-directional sensitivity to both anxiolytic drugs, particularly benzodiazepines and anxiogenic agents used in humans. Generally, anxiolytics are known to increase the percentage number of entry into and duration spent in the open arm of the EPM.

Acute oral administration of SNE (10-300 $mgkg^{-1}$) produced an anxiogenic-like effect in mice as it decreased the percentage number of entry and percentage time spent in the open arm of the maze. Diazepam, in agreement to previously reported studies, produced an opposite effect to that induced by SNE and this effect has been shown to be mediated via the GABAergic system.

Ethological parameters indicative of risk assessment, such as stretch-attend posture and exploratory head dipping, which have been validated to be predictive of anxiety (Rodgers *et al.*, 1997; Rodgers and Cole, 1994; Rodgers and Johnson, 1995), were also measured in this study. These parameters have also been shown in the elevated zero-maze to distinguish between anxiogenesis or sedation (Shepherd *et al.*, 1994). SNE induced anxiogenic-like effects by decreasing the number of head dipping whereas in the diazepam treated group, as expected, the number of head dipping increased (Rodgers *et al.*, 1997). However, both SNE

and diazepam decreased the number of stretch-attend postures thus suggesting anxiolysis (Rodgers *et al.*, 1997). This indicates that the anxiogenic-like effect observed may not really be so but rather due to sedation. The total number of entries into the arms has been used as an indicator of locomotor activity of rodents in the EPM and that anxiolytic agents have been shown to generally increase this parameter (Cruz *et al.*, 1994; File and Aranko, 1988; Lister, 1987). Since the total number of entries into both the closed and open arms was decreased by SNE in comparison with diazepam, SNE is more likely an anxiogenic than anxiolytic. In contrast, a decrease in total entries has also been attributed to sedation or locomotor impairment rather than anxiogenesis (Weiss *et al.*, 1998). In these instances, measures of the number of stretch-attend posture have been used to dissociate anxiogenesis from sedation or locomotor impairment. SNE reduced both total arm entries and the number stretch-attend postures thus indicating that the effects observed could be due to sedation and / or locomotor impairment rather than anxiogenesis (Dubinsky *et al.*, 2002). A classical example is that, the anxiogenic drug m-chlorophenylpiperazine (mCPP), which reduces total entries in the EPM, was shown in the zero-maze to reduce the time spent on the open areas but to increase the number of stretched–attend postures, supporting the conclusion that the effects of mCPP in this test are due to anxiogenesis rather than sedation or locomotor impairment (Shepherd *et al.*, 1994). In addition, SNE has also been shown to produce sedation and motor incoordination in similar doses as used in this study.

The light/dark box paradigm is an ethologically-based approach-avoidance conflict test and is widely used to investigate drugs that affect anxiety (Chaouloff *et al.*, 1997; Costall *et al.*, 1989a; Crawley *et al.*, 1997). Anxiolytic drugs have been known to increase the number of entry and duration spent in the light compartments. Analysis of the results obtained from this test indicates that SNE possess an anxiogenic-like effect since it decreased the number of entries into the lit compartment and also reduced the time spent in this compartment. Moreover, the number of transitions has been used as an index of activity-exploration and is more sensitive to sedation or psychostimulant effects of drug treatment (Hascoet and Bourin, 1998). Even though anxiolytics are known to generally increase this parameter, sedation, stimulation or changes in exploration induced by anxiolytics have been known to cause problems (Imaizumi *et al.*, 1994). For instance, diazepam and alprazolam at sedative doses have been reported to cause decreased number of transitions whereas increased

transitions were seen with caffeine (Hascoet and Bourin, 1998). Thus the gradual decrease in the transitions observed by SNE could be due to sedation rather than anxiogenesis.

The effects of oral administration of SNE to mice in the observation cages of the versamax animal activity systems (VAMS) indicate that SNE decreased locomotor activity and produced anxiogenic-like effects dose dependently. VAMS produces a set of responses, demonstrated by test animals, which can be characterized as locomotor, anxiety or physiological arousal. VAMS is basically a modified open field paradigm test and parameters scored in this test are the same as that of a typical open field. The advantage of this system over the conventional open field paradigm is that scoring of parameters are done by the computer thus excluding human errors of omission which can happen with conventional open field test. Also, the test animals used were made to acclimatize in the VAMS for three hours each day for three days before beginning the actual test, and on the test day too, an hour of primary, drug-free period was allowed before the two hour post treatment assessment was done. This ensures that contrasting behavioral effects of drug treatment are vividly observed and free of any bias due to the animal tested.

Locomotor activity is measured as horizontal and vertical activities, and the total distance travelled by the animal in the observation chamber/cage over a two- hour post treatment. SNE significantly and dose-dependently decreased horizontal and vertical motor activity and the total distance travelled. A drug-induced decrease in spontaneous horizontal motor activity is regarded as an indication of sedation (Kinnard and Carr, 1957). SNE has also been shown to have sedative effects (in the pentobarbitone- induced sleeping time assay) confirm the sedation as the cause of the reduced locomotor activity observed.

Typical anxiety parameters that can be obtained from VAMS include the margin distance and time at the margins, center distance travelled and time spent at this area and the total time spent at the left and right front or rear corners of the observation field. This modified open field measures anxiety- related behavior characterized by a normal aversion of experimental animals to brightly lit environment (Choleris *et al.*, 2001; Mechan *et al.*, 2002). Thus, when test animals are removed from their acclimatized cages and placed in

novel environments, they express anxiety and fear by causing alterations in all or some of the parameters, such as decrease in ambulatory and exploration in the center of the field with increased peripheral movements or thigmotaxis (Bhattacharya *et al.*, 1987). These parameters are attenuated by classical anxiolytics but potentiated by anxiogenics. Thes results obtained suggest the SNE is anxiogenic-like since it reduced entry into the central area and increased the time spent at the margins. Since the general activity of the mice pretreated with SNE was reduced, it is quite likely that the observed effect were due to sedation but not anxiogenesis.

Physiological arousal and risk assessment parameters such as grooming and stretch attend postures (supported or unsupported) respectively are provided together as stereotype counts and time spent stereotyping by VAMS since the computer could not differentiate one from the other. If the animal breaks the same beam (or set of beams) repeatedly then the monitor considers that the animal is exhibiting stereotypy. This typically happens during grooming, head bobbing, and stretch-attend postures etc. An increase in physiological arousal indicates anxiolysis whereas a decrease connotes anxiogenesis but decrease in risk assessment like stretch-attend posture indicates anxiolysis. The results indicate that SNE dose-dependently decreased both the stereotype counts and duration. Since the stereotype measurement does not differentiate physiological arousal from risk assessment, the observed effects by SNE can be due general decrease in activity rather than anxiogenesis. In summary it would be difficult to fully determine whether SNE is anxiolytic or anxiogenic because of confounding effects of SNE-induced changes in locomotor activity. However, it seems that doses lower than 10 $mgkg^{-1}$ of SNE may exhibit anxiolysis which is masked by sedation or impaired motor coordination as the dose increases.

4.5 THE ANTIOXIDANT AND FREE RADICAL SCAVENGING EFFECT OF S. NODIFLORA

The results obtained indicate that the hydro-ethanolic extract of *Synedrella nodiflora* possess potent antioxidant and free radical scavenging effects and this property has been found to augment its anticonvulsant property.

Polyphenols (electron-rich compounds) have the ability to go into electron-donation reactions with oxidizing agents to form stable species (Kang *et al.*, 2005) and thus inhibit or delay the oxidation of different biomolecules (Amarowicz, 2005; Seidel *et al.*, 2000). Hence various plant phenols such as vitamin E (α-tocopherol), exhibit antioxidant properties (Kang *et al.*, 2005; Ozgova *et al.*, 2003a; Seidel *et al.*, 2000).

Phenolic antioxidants are potent free radical terminators and this is thought to be due to the ability to donate hydrogen to free radicals and their presence is a good marker of potential antioxidant activity. The high potential of phenolic compounds to scavenge free radicals may be explained by their phenolic hydroxyl groups. Detection of phenols in the *S. nodiflora* extract was a preliminary evidence of its possible antioxidant activity. The total phenol was assayed based on the reduction of phosphomolybdate-phosphotungstate salts to form a blue complex that is detected quantitatively at 700 nm. The total phenolic content of the extract expressed as the tannic acid equivalents increased concentration dependently.

The phosphomolybdenum method of assay of the total antioxidant capacity was based on the reduction of Mo (VI) to Mo (V) by the antioxidant compound and the formation of a green phosphate/ Mo (V) complex with a maximal absorption at 695nm. Chemical constituents that might contribute to the total antioxidant capacity include carotenoids, flavonoids and cinnamic acid derivatives (Taga *et al.*, 1984). Also a high correlation was obtained between the total antioxidant capacity and the total phenol content suggesting that the phenols detected may basically be responsible for the antioxidant effect found in SNE.

A number of *in vitro* models have been used for the assessment of antioxidant properties of pharmacologically active agents. Antioxidants may be classified according to their chemical nature and mode of function. Based on their mode of action, three types have been found to be consistent; enzyme antioxidants; the preventive oxidants (Cui *et al.*, 2004), and the scavenging or chain-breaking oxidants (Scheibmeir *et al.*, 2005) DPPH is a used as a reagent to evaluate free radical scavenging activity of antioxidants (Oyaizu, 1986), It is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule (Soares *et al.*, 1997). The reduction capability of DPPH is

determined by the decrease in the absorbance at 517 nm induced by antioxidants. The extracts ability to concentration- dependently reduce DPPH forming yellowish-coloured diphenylpicylhydrazine suggest that SNE is a free radical scavenger and acts so by donating an electron or hydrogen radical.

The extract showed a concentration dependent increase in its reducing power. This test investigates the ability of an agent to transform Fe^{3+} to Fe^{2+} (Oyaizu, 1986). Other authors have observed a direct correlation between antioxidant activity and reducing power of certain plant extracts and have associated this effect with the presence of reductones (Duh, 1998; Tanaka *et al.*, 1998). Reductones are also reported to react with certain precursors of peroxides, thus preventing peroxide formation. However activity of antioxidants has been attributed by various mechanisms, among which some of them are prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity, and radical scavenging (Diplock, 1997).

The extract showed an ability to inhibit lipid peroxidation in linoleic acid antoxidation assay. In biological systems, lipid peroxidation generates a number of degradation products, such as MDA, and it is found to be an important cause of cell membrane destruction and cell damage (Yoshikawa *et al.*, 1997). Lipid peroxidation is an oxidative alteration of polyunsaturated fatty acids in the cell membranes that generates the aforementioned degradation and is considered a marker of oxidative stress (Janero, 1990). Most researchers use the homogenised brain as their source of polysaturated fatty acid (PUFA) base (Woode *et al.*, 2007). Linoleic acid autoxidation assay was chosen due to it relatively simple and cost effective methodology (Mitsuda *et al.*, 1967).

Excessive production of oxygen free radicals and other radical species have been suggested to participate in neuronal pathology (Flamm *et al.*, 1978; Granger *et al.*, 1986) as well as in age related neuronal disorders and other forms of neurodegeneration (LeBel and Bondy, 1991; LeBel *et al.*, 1992). However, the extreme reactivity of oxygen radicals has made their direct detection and quantification difficult, and therefore free radical involvement in pathologic conditions has generally been inferred from the measurement of indirect

markers of oxidative stress such as the extent of lipid and protein oxidation and the activities of free radical scavenging enzymes (Bruce *et al.,* 1995). Histological investigations of epileptic brains show neuronal cell loss especially in hippocampal structures (DeGiorgio *et al.*, 1992; Meldrum, 1993). Neuronal cell death results from the increased activity of glutamatergic systems which play a crucial role in epilepsy as reported from animal experiments (Rauca *et al.,* 1999). It is well known from experiments regarding ischemic cerebral injury that the enhanced activity of glutamatergic systems induces an increased formation of free oxygen radicals which may amplify again the basal release of excitatory amino acids including an increase in intracellular calcium which finally results in neuronal cell death (Gilman *et al.*, 1994; Grammas *et al.,* 1993). Recent findings indicate that a linkage exists between the generation of clonic–tonic seizures and the increased formation of free radicals in the brain (Rauca *et al.*, 1999). These assertions suggest that antioxidants can offer some adjunct support to the treatment of epilepsy and that anticonvulsants with antioxidant properties may be beneficial. SNE was able to inhibit lipid peroxidation in PTZ kindled rat brains. Thus it can be suggested that this property exhibited by SNE in vivo could have played a part in the prevention of epileptogenesis induced by the PTZ.

Some AEDs like Zonisamide has been known to have free radical scavenging effects (Oommen and Mathews, 1999) and contrastly sodium valproate is known to induce hepatoxicity which can be alleviated by free radical scavengers (Buchi *et al.,* 1984; Seckin *et al.,* 1999). Thus, SNE's ability to scavenge free radicals and prevent oxidation can augment its role as antiepileptic and analgesic.

4.6 TOXICITY AND SIDE EFFECT PROFILE OF SNE

Oral administration of SNE even to a dose of 10,000 mg kg^{-1} yielded no deaths in the mice treated. These results indicate that SNE is relatively safe. Belmain *et al,* 2001 undertook a study of the insecticidal and vertebrate toxicity of various extracts from plants including *S. nodiflora*. Their findings indicated that *S. nodiflora* exhibited some level of insecticidal effect as reported by other studies (Martin-Rathi *et al.,* 2005). On the vertebrate toxicity, it

was found that none of the extracts from the plants under study exhibited any neurotoxicological or neurobehavioural effects in the rats used over the study period. This confirms that assertion that the hydro- ethanolic extract of *S. nodiflora* possess a good safety profile as far as the therapeutic doses (10-1000 mg kg^{-1}), used in this study are concerned.

The pentobarbitone –induced sleeping time showed that SNE possess sedative effects. This is not surprising, since most anti-epileptics, especially those possessing GABAergic properties like diazepam and phenobarbital produce sedation in patients. More so, SNE exhibited skeletal muscle relaxant effect as indicated by its effect in treated mice on the rotarod. These side-effects exhibited by SNE could have therapeutic value as some anticonvulsants such as diazepam have been used as a sedative and spasmolytic.

4.6.1 General Discussion: Summary

Orally administered extract of *S. nodiflora* in murine models of experimental epilepsy indicates that the extract possess active constituents with anticonvulsant effects possibly mediated via GABAergic (as indicated both the PTZ and picrotoxin-induced seizure tests) or antioxidant mechanisms (also illustrated by the pilocarpine-induced seizures and PTZ-kindling). The extract also showed sedative effects by enhancing pentobarbitone-induced sleep in rats. Moreover, SNE reduced the time spent on the rota-rod at 24 rpm in mice and competitively antagonised acetylcholine-induced contractions in the isolated chick biventer cervicis preparation thus exhibiting skeletal muscle relaxant effect *in vitro*. All these effects were similarly exhibited by reference drugs such as diazepam and phenobarbitone which are known to exert these effects via GABAergic mechanisms. Even, though the exact mechanisms by which SNE exhibits these effects are yet to fully investigated, it would not be surprising to find out that they are mediated via the GABAergic system.

SNE also produced antinociceptive effects in the formalin and acetic acid induced writhing test. In the formalin test, SNE was effective in both the first and second phases of this paradigm thus SNE employs both central and peripherally mediated mechanisms in its antinociceptive effect. Further determinations reveaed that these effects by SNE were

antagonised by caffeine but sparsely by naloxone, thus the possible involvement of adenosinergic mechanisms in the antinociceptive effects exhibited. Similar effects were obtained by the acetic acid induced writhing assay. These indicate, as previously reported, the central analgesic effect of SNE.

In vitro antioxidant assay of SNE suggests the presence of active principle(s) with free radical scavenging effects, increased reducing power effect and inhibits lipid peroxidation. Antioxidants have found to offer some adjunct support to the treatment of epilepsy and that anticonvulsants with antioxidant properties are beneficial. Thus the antioxidant effects of SNE may augment it antioconvulsant effect.

The extract exhibited anxiogenic-like effect in all the murine models of anxiety used, namely; EPM, light/dark test and versamax animal monitor. Generally, the locomotor activity of mice pretreated with SNE in all the models was reduced. SNE effects centrally have been proposed to be GABAergic or adenosinergic and as such a CNS depressant. Also it exhibits sedation and cause motor incoordination at high doses in rodents. A decrease in locomotor activity could either be due to anxiogenesis or sedation/ impaired motor effect. Thus, considering all other effects of SNE, it is possible that the observed effects in these models of anxiety are due to sedation and / motor impairment rather than anxiogenesis.

Oral administration of SNE at an extreme dose such as 10 g kg^{-1} failed to elicit any deaths in mice treated over a 24h period and the LD_{50} is estimated as > 10,000 mg kg^{-1}. This confirms previous reports that the plant extract is relatively safe in rodents. More so, since the leaves of the plant is eaten as food with no known documented or reported adverse effects, the plant extract can also be said as less toxic in humans as well. However, sedation and impaired motor effects are likely to occur when the plant is used in traditionally in the management of epilepsy and pain.

Chapter 5

CONCLUSIONS

5.1 GENERAL CONCLUSION

In conclusion, the research findings suggest that the hydro ethanolic extract of the whole plant of *S. nodiflora* has anticonvulsant effect in the acute and chronic seizure models of epilepsy used and indicates a possible enhancement of GABA activity; exhibits central analgesic effect possibly mediated through adenosinergic mechanism and a peripheral anti-inflammatory activity and a potent antioxidant and free radical scavenger, a muscle relaxant and has anxiogenic-like effects.

5.2 RECOMMENDATIONS

1. The active constituent(s) responsible for the neuropharmacological effects observed should be isolated and characterized and studied further.

2. A product from the extract can be formulated and used as adjunct therapy in epilepsy management. This can be done when drug–herb interaction studies have been done to guide clinicians in the usage of the herb as such.

3. Acute toxicity studies in rats and other animal species should be carried out on SNE and must be also extended to 48 hours. Sub acute and chronic toxicity studies should be done to clearly substantiate the reported claims of less toxicity

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