

**AN EVALUATION OF PLANTS
USED IN EASTERN NIGERIA IN
THE TREATMENT OF
EPILEPSY AND CONVULSION**

by

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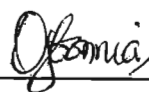
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PREFACE

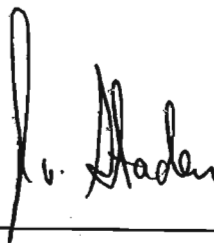
The experimental work in this thesis was carried out under the supervision of Professor J. van Staden in the Research Centre for Plant Growth and Development, School of Botany and Zoology, University of Natal Pietermaritzburg.

The studies have not been submitted in any form to any other University and is the result of my own original investigation, except where the work of others are acknowledged.



Steve Okwudili Ogbonnia

I certify that the above statement is correct.



Professor J. van Staden
(SUPERVISOR)

2002

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ABSTRACT

Schumanniohyton magnificum and *Glypheae brevis* are important medicinal plants growing wild in the West African rain forest. They are used in folkloric medicine in the treatment of epilepsy and convulsion as well as for some other diseases. The purpose of this work was to investigate the aspect of folkloric use in order to support folkloric claims and document the findings. The extracts were prepared from ground plant material by a continuous extraction method.

Five hundred grams of ground plant material were continuously de-fatted with 2 L petroleum ether (60°- 80°) in a Soxhlet apparatus for about 5 h. The resulting marc was dried and the chemical constituents extracted hot in a Soxhlet apparatus for about 8 to 10 h with 2 L aqueous ethanol (70%). The efficacy of the extraction method was confirmed using standard bioassays and phytochemical analyses.

The anti-convulsant activity of the crude extracts was evaluated *in vivo* against chemically induced convulsions using three different animal models, namely the strychnine, the picrotoxin and the pentylenetetrazole tests. The acute and delayed toxicity test results showed that in all the animal models investigated very high doses, about four times higher than the protective doses of the extracts, were required to kill 50% of the population of animal used.

Phytochemical assays of the extracts indicated the presence of alkaloids only in *S. magnificum* root extract and glycosides in extracts from both species. The glycosides were positive to Baljet, Xanthydrol and Keller-Kiliani tests for cardiac glycosides.

S. magnificum and *G. brevis* chemical constituents were initially isolated with a sequential fractionation method starting with a highly non-polar solvent and gradually increasing to a more polar solvent. The fractions were pooled on the basis of TLC similarity profiles when viewed under the UV light at 254 and 366 nm and were found to have two and four major UV absorbing fractions for *S. magnificum* and *G. brevis* respectively.

Radio-receptor binding tests were used to assess the anti-convulsant activities of the hydro-alcoholic crude extracts, the organic and aqueous fractions of the crude extracts, partially purified components and pure components in *in vitro* tests against some standard GABA_A receptor antagonists, muscimol and isoguvacine respectively. The anti-convulsant activities resided in the aqueous fractions of the hydro-alcoholic crude extracts of both plants. The purely organic fractions of *G. brevis* demonstrated no activity while all the fractions of the aqueous component demonstrated some degree of activity. The anti-convulsant activity of *S. magnificum* was found only in one fraction-Fraction 1. This Fraction was further investigated and one of the components appear to be responsible for the activity. The structure of the active constituent was 5,7-dihydroxy-2 methylbenzopyran-4-one, a noreugenin. A second bioactive compound, schumanniofoside, was identified from Fraction M_{5,2} from *S. magnificum*.

CONFERENCE CONTRIBUTION FROM THIS THESIS

OGBONNIA, S.O., VAN STADEN, J. and JÄGER, A.K. 2002. Anti-convulsant activity and phytochemical screening of the roots of *Schumanniphyton magnificum*. **Twenty-eighth Annual Congress of the South African Association of Botanists (SAAB), Rhodes University, Grahamstown, South Africa.** Abstract S. Afr. J. Bot. 68: 267.

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ABBREVIATIONS

CDCl_3	Deuteriochloroform
CD_3COOD	Deuteroacetic acid
CD_3OD	Deuteromethanol
CF_3COOD	Deuterated trifluoroacetic acid
DMSO	Dimethylsulphoxide
DMSO-d_6	Deuterodimethylsulphoxide
D_2O	Deuterowater
D_2SO_4	Deuteriosulphuric acid
ED_{50}	Medium value of the individual effective dose
HPLC	High pressure liquid chromatography
HRMS	High resolution mass spectrometer
i.d	internal diameter
NMR	Nuclear magnetic resonance
Pyridine- d_5	Deuteropyridine
TLC	Thin layer chromatography
TMS	Tetramethylsilane
VLC	Vacuum liquid chromatography

CHAPTER 1

GENERAL INTRODUCTION

1.1 INTRODUCTION

The discovery that plant products are beneficial for human and animal ailments marked the dawn of what is today referred to as modern medicine. Plant products have ever since remained vital to man and animal life, being of great value as a source of potent medicaments, and indispensable as sources of food and energy.

The history of herbal drugs - their invention, introduction and use - are therefore, the story of man's earliest quest for knowledge and wisdom to solve his problems of illness which results in restoration of good health and prolonging of life. There was also the discovery that drugs can be obtained from animal sources. The use of herbal and other natural based medicines therefore has a long history (COLEGATE and MOLYNEUX, 1993) and medicinal plants remain a potential source of potent medicaments.

Primitive man relied solely on herbal medicines. Today traditional healers also find some medicinal plant extracts efficient for the relief of various pains and ailments. Many of the ancient natural product drugs are still in use today (MANN, 1978). These include drugs such as *Digitalis* leaves (foxglove) which has a very long history and is a worldwide remedy for congestive heart failure. The roots of *Rauwolfia serpentina* has been used from time immemorial in Indian traditional medicine to treat the mentally ill. Extracts from *Rauwolfia vomitoria* roots are used in Nigeria and other African countries as traditional medicine to sedate mentally disturbed patients and in treatment of other diseases either alone or in combination with other plants/herbs (DALZIEL, 1956; KOKWARO, 1993).

Opium, ephedra alkaloids, castor oil, mint and rosemary oil also are some important and cherished ancient drugs still much in use today (EVANS, 1989). In the literature there are many references to the use of plant extracts in the healing of a variety of diseases; for application as agents of death ranging from that of calabar bean and hemlock

alkaloids; use as judicial poisons; and utilization as South American arrow poisons (MANN, 1978; TYLER *et al.*, 1988; HERBERT, 1989).

As human society developed with its accompanying population increase, there were manifestations of different types of ailments. This inadvertently led to an increase in the efforts to discover more plants/herbs endowed with the powers of curing more diseases. Because it was not known why potions worked the way they do, early medicine was linked with black magic, and sorcery. For this reason the details of what to do with each herb, and for which diseases, were often kept secret by the local priests (SOFOWORA, 1993). The priests often performed dual roles as both priest and medicine man. In modern times, it has been established that the physiological and pharmacological activities of plant and herb extracts are due to the organic chemicals in them. Many of these organic compounds have been isolated from plants and micro-organisms and are employed in the treatment of various human and animal diseases with success. These organic compounds form part of a large group known as 'natural products' or 'secondary metabolites'. Many of these compounds have useful physiological and pharmacological properties and have been successfully employed in the treatment and management of certain ailments that were resistant to, or could not be treated with, synthetic drugs (SOFOWORA, 1993). Epilepsy is one such disease for which synthetic drugs could only offer symptomatic relief. It is frequently treated with herbal drugs, resulting in permanent recovery. Presently the focus for the treatment of epilepsy is on traditional medicine or drugs of plant/herb origin. This prompted this research on extracts from *Schumanniphyton magnificum* and *Glyphaea brevis* extracts.

1.2 LITERATURE REVIEW

This review concentrates on epilepsy and the test plants namely, *Schumanniphyton magnificum* Harms and *Glyphaea brevis* (Spreng) Moraches that are used either singly or in combination in the treatment of epilepsy and convulsion in Igboland the south eastern area of Nigeria. It also covers some of the secondary metabolites or natural products and synthetic products that exhibit some anti-convulsant activities. Some chemical properties of the synthetic drugs are briefly outlined.

1.2.1 Epilepsy

The term epilepsy is derived from the Greek word 'Epilambanein' meaning 'to seize'. It is one of the most dreadful non-communicable diseases. It was succinctly described by one authority as the only disorder, disease or disability where the sufferer is more handicapped by the attitude of the Society than by his own ability (KIFFIN and PENRY, 1976). This societal attitude towards the sufferer can be attributed to the seizures through which epilepsy is manifested.

Many definitions have been given for epilepsy. It was defined by JASPER *et al.* (1969) as a seizure of the mind and senses together with sudden fall. In some cases it is associated with convulsions and in others without; in certain cases as a group of diverse disorders of brain function or as diseases with a common central nervous system manifestation with the occurrence of seizures (PARKER, 1988). Seizures comprise of abnormal motor activity, abnormal sensory activity, abnormal autonomic activity, and abnormal psychic activity and was defined by Hughlings Jackson, "as a state produced by an abnormal discharge within the central nervous system" (VIDA, 1977). A seizure may manifest itself as a transient alteration of behaviour due to disordered, synchronous and rhythmic firing of brain neurons (ELIASSON *et al.*, 1974).

Epilepsy was once regarded and described as a 'sacred disease', because its causes were largely unknown and attributed to the supernatural in ancient times. This belief continues even today in some clans. TEMKIN (1971) in his authoritative work, 'The Falling Sickness' gave an account of the earliest known treatise on epilepsy. The Hippocratic writing dealing with the subject dates back to about 400 BC. In one of the works titled "On the Sacred Disease", there is a survey on the beliefs and practices and a vigorous attack on magicians and charlatans who called the disease 'sacred' as a shelter to hide their own ignorance and fraud.

In modern times, with advances in medical and biological sciences, the possible causes of epilepsy are being uncovered. It has been established that epilepsy, can be associated with an altered physiological state of the central nervous system (CNS), which may result from any disordered function of the body capable of changing the ionic milieu, metabolism or structure of the nerve cells within the central nervous system (ELIASSON *et al.*, 1974). This may occur as a result of a brain disorder in which

abnormal bursts of electrical activity occur in the cells of the brain, resulting in seizures. It has to be pointed out that not all nerves within the central nervous system can produce clinical features of epilepsy.

Epilepsy is clinically characterized by recurrent episodes of convulsions which may be brief and associated with other stereotyped motor behaviour in association with disordered perception and impairment or actual loss of consciousness (ELIASSON *et al.*, 1974; PARKER, 1988). The clinical sign of epileptic seizure depends upon the region of the brain where the functions are being impaired by the seizure activity. When a seizure originates from a known and identifiable central nervous system dysfunction it is referred to as symptomatic, otherwise it is said to be idiopathic. It has to be noted that not all seizures are epileptic. Epilepsy associated with loss of consciousness can be grouped into three basic types: grandmal, petitmal and psychomotor epilepsy (ELIASSON *et al.*, 1974). Grandmal epilepsy can be either generalized or akinetic. Petitmal epilepsy is said to be non-focal in nature while the focal types comprise of focal motor, focal sensory, focal onset but generalized and focal temporal epilepsy (JENNETT, 1962). Epilepsy is usually classified according to seizure type.

1.2.2 Classification of Epilepsy

Epilepsy with no identifiable cause is known as primary, idiopathic or cryptogenic epilepsy while epilepsy resulting from an identifiable precipitating factor is known as secondary or organic epilepsy (PARKER, 1988). Based on this, epilepsy is therefore, classically divided into two major groups, but different types of epileptic seizures are classified on the basis of the affected cerebral areas and the consequent clinical symptoms (SWINYARD, 1985; PARKER, 1988).

Based on seizure type the international classification of epilepsy is as follows: (JENNETT, 1962; O'LEARY and GOLDRING, 1975; ASHWORTH and SAUNDERS, 1985).

- (I) Partial (focal, local) seizures comprise of:
 - A. Simple partial seizure (consciousness not impaired), consisting of:
 - (i) Motor signs (includes Jacksonian seizures);

- (ii) Somatosensory or special-sensory symptoms (e.g tingling, buzzing);
 - (iii) Autonomic symptoms or signs(e.g pallor, sweating); and
 - (iv) Psychic symptoms (disturbance of higher cerebral functions).
- B. Complex partial seizures (psychomotor, temporal lobe), consisting of:**
- (i) Simple partial onset followed by impairment of consciousness; and
 - (ii) Impairment of consciousness at onset, which may be: Cognitive symptoms, Affective symptoms, Psychosensory symptoms and Psychomotor symptoms.
- C. Partial seizures evolving to generalized seizures (may be generalized tonic-clonic, tonic, or clonic). This may be:**
- (i) Simple partial seizure (A) evolving to generalized seizure;
 - (ii) Complex partial seizure (B) evolving to generalized seizure; or
 - (iii) Simple partial seizure evolving to complex partial seizure to generalized seizure.
- (II) Generalized (bilateral symmetrical; convulsive and non-convulsive) seizure is made up of the following; Absence seizure (petitmal, stare); Myoclonic seizure (sudden, brief jerks); Clonic seizure (muscle contraction and relaxation); Tonic seizure (muscle contraction); Tonic-clonic seizure (grandmal); Atonic seizure (drop attack); Akinetic seizure; and Infantile spasms;
- (III) Unilateral seizure; and
- (IV) Unclassified seizure (e.g. neonatal).

1.2.3 Anti-convulsant and Epilepsy Treatment

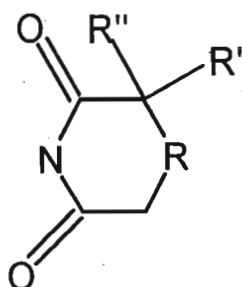
An anti-epileptic drug is one that is used medically to control epilepsy, not all of which are convulsive in humans. While the term anti-convulsants, strictly speaking, designates an agent that blocks experimental induced seizures in laboratory animals (FOYE, 1990), in the present context, the two terms are taken to mean the same thing and are used interchangeably.

The treatment of epilepsy comprises, and includes partly, general management and partly the administration of drugs to influence the attack, to arrest the occurrence or failing to do this, to render the attack less frequent and less severe (GARDNER *et al.*, 1977). Unfortunately, synthetic therapeutic agents currently employed for treatment of epilepsy offer only symptomatic relief. They only inhibit seizures as neither effective prophylaxis nor a cure is available (SWINYARD, 1985). Compliance with the medication is a problem because of the need for long term therapy together with many side effects which may manifest themselves (PARKER, 1988). Many patients may remain seizure free on prophylactic drug therapy, however, existing synthetic anti-epileptic drugs have considerable potential for concentration-dependent and idiosyncratic toxicity. There are, however, some diagnosed and confirmed cases of epilepsy that were treated with secondary metabolic products or natural product drugs resulting in permanent recovery (UGBOAJA, 1996). Natural product drugs have the advantage of not possessing serious side effects as is associated with the synthetic drugs currently being used for the treatment of epilepsy and convulsion. These secondary products in the form of essential oils, alkaloids, and/or glycosides are currently being investigated for use in the treatment of epilepsy (De BARROS *et al.*, 2000). Hence, the need to carry out scientific investigations on some of the plants and herbs employed for this purpose.

1.2.4 Classification and Chemistry of Synthetic Anti-convulsants

The ideal anti-epileptic drug, among other things, should completely suppress seizures in a dose that do not cause sedation or other undesirable central nervous system toxicity (FOYE, 1990). It should be well tolerated and highly effective against various types of seizures and devoid of undesirable side effects on vital organs and functions (FOYE, 1990).

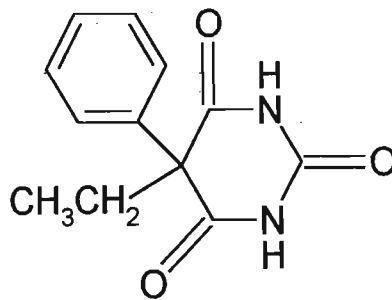
The anti-convulsants are classified, based on the structure of the parent drug. Several major groups of anti-convulsants have common structural features (FOYE, 1990; DELGADO and REMERS, 1998) and are classified according to their parent structure. The Structure Action Relationships (SAR) amongst anti-convulsants are shown overleaf.



R	Structure common to
O -C -NH	Barbiturates
-NH-	Hydantoins
- O -	Oxazolidine diones
- CH ₂ -	Succinimides
-NH ₂	Phenacemides

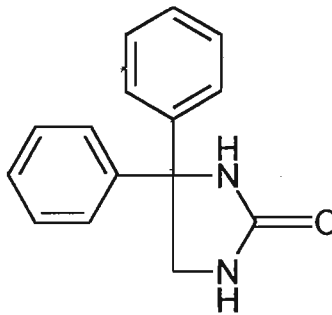
- (a) **Bromide salts:** Sodium bromide or potassium bromide (NaBr or KBr) were the first anti-convulsants to be employed in the treatment of epilepsy (VIDA, 1977). This class of anti-convulsant has sedative effects and other unwanted effects and is therefore no longer used.
- (b) **Barbiturates:** Drugs in this class are based on the barbituric acid structure. Many barbiturates display sedative-hypnotic activity, only a few have anti-convulsant properties (PARKER, 1988). Phenobarbital, mephobarbital and marginally, methobarbital display adequate anti-convulsant selectivity for use as anti-epileptics.

Phenobarbitone (5-ethyl-5-phenylbarbituric acid) (1) was the first drug of this class to be used in the treatment of epilepsy, and to date remains the drug of choice for epilepsy treatment. Phenobarbitone is not active in the treatment of absence seizures and it has side effects of being sedative (RANG and DALE, 1987).



Phenobarbitone (1)

- (c) **Hydantoin derivatives:** These compounds are represented by phenytoin (2): It is highly effective in reducing the intensity and duration of electrical induced convulsion but ineffective against leptazol-induced convulsion.

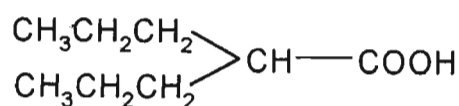


Phenytoin (2)

- (d) **Oxazolidine diones:** These compounds are widely used to control epileptic absences (FOYE, 1990). Trimethadione, a representative of this class, is ineffective against other generalised major (grandmal) and partial seizures. In animals it is characterized by its effectiveness in pentylenetetrazol (PTZ) tests and it has limited value or is not effective in the electroshock seizure tests (maximal electroshock seizure test - MES) (VIDA, 1977; FOYE, 1990). Oxazolidine diones have the disadvantage of being toxic.

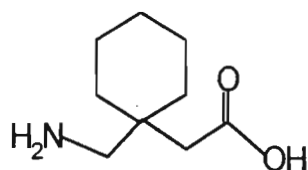
- (e) **Succinimides:** These are widely accepted for the treatment of epileptic absence conditions. Ethosuccimide is the most effective of the succinimides, it is less toxic and was developed by modification of the barbituric acid ring. It is active against leptazol-induced convulsion and against epileptic absence seizure in man. It has some undesirable side effects such as drowsiness, ataxia, rashes, hepatic and renal dysfunction.
- (f) **Primidone:** Is derived from a reduced barbituric acid parent structure and it closely resembles phenobarbitone pharmacologically. Part of its action may be attributed to the formation of barbiturate as an active metabolite (PARKER, 1988).
- (g) **Miscellaneous agents:** Many carboxylic acids have anti-convulsant activity, although often of low order potency, possibly in part because of extensive dissociation at a physiologic pH producing poor partitioning across the blood-brain barrier (FOYE, 1990; DELGADO and REMERS, 1998). In this class are:
- (i) *Valproate (2-propylpentanoic acid) (3):* This is a monocarboxylic acid, chemically unrelated to any class of anti-convulsants and was accidentally discovered in 1963. It inhibits most types of experimental induced convulsions and has proved to be effective in many clinical types of convulsion. It causes a significant increase in γ -aminobutyric acid (GABA) content of the brain and hepatotoxicity which is considered one of its serious side effects (RANG and DALE, 1987).

It is metabolised by conjugation of the carboxylic acid groups and oxidation of one of the hydrocarbon groups.



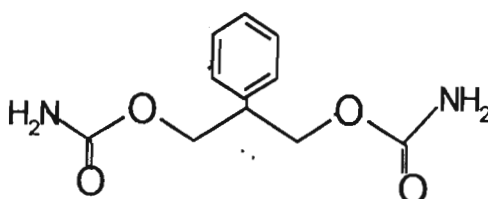
Valproate (3)

- (ii) *Gabapentin (Neurontin) (4)*: This is a relative of GABA with increased hydrophilic characteristics. Its mechanism of action appears not to involve an interaction with GABA_A receptor. It may involve the L-system amino acid transporter protein. It was introduced as adjunctive therapy for refractory partial seizures and secondary generalised tonic-clonic seizures.



Gabapentin (4)

- (iii) *Felbamate (Felbatol) (5)*: This has been used successfully in refractory patients with generalised tonic-clonic seizures and complex partial seizures. The mechanism of action may involve interaction with a strychnine insensitive receptor on the N-methyl-D-aspartate (NMDA) receptor. NMDA resembles the nicotinic receptor since it is constituted of five subunits. It only differs in its cationic specificity, gating both Na⁺ and Ca²⁺ and also in its mode of action (GILLHAM *et al.*, 1997).

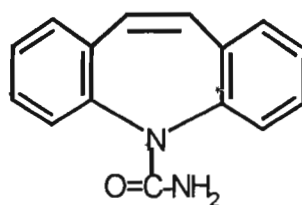


Felbamate (5)

- (iv) *Benzodiazepines*: Diazepam, a benzodiazepine, when given intravenously is effective in the treatment of status epilepticus, a life threatening condition in which epileptic seizure always occur without a break (VIDA, 1977; RANG and DALE, 1987).

- (v) *Lamotrigine*: Is effective against refractory partial seizures. It is a glutamate antagonist and it is said to act by modulating sodium channels and prevent glutamate release. Compounds of this type reduce neuronal cell death in ischemia.
- (h) **Ureas and monoacylurea**: These two chemical classes have a long history of producing compounds with anti-convulsant activities (DELGADO and REMERS, 1998). Most of the anti-convulsants contain an ureide structure but there are some e.g phenacemide (phenylacetylurea) with an open chain structure lacking the heterocyclic ring common to ureides that has anti-convulsant activity. In this class are :

- (i) *Carbamazepine: 5H Dibenz(b,f)azepine-5-carboxamide (6)*: It may be viewed as an ethylene bridge 1,1-diphenylurea or an amido-substituted tricyclic system. It has a hydrophobic moiety joined to a rather simple non-ionic polar H-bonding group and two phenyls on the nitrogen. DELGADO and REMERS (1988) pointed out that either view fits a very general activity pattern for anti-convulsants, namely, the hydrophilic moiety joined to a rather simple non-ionic polar H-bonding group. The two phenyls on the nitrogen fit the pattern of antigeneralised tonic-clonic activity (FOYE, 1990). It is a useful drug of choice in generalised tonic-clonic and partial seizures (RANG and DALE, 1987).



Carbamazepine (6)

- (ii) *Phenacemide (phenylacetylurea)*. This is a broad spectrum agent that finds some use in psychomotor epilepsy.

1.2.5 Mechanism of Action of Anti-convulsant Drugs

Anti-convulsants act principally in two ways:

- (i) By stopping the spread of the seizure which appears to depress consistently the hyperpolarising effect of GABA on a variety of neurones, including those in the thalamus nucleus and neurones in the cerebral cortex (FOYE, 1990). It has been proposed that the blocking of sustained high frequency repetitive firing (SRF) may underline the action of phenytoin, phenobarbital, and valproic acid against generalised tonic-clonic seizures in humans and against maximum electrical seizures in animals (RANG and DALE, 1987; FOYE, 1990).

Enhancement of GABAergic transmission may underlie the actions of benzodiazepines and valproic acid against absence seizures in humans and against pentylenetetrazole-induced seizures in experimental animals (VIDA, 1977).

- (ii) Anti-convulsant drugs may act by elevating the threshold of seizures. This is the type of seizure induced by pentylenetetrazole. No currently used synthetic anti-convulsant possess both mechanisms of action (VIDA, 1977). Therefore, no currently used anti-convulsant can be employed in the management and treatment of all types of epilepsy and convulsion.

1.2.6 Neurotransmitters and Drug Action in the Central Nervous System (CNS)

Of all the chemical transmitters found in the brain and other parts of the central nervous system (CNS) GABA appears to be the most abundant transmitter substance in the mammalian brain (PAREDES and AGMO, 1992). This neurotransmitter is related to many neurological and psychiatric disorders, including epilepsy, anxiety and pain (KUBOVA, 1999).

In connection with epilepsy, much of the current discussions about central neurohumoral transmission centers around GABA and the monoamines (HARRIS and MAWDSLEY, 1974). It is now established that a general phenomenon as regards brain excitability and seizure liability must involve a great number of neurochemicals acting as neurotransmitters. There is evidence that GABA and monoamine are fundamentally involved (KRNJEVIC, 1974). This finding has led to extensive use of a few GABAergics

in the treatment of epilepsy and spasticity (HARRIS and MAWDSLEY, 1974; PAREDES and ÁGMO, 1992).

GABA receptors in CNS are pharmacologically different from those causing inhibition of transmitter release, in that, the latter cannot be blocked by bicuculline. This distinction has given rise in the nomenclature to the use of GABA_A and GABA_B sites, respectively for these two species of receptors (BOWERY *et al.*, 1981; SIMMONDS, 1983). GABA_A receptor is part of a macromolecular complex coupled to a Cl⁻ ionophore. The complex has binding sites for benzodiazepines (GUIDOTTI *et al.*, 1983; FONNUM, 1987; SAANO 1987; BRIONI *et al.*, 1989), barbiturates (PETERS *et al.*, 1988), and steroids (GEE *et al.*, 1987; HARRISON *et al.*, 1987). Structurally the GABA_A receptor is composed of two alpha and two beta subunits (PAREDES and ÁGMO, 1992). The beta unit has a molecular weight of between 55000 and 58000 daltons (SCHWARTZ, 1988) and it is on this that the GABA binding site is located (COSTA, 1978; SCHWARTZ, 1988). Functionally GABA_A receptors are located both pre- and post-synaptically. Activation of GABA_A receptors causes the opening of a Cl⁻ ionophore, producing inhibition of neural activity either due to hyperpolarization or to the reduction in membrane resistance (BURMANN, 1988). Compounds like isoguvacine, muscimol and piperidine-4-sulphonic acid act as GABA_A agonists while bicuculline acts as a specific antagonist. The structure of the GABA_B receptor is as yet unknown but it seems to be located both pre- and post-synaptically as GABA_A (PAREDES and ÁGMO, 1992).

GABA became associated with epileptogenesis when it was discovered that reduction in GABA synaptic function produced convulsions. It was also noticed that direct blockage of the GABA_A receptor with picrotoxin and bicuculline (PIREDDA *et al.*, 1985) or inhibition of GABA synthesis by GAD inhibitors produced seizures (TAPIA, 1974; WORMS and LLOYD, 1981). GABA deficiencies have been observed in animal models of epilepsy and GABAergic alteration associated with convulsion has been described in different species including mice (FRANSEN *et al.*, 1987). There is also strong evidence that alteration of GABA synaptic activity may be important in certain types of human epilepsy. This is supported by the finding that in patients with different types of epilepsy there is decreased glutamic acid decarboxylase (GAD) activity and reduced GABA levels (WOOD, 1975).

It is noteworthy that GABA agonists have a low profile of anti-convulsant activity (PAREDES and ÁGMO, 1992). Muscimol, a GABA_A agonist is not effective against seizures in animal models (RASTOGI and TICKU, 1986). GABA is destroyed by transamination in which the amino group is transferred to α -oxoglutaric acid to yield glutamine with the production of succinic semialdehyde and consequently succinic acid. The reaction is catalysed by GABA-transaminase (GABA-T), a widespread enzyme located in mitochondria. GABAergic neurones have an active GABA uptake system, and it is this, rather than GABA-T that removes GABA after it had been released (RANG and DALE, 1987).

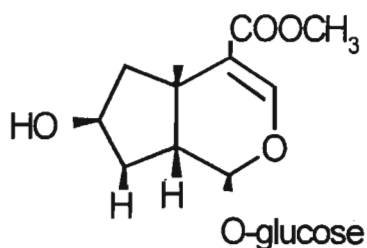
It was thought that GABA-like substances may prove effective in controlling epilepsy and other convulsive states. This is because GABA itself does not penetrate the blood - brain barrier, therefore a search began for lipophilic GABA analogues. One such substance is a p-chlorophenyl derivative of GABA (baclofen) which was introduced in 1972. It was pointed out above that a remarkable relationship exists between GABA_A receptors and the action of the benzodiazepine group of drugs which have powerful sedation and anxiolytic effects (MENNINI *et al.*, 1993). These drugs potentiate the effect of GABA on GABA_A receptors in such a way that GABA actions are facilitated and its pharmacological activities enhanced.

1.2.7 Natural Products in Epilepsy

A number of natural product drugs from different morphological groups and of various organic compound classes such as essential oils, alkaloids and glycosides have been used singly or in combination in the treatment of epilepsy by traditional healers.

- (i) **Essential oils:** These are obtained mainly from the aromatic herbs, which were the most prized treasures of the ancient world and are widely used drugs known as aromatherapy. Many essential oils have been screened for their anti-convulsant activity (NWAIWU and AKAH, 1986). Essential oils are complex mixtures which consist mostly of monoterpenes and sesquiterpenes. Some important chemical constituents of essential oils include linalol, camphor, citral, eugenol, β -citronellol and terpinen-4-ol (PINE, 1987).

- (ii) **Monoterpenes:** Monoterpenes are important constituents of essential oils. These may occur in the form of aliphatic or cyclohexanoids or carbocyclic five member rings as found in iridoids (LUCKNER, 1984). They are all C₁₀ compounds, may contain one or two isolated double bonds, may be saturated or be aromatics (TYLER *et al.*, 1988). Functional groups such as ketones, aldehydes, esters and carboxylic acids may be present. Most compounds of this group are volatile and lipophilic and have a very old history of use as medicines and condiments hence the name essential oil (EVANS, 1989). They also have applications in industry especially in the food industries as flavours. Some commercial and medicinal important monoterpenes include camphor, menthol, anise oil, carvone and thymol (VICKERY and VICKERY, 1983; EVANS, 1989).
- (iii) **Iridoids (7):** Iridoids are derived from iridodial and have carbocyclic five membered rings (TEDDER *et al.*, 1972). They are of two types (a) loganin and (b) secologanin. Most iridoids occurring in plants are glucosides derived from the O-heterocyclic form of iridodial while some contain heterocyclic nitrogen atoms (Iridoid alkaloids).



Iridoids (7)

Most plant iridoids are bitter substances which may repel predators but attract certain specialised animals. Some iridoids are employed in the production of liquors and medicines for treatment of epilepsy and other diseases (GOODWIN and MERCER, 1983). Non-glycosylated iridoids e.g. iridodial and dolichodial are constituents of the defense mechanism of ants (GOODWIN and MERCER, 1983). They also serve as precursors of indole and isoquinoline alkaloids (ROBINSON, 1981; LUCKNER, 1984).

- (iv) **Sesquiterpenes:** This comprises the largest group of the known terpenes and may be aliphatic or cyclic isoprenoid C₁₅ compounds which may be saturated or contain isolated double bonds (FINAR, 1975). Often oxo, hydroxy, aldehyde, carboxylic acids, and lactones may be present and most of them are volatile and lipophilic. Sesquiterpene alkaloids also occur in some families of higher plants. Compounds containing sesquiterpenes are of commercial interest and are frequently employed as medicines. In higher plants and insects certain representatives of sesquiterpenes e.g. abscisic acid, xanthoxin and juvenile III, may act as hormones (GOODWIN and MERCER, 1983).
- (v) **Alkaloids Used in Epilepsy:** *Piper methysticum*, (Piperaceae) popularly known as kava kava, is a highly desired medicinal plant that has been at the center of social and ceremonial life in the Pacific Islands (PISCOPO, 2001). The roots of this plant contain alkaloids amongst other chemical constituents used in the treatment of anxiety disorders, insomnia, restlessness and epilepsy. Chemical compounds isolated include alkaloids, flavones and flavonoids such as flavokawains, plant alcohols, phytosterols, ketones and organic acids (SHULGIN, 1973). The lactones are typically 4-methoxy-2-pyrone with phenyl or styryl substitutes at the 6-position and are similar in structure to myristicin, which is found in nutmeg. The lactones do have psychoactive effects. Roots of *Delphinium denudatum* popularly known as 'Jadwar' possess anti-convulsant properties and is used in the Unani medicine system (SAID, 1970). Generally the roots are considered to be toxic due to the presence of diterpenoid alkaloids. The roots singly or in combination with other drugs are used by traditional healers in the treatment of epilepsy and other diseases (BENN and JACYNO, 1983).

1.3 CHARACTERISTICS OF TEST PLANTS

1.3.1 *Schumanniohyton magnificum* Harms (Figure 1)

Local name	:	Mgbammili (Igbo)
Family	:	Rubiaceae
Geographical source	:	Obtained from Okija, Ihiala Local Government Area, Anambra State, Nigeria.



Figure 1: *Schumannia magnificum* Harms

General Family Characteristics

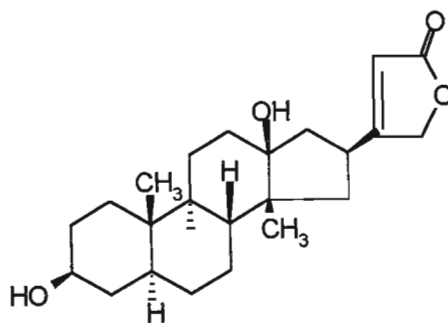
Many useful medicinal plants are found in this family and they have been reported to contain various types of organic chemical constituents in different species of the family. Alkaloids with different structures such as an indole, oxindole, quinoline are found in cinchona plants, and purine alkaloids have been reported to occur in the family (EVANS, 1989). Anthraquinones occur both in the free state and as glycosides in *Morinda*, *Rubia* and *Galium*. Anthocyanins are reported present in cinchona and aucubin glycosides in asperulin. Cyclitols (e.g. quinic acids) and coumarins are found in *Coffea* while phlobatanins and catechins are found in *Uncaria*. Diterpenoids, triterpenoids, and iridoid glycosides have been isolated from genipa (TYLER *et al.*, 1988; EVANS, 1989). *Morinda reticulata* is an interesting species because it accumulates selenium and is very toxic (EVANS, 1989). Some species of the Rubiaceae are reported to contain pentacyclic triterpenoid saponins. In these saponins the sapogenins are attached to a chain of sugars or uronic acid units often in position 3. Triterpenoid saponins may be classified into three groups represented by phenanthrene glycosides. The principal types of phenanthrene related glycosides are:

(A) Cardiac glycosides; and (B) Saponins

(A) Cardiac glycosides:

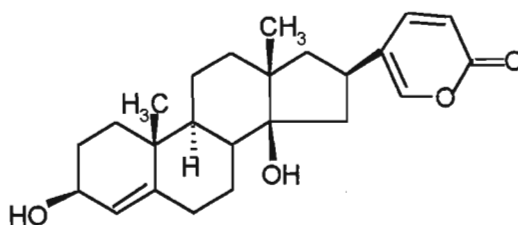
Cardiac glycosides are of medical interest because of their stimulating effect on the heart (GOODWIN and MERCER, 1983). Several of these have been used as arrow poisons to kill animals (HERBERT, 1989). Cardiac glycosides comprise a family of C₂₁ to C₂₄ steroids, linked through C₃ to a chain of sugars which ensure water solubility and easy cleavage. These compounds occur widely in tropical plants, temperate plants and skin secretions of toads especially tropical toads and frogs. There are two types of steroids :

- (i) **Cardenolides Uzarigenin (8)** - the steroids of this class have a five member ring attached to position C₁₇ of the steroid nucleus. *Digitalis* glycosides are notable members of this class of steroid, and are the most widely used cardiac glycosides. The ability of many cardiac glycosides, especially digitonin, to form a complex with steroids containing a 3-β-OH group forming an insoluble crystalline compound is noteworthy.



Uzarigenin (8)

- (ii) **Bufadienolides or bufatoxins** - these are fewer in number and have a six membered heterocyclic ring attached to the C₁₇ position of the steroid. They occur in toads and in the Squill plant family. Cardenolides contain $\alpha\beta$ -unsaturated γ -lactone rings and show a λ max at 220 nm, while scilladienolides or bufadienolides (9) contain an δ -ring which has a conjugated diene and show a λ -max at 300 nm.



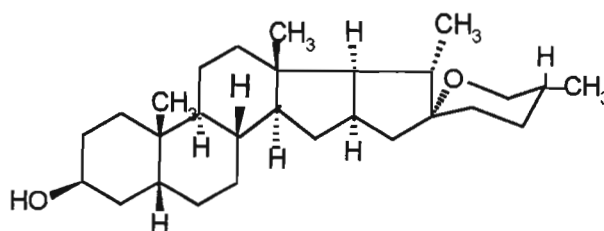
Scillarenin (9)

(B) Saponins:

Two types of saponins are recognized :

- (i) Those with a **Tetracyclic triterpenoid structure (Smilagenin) (10)** - Saponins with this type of structure are another group of non-cardiac steroidal glycosides that have the properties of foaming when shaken with water, like soap solution, hence the name saponin. These compounds that are capable of inducing this frothing behaviour are, more often than not, characterised structurally by the presence of a oligosaccharide unit(s) appended to a steroid or triterpenoid aglycone (DAVIES *et al.*, 1964;

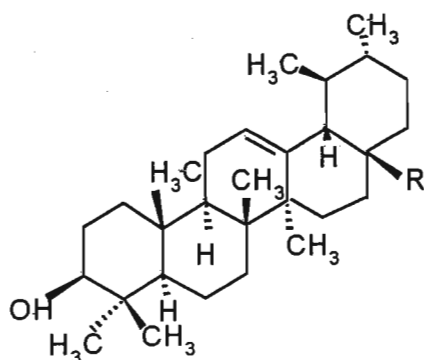
COLEGATE and MOLYNEUX, 1993). Saponins are of interest for various reasons, but as a natural product, the biological activity is of primary importance. These compounds have pronounced haemolytic activity. Another bioactivity commonly associated with saponins is mulloscidal (snail killing) activity. They interact with membranes of living cells which may result in destruction of the membrane with cell death (non-reversible effect) or a transient change on the membrane structure followed by specific biological effects (MELZIG and BADER, 2001). It is now well recognized that saponins are important defensive compounds with a variety of anti-microbial and anti-fungal activities. Saponins are widely distributed in plants, they are poisonous and haemolytic when administered intravenously as an aqueous solution in animals, but usually non-toxic when given orally. They are mostly used as fish poisons (TYLER *et al.*, 1988; EVANS, 1989). Saponins have a high molecular weight and present some difficulties in their isolation in a state of purity. They are hydrolysed by acids to give an aglycone, various sugars and related uronic acids (TEDDER *et al.*, 1972; COLEGATE and MOLYNEUX, 1993). The aglycones - saponins - are characterised by the presence of spiroketal side chains. Accordingly the structures are derived from cyclopentanoperhydrophenanthrene (FINAR, 1975; PINE, 1987). They are neutral in nature.



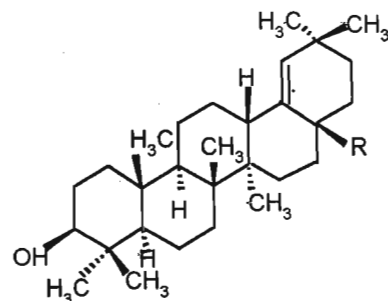
Smilagenin (10)

(ii) **Pentacyclic triterpenoids :**

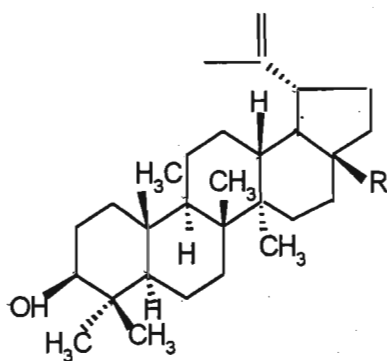
These are acidic in nature. They can be classified into three groups represented by α -amyrin (11), β -amyrin (12) and lupeol (13).



α -amyrin (11)



β -amyrin (12)



Lupeol (R= CH₃) (13)

Natural sapogenins differ only on the configuration at carbon atoms 3, 5 and 25. In the spirostane series the orientation at C₂₂ need not be specified. Mixtures of the C₂₅ epimers as in diosgenin (D⁵,25 α -spirosten-3 β -ol) and γ -amogenin (D⁵,25 β -spirosten-3 β -ol) are of normal occurrence (EVANS, 1989).

Ethnobotanical Uses:

Schumanniophyton magnificum grows wild in Igboland, eastern region of Nigeria, but because of its multiple medicinal value, it is now widely cultivated. *Schumanniophyton magnificum* is a highly valued medicinal plant in this area and has been found useful in treatment of quite a number of diseases, hence the justification of its local name, Mgbammili meaning 'water giver', since water is associated with life. The plant-drug has been used locally by traditional medical practitioners for its effect on blood clotting (HOUGHTON and SKARI, 1994) and as an anti-snake bite agent (AKUNYILI and AKUBUE, 1986; HOUGHTON and HARVEY, 1989; HOUGHTON *et al.*, 1992; MARTZ, 1992). It has also been screened for its inhibition of venom-induced haemolysis

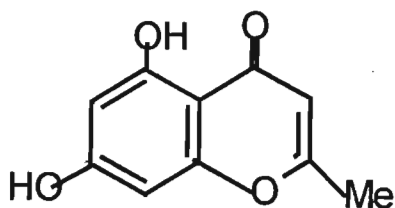
(HOUGHTON and TAFESSE, 1993). It is used in the treatment of viral infections and has been screened for such activities (HOUGHTON *et al.*, 1996). It is used widely in traditional medicine for the treatment of schistosomiasis and its anti-schistosomiasis effects have been studied by OKUNJI and IWU (1988). Natives use it for its anti-leishmania activity and it has been investigated for such activity (IWU *et al.*, 1992). The plant bark is used as a remedy for dysentery and as an enema. Some tribes use it after circumcision (TANE *et al.*, 1990). So far no work has been reported on its anti-epileptic and anti-convulsant activities.

Chemical Constituents found in *S. magnificum*

Schumanniphyton magnificum contains chromone alkaloids (HOUGHTON and YANG, 1985), chromone glycosides (TANE *et al.*, 1990), a peptide (HOUGHTON and OSIBOGUN, 1992), and schumanniofoside, a glycoside (AKUNYILI and AKUBUE, 1986).

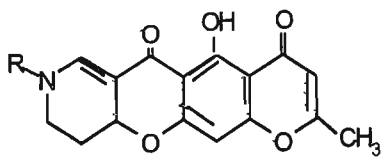
Chromones:

The chromone aglycone, noreugenin (14), results from the hydrolysis of schumaniofoside A. It has a molecular formula of $C_{10}H_8O_4$, melting point 277-278°C and molecular mass of 192. The sugar component is glucose (TANE *et al.*, 1990).



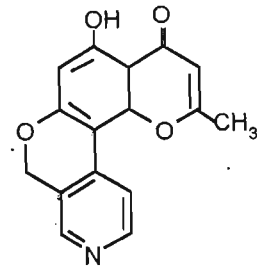
Noreugenin (14)

OKOGUN *et al.* (1983) and HOUGHTON and YANG (1985) have isolated and characterized several alkaloids from *S. magnificum* with the structures shown below.

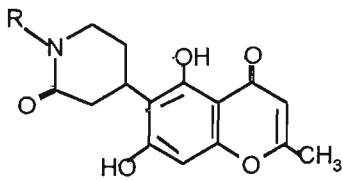


R = H Anhydroschumannificine
 CH₃ N-Methyl anhydroschumannificine

(15)

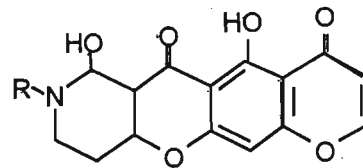


(16)



R = H
 R = CH₃ Piperidin-2-ones

(17)



R = H Schumannificine
 R = CH₃ N-Methylschumannificine

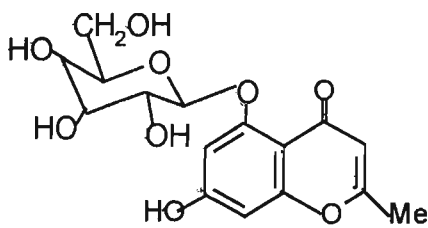
(18)

Chromone glycosides:

The glycosides isolated were schumaniofiosides

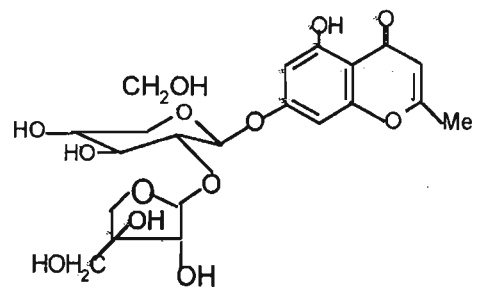
(A) 2-methyl-5,7-dihydroxychromone-5-O-β-D-glucopyranoside, and

(B) 2-methyl-5,7-dihydroxychromone 7-O-β-D - glucopyranosyl-(1→2) apiofuranopyranoside.



Schumaniofioside A

(19)



Schumaniofioside B

(20)

Schumanniosides A and B were isolated from a 90% ethanol extract of the powdered root bark of *S. magnificum* (TANE *et al.*, 1990; KASHIWADA *et al.*, 1990). Sixty grams of the extract were subjected to column chromatographic separation with silica gel and eluted with a CHCl₃-MeOH (17:3) solvent system. The fractions having the same TLC characteristics were pooled and were finally purified using preparative TLC.

Schumannioside A was obtained as white crystals from MeOH, mp 162-163°C (found: [M]⁺ m/z 354 and ¹H NMR at 60 MHz as indicated in the literature. Schumannioside B was obtained as white crystals from MeOH, mp 216-217°C. ¹H NMR at (60 MHz) spectra are given in the literature (TANE *et al.*, 1990; KASHIWADA *et al.*, 1990).

1.3.2 *Glyphaea brevis* (Speng) Moraches (Figure 2)

Local name	:	Aloanyasi (Igbo)
Family	:	Tiliaceae
Geographical source	:	Obtained from Atani, Ogbaru Local Government Area, Anambra State, Nigeria

General Family Characteristics

Glyphaea brevis usually is a small tree when fully grown. In this family is found the Lime tree flower (British Pharmacopoeia 1993, Addendum) which is the dried inflorescence of *Tilia platyphyllos* or *T. cordata* and which is used for treatment of respiratory tract infections and as a nervine and tonic. Its constituents include volatile oils with over ninety components, flavonoids and phenolics. Cardiac glycosides are reported from *Corchorus*, alkaloids are absent (EVANS, 1989).

Ethnobotanical Uses

Flowers of several *Tilia* species have been employed as a folkloric remedy for treatment of colds, headaches, indigestion and nervousness (EVANS, 1989; TYLER *et al.*, 1988). *Glyphaea brevis* is used in the treatment of sleeping sickness in the Ivory Coast, as an aphrodisiac in Guinea, and for treatment of eye infections in Nigeria. It is also used extensively as a chewing stick in Nigeria (BHAT *et al.*, 1990).



Figure 2: *Glyphaea brevis* (Speng) Moraches

Members of the family *Tiliaceae* contains glycosides especially cardiac glycosides, but alkaloids are conspicuously absent. So far no investigation has been carried out on the anti-convulsant activities of this species nor on the nature of its chemical constituents.

1.4 AIMS AND OBJECTIVES

The purpose of this study was primarily to determine the anti-convulsant activities of *Schumanniohyton magnificum* and *Glyphaea brevis*, which have long been employed by traditional medical practitioners in the treatment of epilepsy and convulsion especially in children in Nigeria.

There is no documented work on the plants in this regard. There is a need, therefore, on one hand to carry out detailed scientific research on the plants and the other hand to document the findings in order to support the use of the traditional remedy prepared from the plants.

Specific objectives involved isolation of the chemical constituents responsible for this pharmacological activity. General objectives were to investigate the anti-epileptic and anti-convulsant properties of the test plant extracts and the isolated chemical constituents and to carry out toxicity test on the crude extracts and isolated compounds where possible.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

Test plants

- (i) *Schumanniophyton magnificum* (roots)
- (ii) *Glyphaea brevis* (leaves)

The plants were collected from the localities as indicated in Chapter 1, during the rainy season before the end of October 2000, when a high yield of secondary metabolic products are expected as the season and time of collection affect the yield (TYLER *et al.*, 1988; EVANS, 1989). Plants were identified by Mr T.I. Adeleke of the Department of Pharmacognosy, School of Pharmacy, University of Lagos. Voucher specimens were deposited in the School Herbarium. Identification was confirmed by the Forestry Research Institute, Ibadan, Nigeria.

Processing of Plant Material

It was necessary that enough of the material be collected for the intending investigation. A decision therefore had to be made as to whether the medicinal plant was to be harvested from the wild or cultivated sources or both (SOFOWORA, 1993).

Schumanniophyton magnificum is an ornamental medicinal plant in Igboland. It is now cultivated while *Glyphaea brevis* grows wild. The plant material was collected from the wild before noon on the day of collection, and was thoroughly washed, especially the roots, with copious amounts of water to remove sand and other foreign matter and spread on a mat for excess water to drain off. Material was then dried between 30-40°C in an oven (HARBORNE, 1998).

Dried material was milled using a Christy Machine Type 8 Laboratory Mill.

2.2 METHODS

General Apparatus

All extracts were concentrated with a rotary evaporator (Büchi K4R Electronic). NMR spectra were recorded using a Varian 500 instrument with tetramethylsilane as internal standard. Liquid chromatographic columns were packed with silica gel 60 (0.063 - 0.200 mm, Merck) and Sephadex LH₂₀ (Pharmacia) respectively. Fractions were monitored by TLC strips of pre-coated plates of silica gel 60 containing a fluorescence marker (Merck, PF₂₅₄). Ultraviolet light at 254 nm and 366 nm were used for spot detection. Compounds of interest were scraped off TLC plates, placed in conical flasks and recovered by dissolving with redistilled methanol and filtered through sintered glass funnels no. 3 or 4.

Extractions

The study of natural product chemistry usually starts with the extraction of bio-active principles from plant or animal sources. The extraction process is the most crucial stage in the study, and is considered one of the most intensive areas of secondary metabolite research today (SOFOWORA, 1993). It is therefore very important that the extraction method be efficient, and the efficacy of the method verified using standard assay procedures (EVANS, 1989; SOFOWORA, 1993). The ability of a solvent used for extraction to extract the components of the solute must be considered. The more efficient the extraction step, the greater is the range of compounds present in an extract (COLEGATE and MOLYNEUX, 1993). The extraction of the active constituents may be performed for the following reasons:

- (a) To identify a unknown compound responsible for certain bio-activity (LUCKNER, 1984) or to isolate a certain compound known to be produced by a particular organism;
- (b) To verify if a group of compounds within an organism have some similarities with that from another plant (COLEGATE and MOLYNEUX, 1993) and to characterise all isolated compounds that may be of interest, especially secondary metabolites confined to the organism (LUCKNER, 1984; COLEGATE and MOLYNEUX, 1993);
and

- (c) It may be necessary to identify all the metabolites produced specifically by one natural source that are not produced by a different 'control' source, for example as in the determination of the metabolic products of two species of the same genus or the same organism cultivated under different conditions (GOODWIN and MERCER, 1983; COLEGATE and MOLYNEUX, 1993).

Fresh plant material can be extracted or the plant material may be dried at a very low temperature and preserved for a long time before extraction. Air or freeze dried samples are sometimes sequentially extracted with a variety of solvents, starting with a highly lipophilic solvent and moving to an highly polar solvent if crude fractionation of the metabolites is sought. Generally organic polar solvents such as ethylacetate and methanol are used. It has been observed that alcoholic solvents efficiently penetrate cell membranes, permitting the extraction of large amounts of endocellular components (SILVA *et al.*, 1998). In general aqueous alcoholic solvents seem to possess optimum solubility characteristics for initial extraction (KINGHORN, 1985).

(i) Alkaloid Extraction

The plant alkaloids, so called because of their basic properties have attracted considerable attention on account of their medicinal, and in many cases their highly poisonous properties (OSLOW, 1929). The alkaloids are, as a rule in the form of a base, insoluble in water but soluble in organic solvents such as ethylacetate, chloroform or as a salt soluble in water (CORDELL, 1981; EVANS, 1989). For this reason various methods are employed for extraction of alkaloids, but the exact course of events depends on the alkaloids in question. Alkaloids can be extracted from the ground plant material with aqueous alcohol (usually 70% alcohol), with dilute acid or with a base (EVANS, 1989).

The crude extract or mixtures obtained are purified by solution in acid and filtration from insoluble materials. The alkaloids are then precipitated with alkali and extracted with an immiscible solvent from alkaline solution.

Alternatively, the ground plant material is moistened with water and mixed with lime which combines with acid tannins and other phenolic substances, and set free alkaloids (EVANS, 1989; COLEGATE and MOLYNEUX, 1993). Extraction is

therefore carried out with organic solvents, the concentrated organic liquid shaken with aqueous acid and allowed to separate. The alkaloid salts are in aqueous solution while impurities remain behind in the organic solvent.

Individual alkaloids are sometimes separated from each other through differences in the solubilities of their bases and their salts (COLEGATE and MOLYNEUX, 1993).

(ii) Extraction of Glycosides/Terpenoids

The drying of plant material containing glycosides is carried out under controlled conditions to avoid major chemical changes occurring (HARBORNE, 1998). Glycosides are water soluble, the precise mode of extraction depends therefore on the texture and water content of the material extracted (TYLER *et al.*, 1988; HARBORNE, 1998). The classical chemical procedure constitute continuous extraction from dried plant material in a Soxhlet apparatus using a range of solvents. Glycosides have been extracted with 90% methanol (FUJIMOTO *et al.*, 1986), ethanol (MISRA *et al.*, 1991; BARRERO *et al.*, 2000), aqueous methanol or aqueous ethanol (70%) (TYLER *et al.*, 1988; EVANS, 1989; HARBORNE, 1998).

Petroleum spirit, ether or chloroform may be used in the extraction of lipids and terpenoids (GOODWIN and MERCER, 1983; HARBORNE, 1998). The concentrated extracts often deposits crystals on standing, which may be collected by filtration and their homogeneity tested by chromatography using several solvents (COLEGATE and MOLYNEUX, 1993).

Experimental

The classical chemical procedure of obtaining organic constituents from dried plant materials is that of continuous extraction (HARBORNE, 1998). The ground plant material (500 g) is extracted hot using a Soxhlet apparatus.

The material packed in the extractor chamber is first continuously de-fatted with petroleum spirit (60-80°C) until there are no more signs of chlorophyll and fats (EVANS, 1989; De TOMMASIX *et al.*, 1998). The resulting marc is then dried, returned to the extractor chamber and then subsequently continuously extracted with ethanol (95%) until all the chemical constituents are assumed extracted (KINGHORN, 1985; HUSSEINZADEH and MADANIFORD, 2001). The extracts are then concentrated under vacuum using a rotary evaporator and dried under a fan to constant weight.

(i) *S. magnificum*

Total weight of material extracted	= 504 g
Total weight of dried extract	= 95 g
Yield	= 7.9 % of the total weight extracted.

The above process was also employed for the extraction of *G. brevis* leaf powder.

(ii) *G. brevis*

Total weight of material extracted	= 1012 g
Total weight of the dried extract	= 115 g
Yield	= 11.4% of the total weight extracted.

2.3 DISCUSSION

The process of extraction of active constituents from natural sources constitutes a crucial and sensitive stage in the study of natural products, be it for the purpose of physiological evaluation through bioassay, or identification of the chemical constituents by phytochemical screening. This is because the active constituents may suffer some damage such as deterioration or breakdown resulting from hydrolysis in case of glycosides during extraction. There may be rearrangement and isomerization in the presence of alkali. For stereoactive constituents activity may be lost owing to racemization. All this must be guarded against. The active constituents should be stable in the extracting solvent which should have the capacity to extract a wide range of chemical constituents present in the material investigated (SILVA *et al.*, 1998). The solvent employed for extraction depends on the chemical nature of the constituents

being extracted. Tetracyclic saponins can be extracted with 90% methanol but methylation of the active constituent was observed leading to confusion regarding the identity of the glycone (FUJIMOTO *et al.*, 1986). This ambiguity can be resolved by maintaining the pH of the extract at 7 and by adding small amounts of pyridine occasionally. SEN and CHANDHURI (1991) employed chloroform to extract the active constituents from plant sources, though this had some limitations. Chloroform being a non-polar solvent will extract non-polar and basic chemical constituents only. Chloroform, ethylacetate and methanol were sequentially used to extract the active constituents from plant material (JIANG *et al.*, 1991). This method appeared to be effective and a wide range of chemical constituents were extracted. Triterpenoid saponins were extracted with ethanol (MISRA *et al.*, 1991) and saiko saponins from the roots of *Buleurum gibrattarium* and *B. spinosum* with absolute ethanol (BARRERO *et al.*, 2000).

In the present work an ethanolic solution (70%) was employed for the extraction of the chemical constituents from the two plant samples (KINGHORN, 1985). This is because hydrophilic organic solvents have the capacity to penetrate both endo- and exo-cellular compartments of the plant materials (LUCKNER, 1984; KINGHORN, 1985). They therefore extract both basic and acidic chemical constituents, extracting a greater range of chemical compounds than any other solvent system. Addition of water to hydrophilic organic solvents such as alcohols makes them more hydrophilic and minimizes their tendency to undergo chemical reactions such as formation of ethers or esters usually experienced with absolute alcohols (KINGHORN, 1985).

Some solvents can be selective for the type or nature of the active constituents extracted. A good example of this is the basic solvents which precipitate alkaloids from the solution or use of acidic solvents to extract alkaloids in the form of salts (TYLER *et al.*, 1988; EVANS, 1989).

The suitability of the extracting method and solvent used for a particular extraction may be evaluated by the use of standard phytochemical screening methods (FARNSWORTH, 1966). It can also be evaluated using a standard bioassay procedure for the expected or anticipated physiological activity of the chemical constituent(s) being sought.

CHAPTER 3

PHYTOCHEMICAL AND ANIMAL STUDIES ON CRUDE EXTRACTS

3.1 INTRODUCTION

Animal studies: *in vivo* vs. *in vitro* methods

Animal studies or biological assays in general have been described by FINNEY (1964) as the methods for the estimation of the nature, constitution or potency of a material (or process) by means of its reaction that follows its application to living matter. Biological assays also aim at establishing the efficiency of the extraction method or procedure of the active chemical constituent(s) from their natural sources. It also demonstrates if chemical constituents obtained from extraction have some physiological and pharmacological activities against the diseases being investigated (SOFOWORA, 1993).

The assay may be qualitative or quantitative in nature. Quantitative bioassays are similar to quantitative chemical analysis in that their function is to provide numerical assessment of some property of the material being assayed, that is determination of the quantity of the material that will elicit the desired physiological response. In *in vivo* assays live animals are usually used while in *in vitro* tests animal tissues or other biological materials such as micro-organisms are used. The anti-convulsant activities of the extracts were evaluated using both *in vivo* and *in vitro* tests.

3.2 MATERIALS AND METHODS

3.2.1 Materials

- (i) Test plant extracts.
- (ii) Albino mice (both sexes, 20 - 30 g).
- (iii) Chemicals: Pentylenetetrazole (PTZ), picrotoxin and strychnine were used. They were obtained from Sigma Chemical Company, USA.
- (iv) Perspex transparent mice cages.

3.2.2 Methods

Both male and female albino mice were used and were supplied by the Animal Center, College of Medicine, University of Lagos, Idi-Araba, Lagos in compliance with the animal Ethics Committee of the Center. The animals were randomly allotted into groups of five or seven mice per group and were allowed free access to food and water (NWAIWU and AKAH, 1986; AMABEOKU *et al.*, 1998). Each animal was used for one seizure experiment only. Pentylenetetrazole (PTZ) and picrotoxin solutions were prepared by dissolving a calculated amount with distilled water or physiological saline. The strychnine solution was prepared by placing a calculated amount of strychnine in a 100 ml volumetric flask, dissolved with 50 ml distilled water to which a drop of concentrated hydrochloric acid was added, and then made to volume with distilled water.

3.2.3 Anti-convulsant Activity

The anti-convulsant activity test methods of NWAIWU and AKAH (1986) and AMABEOKU *et al.* (1998) were modified and used to assess the anti-convulsant activity of the extracts of *S. magnificum* and *G. brevis* respectively. Mice were randomly allotted to five groups of five mice per group. A dose of the extract was administered to each group while the control group received 0.3 - 0.5 ml of distilled water or normal saline in place of the extract. The animals were kept singly in transparent perspex cages (20 cm x 15 cm x 15 cm) for at least 30 min to enable them to adjust to their new environment before commencing the experiment.

Seizures were induced in the mice with a dose of pentylenetetrazole (PTZ), picrotoxin or strychnine - 70, 5 and 4 mg kg⁻¹ body weight respectively. The calculated PTZ, picrotoxin or strychnine doses were administered to the animals, and the animals observed for a period of 30 min following the administration of the dose. Seizures were manifested as wild running followed by stunning or clonic convulsion and then tonic convulsion exhibited by tonic hind limb extension accompanied in most cases by death. The time to induce clonic and tonic convulsions were recorded.

The experiments were repeated with oral pre-treatment of mice with the following extract doses 200, 400 and 800 mg kg⁻¹ body weight administered to each group respectively while control animals received 0.5 ml normal saline 30 min prior to subcutaneous (sc) administration of a calculated PTZ, strychnine or picrotoxin dose

respectively. The time to induce clonic and tonic convulsions were recorded. Time-dependent effects of the protective dose of the extract, that is the dose that offered the best protection to the animals, was then evaluated. All experiments were carried out between 08h00 to 17h00 in a quiet laboratory environment.

(i) **Pentylentetrazole (PTZ) Test Results:**

S. magnificum

Table 3.1: Pretreatment of mice with different dosages of *S. magnificum* root extract 30 min prior to subcutaneous (sc) administration of PTZ at 70 mg kg⁻¹ body weight

Dosage applied (mg kg⁻¹)	No. of mice	Average time to onset of seizure ± SEM (min)	Quantal death	Survival (%)
Control	5	3.35 ± 0.28	5/5	0
200	5	5.70 ± 0.35	5/5	0
400	5	6.33 ± 0.54	3/5	40
800	5	8.30 ± 0.42	1/5	80

An extract dosage of 800 mg kg⁻¹ body weight plant extract offered the best protection to the animals (Table 3.1). The time-dependent effect of this dosage was therefore determined by subsequently giving the dosage to four groups of mice with five animals per group at 30, 60, 90 and 120 min intervals (PTZ application) respectively.

Table 3.2: Time-dependent anti-convulsant effect of the protective dosage of a *S. magnificum* root extract (800 mg kg⁻¹ body weight) with PTZ at 70 mg kg⁻¹ body weight

Time before PTZ application (min)	No. of mice	Average time to onset of seizure \pm SEM (min)	Quantal death	Survival (%)
30	5	8.30 \pm 0.42	1/5	80
60	5	12.72 \pm 1.09	1/5	80
90	5	6.35 \pm 0.30	4/5	20
120	5	4.60 \pm 0.18	5/5	0

The protective activity of the extract was at its peak when it was administered 60 min prior to PTZ application and gradually declined with increased time before drug application. When administered 90 min prior to PTZ application only 20% protection was observed and at 120 min prior to PTZ application, the extract offered no protection to the animals (Table 3.2).

***G. brevis*:** The same procedure was adopted as for *S. magnificum* root extract

Table 3.3: Pretreatment of mice with different dosages of *G. brevis* leaf extract 30 min prior to subcutaneous (sc) administration of PTZ at 70 mg kg⁻¹ body weight

Dosage applied (mg kg ⁻¹)	No of mice	Average time to onset of seizure \pm SEM (min)	Quantal death	Survival (%)
Control	7	2.24 \pm 0.14	7/7	0
200	7	3.20 \pm 0.42	7/7	0
400	7	6.20 \pm 1.19	3/7	60
800	7	8.73 \pm 0.80	3/7	60

Non-lethal dosages of 400 and 800 mg kg⁻¹ offered the same degree of protection to the mice (60%). The 800 mg kg⁻¹ dosage prolonged the onset of seizures and delayed the time to death (8.73 \pm 0.80 min) compared with a 400 mg kg⁻¹ dose (6.20 \pm 1.19 min) (Table 3.3).

Table 3.4: Time-dependent anti-convulsant effect of the protective dosage *G. brevis* leaf extract (800 mg kg⁻¹ body weight) with PTZ at 70 mg kg⁻¹ body weight

Time before PTZ application (min)	No. of mice	Average time to onset of seizure ± SEM (min)	Quantal death	Survival (%)
30	7	8.73 ± 0.80	3/7	60
60	7	12.05 ± 0.30	1/7	85
90	7	15.20 ± 0.05	0/7	100
120	7	9.25 ± 0.06	2/7	71

The protective effect of *G. brevis* leaf extract was at its peak when administered 90 min prior to PTZ application. A 100% protection was conferred on the animals. With time the activity declined as shown by 71% protection offered when administered at 120 min before PTZ application (Table 3.4).

(ii) Picrotoxin Test Results:

S. magnificum

Table 3.5: Pretreatment of mice with different dosages of *S. magnificum* root extract 30 min prior to subcutaneous (sc) administration of picrotoxin at 5 mg kg⁻¹ body weight

Dosage applied (mg kg ⁻¹)	No. of mice	Average time to onset of seizure ± SEM (min)	Quantal death	Survival (%)
Control	5	6.30 ± 1.80	5/5	0
250	5	16.05 ± 3.50	3/5	40
500	5	22.50 ± 5.30	1/5	80
750	5	27.01 ± 0.01	1/5	80
1000	5	- -	0/5	100

A dosage of 1000 mg kg⁻¹ body weight of *S. magnificum* root extract offered maximum protection to the mice (Table 3.5). The time-dependent effect of this dosage was therefore further investigated.

Table 3.6: Time-dependent anti-convulsant effect of a protective dosage (1000 mg kg⁻¹ body weight) of *S. magnificum* root extract with picrotoxin at 5 mg kg⁻¹ body weight

Time before Picrotoxin application (min)	No of mice	Average time to onset of seizure \pm SEM (min)	Quantal death	Survival (%)
30	5	-	0/5	100
60	5	-	0/5	100
90	5	24.0 \pm 2.12	2/5	60
120	5	17.0 \pm 1.74	3/5	40

The effect of the protective dosage of *S. magnificum* root extract was at its peak when given 60 min prior to picrotoxin application as 100% protection was offered. The protective capacity gradually declined after this time (Table 3.6).

(iii) Strychnine Tests Results:

S. magnificum

Table 3.7: Pretreatment of mice with different dosages of *S. magnificum* root extract 30 min prior to subcutaneous (sc) application of strychnine at 4 mg kg⁻¹ body weight

Dosage applied (mg kg ⁻¹)	No. of mice	Average time to onset of seizure \pm SEM (min)	Quantal death	Survival (%)
Control	5	1.76 \pm 0.16	5/5	0
200	5	2.37 \pm 0.11	5/5	0
400	5	3.17 \pm 0.13	4/5	20
600	5	3.94 \pm 0.15	3/5	40
800	5	4.50 \pm 0.10	1/5	80

A dose of 800 mg kg⁻¹ offered the best protection to the animals (Table 3.7).

Table 3.8: Time-dependent anti-convulsant effect of a protective dosage of *S. magnificum* root extract (800 mg kg⁻¹ body weight) with subcutaneous (sc) administration of strychnine at 4 mg kg⁻¹ body weight to mice

Time before sc strychnine application (min)	No. of mice	Average time to onset of seizure \pm SEM (min)	Quantal death	Survival (%)
30	5	4.50 \pm 0.10	1/5	80
60	5	5.34 \pm 0.45	1/5	80
90	5	3.37 \pm 0.18	4/5	20
120	5	2.26 \pm 0.23	5/5	0

The protective capacity was at its peak 60 min after subcutaneous (sc) administration of strychnine, offering 80% protection. Thereafter the protective capacity rapidly declined (Table 3.8).

***G. brevis*:**

The same procedure was used as above for the *S. magnificum* extract.

Table 3.9: Pretreatment of mice with different dosages of *G. brevis* leaf extract 30 min prior to subcutaneous (sc) application of strychnine at 4 mg kg⁻¹ body weight

Dosage applied (mg kg ⁻¹)	No. of mice	Average time to onset of seizure \pm SEM (min)	Quantal death	Survival (%)
Control	5	1.60 \pm 0.07	5/5	0
200	5	3.00 \pm 0.43	5/5	0
400	5	4.60 \pm 0.55	2/5	60
600	5	4.20 \pm 0.65	3/5	40
800	5	3.80 \pm 0.26	4/5	20

A dosage of 400 mg kg⁻¹ offered the best protection. The time-dependent effect of this dosage was subsequently evaluated (Table 3.9).

Table 3.10: Time-dependent anti-convulsant effect of a protective dosage of *G. brevis* leaf extract on mice (400 mg kg⁻¹ body weight) following sc application of strychnine at 4 mg kg⁻¹ body weight

Time before strychnine application (min)	No. of mice	Average time to onset of seizure ± SEM (min)	Quantal death	Survival (%)
30	5	4.60 ± 0.55	2/5	60
60	5	5.59 ± 0.13	1/5	80
90	5	4.18 ± 0.23	3/5	40
120	5	2.76 ± 0.13	5/5	0

The protective dosage was at its peak when given 60 min prior to subcutaneous (sc) administration of strychnine, offering 80% protection to the animals (Table 3.10).

(iv) Evaluation of a Combination of *S. magnificum* and *G. brevis* Extracts

A combination of the extracts in equal proportions (1:1) was evaluated against seizures induced by PTZ and strychnine respectively. The method was the same as with the single dosage evaluations and the results are shown in Table 3.11. Equal proportions at 200 mg kg⁻¹ body weight gave the best results.

Table 3.11: Pretreatment of mice with different dosages of the extract combinations 30 min prior to sc administration of PTZ at 70 mg kg⁻¹ body weight

Dosage applied (mg kg ⁻¹)	No. of mice	Average time to onset of seizure ± SEM (min)	Quantal death	Survival (%)
Control	5	3.30 ± 0.01	5/5	0
200	5	12.38 ± 0.18	2/5	60
400	5	6.25 ± 0.32	2/5	60

(v) **Evaluation of Drug Mixtures Against Strychnine-induced Convulsion**

A mixture of the extracts in equal proportions was evaluated against strychnine-induced convulsion using the same method as described above. The results are presented in Table 3.12.

Table 3.12: Pretreatment of mice with different dosages of extract combination in equal proportions (1:1) 30 min prior to subcutaneous (sc) administration of strychnine at 4 mg kg⁻¹ body weight

Drug dose (mg kg ⁻¹)	No of mice	Average time to onset of seizure ± SEM (min)	Quantal death	Survival (%)
Control	5	2.75 ± 0.02	5/5	0
200	5	5.05 ± 0.61	1/5	80

3.3 ACUTE/DELAYED TOXICITY STUDY ON THE PLANT EXTRACTS

Investigations have shown that plant extracts could produce dose related fatalities in mice or any other animal used in acute/delayed toxicity tests (SOFOWORA, 1993). The signs associated with toxic doses include hyperventilation, coma, clonic-tonic convulsion, urination and enlargement of the scrotum. One or more of these signs may be observed at high dosages. The median or the lethal doses (LD₅₀) was determined for *S. magnificum*, root and *G. brevis* leaf extracts respectively. In each test, a total of forty (40) albino mice of either sex and weighing between 20-30 g were randomly allotted to eight (8) groups, five mice per group. They were orally administered with different dosages of the extracts at 250, 500, 1000, 2000, 4000 and 8000 mg kg⁻¹ body weight respectively. The control group also had 5 mice and received 0.3 - 0.5 ml of physiological saline orally.

The animals were observed for 24 h to record mortality and then for another 24 h and 72 h for signs of delayed toxicity. They were left one per cage under quiet laboratory conditions and observed at 60 min intervals. The time of death was recorded and the results are presented in Tables 3.13 and 3.14.

Results

(i) *S. magnificum*

Table 3.13 : Acute/delayed toxicity tests of *S. magnificum* root extracts in mice

Group	Dosage (mg kg ⁻¹)	MORTALITY AFTER			Death (%)
		24 h	48 h	72 h	
1	125	0	0	0	0
2	250	0	0	0	0
3	500	0	0	0	0
4	1000	0	0	0	0
5	2000	1	-	-	20
6	4000	2	-	-	40
7	8000	3	4	5	100
Control	normal saline	0	0	0	0

GenStat statistical software was used for the following statistical parameter calculation. Using Logit function the regression coefficient 0.001176 was obtained which is also the same as the gradient of the slope. The constant was -4.82. The general regression equation can be written as $Y = 0.001176 X - 4.82$. Y is the number of animals and X is the dosage. The distribution was binomial and chi probability was <0.001. This is highly significant. The effective dosage, which killed 50% of the test animals (LD₅₀) was 4096 mg kg⁻¹ body weight. The (LD₅₀) of 4096 mg kg⁻¹ is far above the effective protective doses of *S. magnificum* root extract respectively in the three animal models (strychnine, pentylenetetrazole and picrotoxin) respectively.

(ii) *G. brevis*

Table 3.14: Acute/delayed toxicity tests of *G. brevis* leaf extract in mice

Group	Dose (mg kg ⁻¹)	MORTALITY AFTER			Death (%)
		24 h	48 h	72 h	
1	250	0	0	0	0
2	500	0	0	0	0
3	1000	0	0	0	0
4	2000	0	0	0	0
5	4000	1	3	3	60
6	8000	2	4	5	100
Control	Normal saline	0	0	0	0

Using GenStat statistical software the regression coefficient, which is the same as the slope of the curve 0.01256, was obtained with the constant 4. The regression equation $y = m x + c$, can be written as: $Y = 0.01256 X + 4$. X is the dosage in milligram (mg) and Y is the number of animals that died. The distribution was binomial and chi probability was <0.0000 3616 which is highly significant.

The effective dose that will kill 50% of the test animals (LD_{50}) is 3979 mg kg⁻¹. This is far above the effective protective dosages in all the animal models investigated.

Discussion

The main aim of the *in vivo* animal studies was to determine if the extracts exerted some anti-convulsant effects or not (FINNEY, 1964; SOFOWORA, 1993). Three animal models were employed for the evaluation of the anti-convulsant activities of *S. magnificum* picrotoxin, strychnine and pentylenetetrazole (PTZ) and two models for *G. brevis*, strychnine and pentylenetetrazole (PTZ). These chemical convulsants induce convulsion in mice through different mechanisms resulting in types of convulsion similar to those observed in human subjects.

Picrotoxin administered subcutaneously acts by blocking presynaptic inhibition mediated by GABA and induces minimal threshold clonic seizure (FOYE, 1990). The drugs effective against picrotoxin-induced convulsions are likely to produce their anti-convulsant effects by enhancing chloride currents through picrotoxin-sensitive chloride channels and by weak inhibition of sodium (Na^+) and N-methyl-D-aspartate receptor (NMDA) channels (SMITH *et al.*, 2001).

Strychnine administered subcutaneously acts by blocking postsynaptic inhibition mediated by glycine in the spinal cord and induces maximal threshold tonic extensor seizure while subcutaneously applied PTZ acts through direct stimulation of the neuronal membrane and induces minimal-threshold clonic seizure (FOYE, 1990). The plant extracts possessed some protective capacities against different types of epileptic seizures induced in different animal models. The combination of the drugs gave some good protection to the mice in relatively small dosages. This additive effect may be the main reason justifying the combined use of the drugs by traditional herbalists.

3.4 PHYTOCHEMICAL SCREENING OF THE EXTRACTS

3.4.1 Introduction

Phytochemical screening is part of the search for the chemical compound(s) that is responsible for the observed or anticipated physiological activity within an extract (SOFOWORA, 1993). This may be achieved partly by the identification and classification of the plant material as chemotaxonomy can be used to determine which group of compounds are likely to be found (VERPOORTE, 1986; SILVA *et al.*, 1998). Extraction methods and solvents used also play important roles in revealing the nature of chemical constituent(s) present, whether acidic, basic or neutral (HARBORNE, 1998). The major aim of phytochemical screening, therefore, is to detect and establish the nature of the chemical constituents in the plant material that are responsible for the observed physiological activity. This is achieved according to FARNSWORTH (1966) and SILVA *et al.* (1998) through a battery of chemical tests which should have at least one of the following attributes:

- (i) The test procedure should be simple, rapid and designed for a minimum equipment requirement ;

- (ii) It should be selective for the compound under test and be quantitative in so far as having a knowledge of the lower limit of detection ; and
- (iii) If possible it should give additional information as to the presence or absence of specific members of the chemical group being evaluated.

Routine chemical tests were therefore employed to determine the nature of organic compounds present; whether alkaloids, glycosides - which may be saponins, anthraquinones, cyanogenic or cardiac in nature; tannins; phlobatannins or carbohydrates. There may be false-positives or false-negatives. These were guarded against by performing confirmatory tests according to FARNSWORTH (1966) when necessary. The phytochemical screening procedures according to FARNSWORTH (1966), ODEBIYI and SOFOWORA (1978 and 1979) as adapted from WALL *et al.* (1952) and SILVA *et al.* (1998) were employed.

3.4.2 Screening of Chemical Constituents

(i) Alkaloids

These are more or less toxic substances which may act primarily on the central nervous system. They may have basic characters, containing heterocyclic nitrogen and are synthesized in plants from amino acids or their immediate derivatives. In most cases, they are of limited distribution in the Plant Kingdom (FARNSWORTH, 1966).

Because of the presence of nitrogen, alkaloids are basic in nature. Alkaloids are generally detected by alkaloidal precipitants which might also produce false-positive reactions with coumarins, purines, polyphenols, amino acids, proteins and other nitrogenous compounds that might occur in plant extracts (CORDELL, 1981). There is also a possibility of false-negative results since not all alkaloids that might be present will give a positive reaction because of the oxidation state of their nitrogen (SILVA *et al.*, 1998).

The following alkaloid tests were used:

- (a) Mayer's Reagent ; and
- (b) Dragendorff's Reagent

About 3-5 mg of the extract were dissolved with 5 ml of 2 N HCl, stirred in a hot water bath for about 5 min and filtered. About 2-3 drops of Mayer's reagent or Dragendorff's reagent were added to 1 ml of the filtrate. Turbidity or precipitate was taken as preliminary evidence for the presence of alkaloids.

To eliminate the false-positive results of non-alkaloids, a confirmatory test was carried out. This employed a modified form of the thin layer chromatographic (TLC) method as described by FARNSWORTH (1966). Dilute ammonia was added in excess to 2 ml of the filtrate of alkaloid salt solution to precipitate the alkaloid, and then extracted twice with 10 ml each of chloroform. The chloroform extracts were combined and dried over anhydrous sodium sulphate, filtered and concentrated on a water bath to about 1-2 ml. This was then spotted on a TLC plate. The presence of alkaloids on the developed chromatogram was detected by spraying with freshly prepared Dragendorff's reagent. A positive reaction constitutes an orange or darker coloured spot against a pale yellow background.

(ii) Saponins

Several types of compounds are taken into consideration whenever saponin testing is being conducted. Of importance are steroidal and triterpenoid saponins and their respective aglycones (FARNSWORTH, 1966).

All known triterpenoid and steroidal saponins are haemolytic, though haemolytic properties are not limited to saponins, other plant constituents may also exhibit this property. Whereas other plant constituents with haemolytic activity may occur both as heterosides and as for triterpenoids and saponins are never found as free sapogenins (FARNSWORTH, 1966; PINE, 1987).

Saponins are surface active agents. They may be detected by vigorous shaking an aqueous solution of the sample, and produce foam which lasts for at least 15 min.

About 5 mg of the extract were dissolved with 2 ml of purified water and shook. It was then allowed to stand, persistent foam indicated the presence of saponins.

(iii) Reducing Sugars

Sugars are defined as polyhydroxyl-aldehydes or ketones. The reducing activity of an aldehyde or ketone manifests in simple sugars and serves as a basis of their chemical identification.

About 3-5 mg of the sample were dissolved with 2 ml purified water, 2 ml of equal volumes of Fehling's solutions I and II were added, and placed in a boiling water-bath for 15-20 min. A brick red precipitate of cuprous oxide indicated the presence of a reducing sugar.

(iv) Soluble Carbohydrate

To 2 ml of 0.20-0.5% aqueous extract, 1 ml of 10% naphthol was added followed by the careful addition of concentrated H_2SO_4 in a slanting position. The presence of a brownish ring at the interface indicated the presence of a soluble carbohydrate.

(v) Glycosides

There are no reliable general tests for glycosides but their presence can be detected by the following procedures:

- (a) Test for reducing sugar as above. A positive result is an indication of the presence of reducing sugars and chances of a glycoside being present. Then a second step can be carried out.
- (b) Hydrolysis of 2 ml of the aqueous extract solution by addition of 2-5 ml 2N HCl and placing in a boiling water bath for 25 to 30 min. Allow to cool and neutralize with excess 2N ammonia solution. The reducing sugar test is performed as above and the results of (i) and (ii) are compared. Appearance of more brick red precipitate after hydrolysis indicates the possible presence of glycosides.

The above test is a general test, therefore specific tests for different types of glycosides must be carried out (FARNSWORTH, 1966; ODEBIYI and SOFOWORA, 1978 and 1979; SOFOWORA, 1993; SILVA *et al.*, 1998).

Specific Tests:

1. Cardiac Glycosides

These are characterised by the presence of a deoxy sugar especially a 2-deoxy sugar in the molecules as glycones. All cardioactive glycosides are classified as steroids having a cyclopentanohydrophenanthrene nucleus, and an α - β unsaturated lactone ring (5 or 6-member) at C₁₇, a β -oriented hydroxyl group at C₁₄, and a *cis* fusion of the C and D rings at C₁₃-C₁₄ (FINAR, 1975; PINE, 1987). The sugar moiety is usually a deoxyhexomethylsugar at position C₃ of the steroid nucleus.

The test for cardiac glycosides is designed to detect either an unsaturated lactone moiety at C₁₇, deoxysugar at C₃ or steroid nucleus. However, a steroid nucleus is not specific for cardiotonic glycosides (FARNSWORTH, 1966).

2. Xanthydrol Test for Deoxysugars

About 0.1 to 0.2 g of the dried extract is dissolved in 1 ml of 0.125% solution of xanthydrol in glacial acetic acid containing 1% HCl. A red colouration indicates a positive result.

3. Keller-Kiliani Test

About 0.1 to 0.2 g of the dried extract is dissolved in 1 ml of glacial acetic acid containing traces of ferric chloride in a test tube. In a slanting position 1 ml of concentrated H₂SO₄ is carefully added. A blue-green colour on the upper acetic layer is an indication of the presence of a deoxysugar.

4. Kedde Test

Solution I: Two percent of 3,5-dinitrobenzoic acid in methanol ; Solution II: KOH (5.7%) in water. One ml of each solution is added to 0.2-0.4 ml of the test sample solution, the development of a bluish to purple colour within 5 min constitute a positive result.

5. **Baljet Test**

Solution I is prepared by dissolving 1 g picric acid in 100 ml ethanol; Solution II is prepared by dissolving 10 g NaOH in 100 ml water. Solutions I and II are combined (1:1) just before use. Two to three drops of the final mixture are added to 3-5 mg of the sample. A positive reaction is indicated by the development of an orange to deep red colour.

6. **Anthraquinone Glycosides**

Anthraquinone glycosides can occur as a O-glycoside or a C-glycoside or as both O and C glycosides in a moiety. Borntrager's test is used for the detection of anthraquinones (EVANS, 1989). To 5 ml of the extract 10 ml of ether is added, thoroughly mixed and allowed to separate. The ether layer, after separation, is mixed with 2 ml of aqueous caustic solution. Pink, red or violet colours indicate the presence of free anthraquinone derivatives.

7. **O-glycoside Test**

About 0.5 g dried extract is dissolved in 5 ml purified water and 5 ml 10% HCl added, and boiled in a water-bath for 15 to 20 min. This is allowed to cool and is then extracted with 10 ml ether. The ethereal layer is shaken with 5 ml of 10% ammonia solution. The formation of a white turbid solution indicates the presence of O-glycosides.

8. **C-glycosides**

About 0.2 to 0.3 g of the extract is dissolved with 5 ml purified water and 2 ml 60% FeCl₃ solution added and hydrolysed by heating in a water-bath. The solution is allowed to cool and extracted twice with 5 ml each CCl₄ (carbon tetra chloride). The CCl₄ extracts are combined and washed with water and then shaken with half the volume of 2 N ammonia. A red colouration in the ammoniacal layer indicates the presence of C-glycosides.

9. Cyanophoric or Cyanogenetic Glycosides

These glycosides release hydrocyanic acid (HCN) on hydrolysis and are of major importance because of their potential danger as poisons to livestock as well as humans (TYLER *et al.*, 1988). They can be detected as follows

About 0.5 g of the dried extract is placed in a test tube and moistened with water. A piece of sodium picrate paper prepared by dipping paper into sodium picrate solution (sodium picrate solution 5 g Na_2CO_3 , 0.5 g picric acid, water to 100 ml), is suspended above the drug by trapping the top edge between cork and test tube wall and then allowed to stand for about 30 min. If cyanogenetic glycosides are present the paper turns brick-red owing to the formation of sodium isopicrate.

(vi) Test for Tannins

Tannins are polyphenolic compounds and give the same chemical tests as phenols.

About 0.2 g of the dried extract was dissolved in 10 ml distilled water and filtered. To 1-2 ml filtrate 2-3 drops of 5% FeCl_3 solution is added. A blue-black, green or blue-green cloudy solution or precipitate indicates a positive reaction for tannins.

(vii) Phlobatannins

About 0.2 g of the extract is dissolved with 1 to 2 ml purified water and an equal volume of 2% HCl added and boiled on a water-bath. Deposition of a red precipitate indicates the presence of phlobatannins.

3.4.3 Results

Table 3.15: Results from Phytochemical tests for alkaloids in extracts of *S. magnificum* and *G. brevis*

<i>S. magnificum</i>		
Test	Observation	Inference / Result
Mayer's reagent	Cream white precipitate observed	Alkaloids suspected present
Dragendorff's reagent	Reddish brown precipitate observed	Alkaloids suspected present
Confirmatory tests FARNSWORTH (1996)	Positive to Mayer's and Dragendorff's tests	Alkaloids appear to be present
<i>G. brevis</i>		
Mayer's reagent	No cream white precipitate observed	Alkaloids not likely present
Dragendorff's reagent	No reddish brown precipitate observed	Alkaloids not likely present
Confirmatory tests according to FARNSWORTH (1966)	Negative to Mayer's and Dragendorff's tests	Alkaloids not present

Table 3.16: Results from tests for reducing sugars in extracts of *S. magnificum* and *G. brevis*

<i>S. magnificum</i>		
Test	Observation	Inference / Result
Fehling's I&II solutions	Brick red precipitate observed	Reducing sugar present; glycosides suspected
After hydrolysis with dilute HCl, above test repeated	More intense brick red precipitate observed	Glycosides appear to be present
<i>G. brevis</i>		
Fehling's I&II solutions	Brick red precipitate observed	Reducing sugar present; glycosides suspected
After hydrolysis with dilute HCl, above test repeated	More intense brick red precipitate observed	Glycosides appear to be present

Confirmatory Tests

To 5 ml aqueous solution of about 0.1 g of the extract, 3 ml 5% FeCl₃ solution and 2 ml 2N HCl were added and boiled in a water-bath for 15-20 min, then allowed to cool and neutralized with excess 2 N ammonia solution, and the reducing sugar test repeated.

An increase in the intensity of brick-red precipitate observed in both samples confirmed the presence of glycosides in *S. magnificum* and *G. brevis* extracts respectively.

Types of Glycoside

Table 3.17: Results from tests for cardiac glycosides in extracts of *S. magnificum* and *G. brevis*

<i>S. magnificum</i>		
Test	Observation	Inference / Result
Xanthydrol test	Red colouration observed	Deoxysugar present; cardiac glycosides suspected
Baljet test	Deep red colouration observed	Methylene group present ; cardiac glycosides suspected
Kedde test	No significant colour change observed	Negative to the test
Keller-Kiliani test	No blue-green colour on the upper acetic acid layer was observed.	Negative to the test
<i>G. brevis</i>		
Xanthydrol test	Red colouration observed	Deoxysugar present; cardiac glycosides suspected
Baljet test	Deep red colouration observed	Methylene group present ; cardiac glycosides suspected
Kedde test	Significant colour change observed	Positive to the test; cardiac glycosides likely to be present
Keller-Kiliani test	Blue-green colour on the upper acetic acid layer was observed.	Positive to the test; cardiac glycosides likely to be present

Table 3.18: Results for tests for anthraquinone glycosides in extracts of *S. magnificum* and *G. brevis*

<i>S. magnificum</i>		
Test	Observation	Inference / Result
O-glycosides	No formation of turbid solution	Negative to the test; O-glycoside not likely present.
C-glycosides	No red colouration formed with ammoniacal layer	Negative to the test; O-glycoside not likely present
<i>G. brevis</i>		
O-glycosides	No formation of turbid solution	Negative to the test; O-glycoside not likely to be present.
C-glycosides	No red colouration formed with ammoniacal layer	Negative to the test; O-glycoside not likely to be present

Table 3.19: Results for tests for cyanophoric or cyanogenetic glycosides in extracts of *S. magnificum* and *G. brevis*

<i>S. magnificum</i>		
Test	Observation	Inference / Result
Sodium picrate paper test	Paper did not turn brick red therefore sodium isopicrate not formed	Negative to the test; cyanogenetic glycosides not likely to be present
<i>G. brevis</i>		
Sodium picrate paper test	Paper did not turn brick red therefore sodium isopicrate not formed	Negative to the test; cyanogenetic glycosides not likely to be present

Saponins

S. magnificum did not form a persistent foam, therefore negative to the test. *G. brevis* did not form a persistent foam, therefore saponins not likely to be present.

Polyphenols

S. magnificum and *G. brevis* gave positive blue-black precipitates with Fe^{3+} salt. The results indicates that the extracts may contain polyphenolic compounds.

pH test

S. magnificum extract was acidic with $\text{pH} < 5.5$ while the *G. brevis* extract was slightly acidic with $\text{pH} > 6.2$.

3.4.4 Discussion

The phytochemical screening results indicate the presence of alkaloids in *S. magnificum* root extracts, and their absence in *G. brevis* leaf extracts. *S. magnificum* was reported to contain chromone alkaloids by HOUGHTON and YANG (1985), OKOGUN *et al.* (1983) and piperidino-chromone alkaloids by HOUGHTON and RAMAN (1998). Alkaloids are reported to be generally absent in the family Tiliaceae of which *G. brevis* is a member (EVANS, 1989). Test for alkaloids basically involves the use of heavy metal salts such as mercuric salts as in Mayer's reagent or bismuth salts as in Dragendorff's reagent. The heavy metallic ions act as chelating agents which react with lone pairs of electrons in nitrogen to form insoluble precipitates regarded as a positive sign for alkaloids. Unfortunately a false positive or a false negative reaction may occur. These were guarded against by performing confirmatory tests as outlined by FARNSWORTH (1966).

The results showed the presence of glycosides in both *S. magnificum* root and *G. brevis* leaf extracts. *S. magnificum* is reported to contain the chromone glycoside, schumanniofoside (AKUNYILI and AKUBUE, 1986; TANE *et al.*, 1990). *G. brevis* and other plants in the Tiliaceae are reported to contain only glycosides (EVANS, 1989). There is no general test for glycosides but the presence of reducing sugars is an indication of the possible presence of glycosides. The presence of reducing sugars was tested for with Fehling's solutions which contains Cu^{2+} ions in an alkaline medium.

Reducing sugars reduce Cu^{2+} to Cu^+ oxide forming brick red precipitates, a positive indication of the probable presence of glycosides. A positive result for reducing sugars was obtained with both *S. magnificum* and *G. brevis* extracts.

A confirmatory test for glycosides involves subjecting the sample to hydrolysis and repeating the above test (FINAR, 1975; TYLER *et al.*, 1988; EVANS, 1989). If more intense precipitates are obtained compared with the first test then the presence of glycosides is confirmed. Confirmatory tests were positive for both plant extracts.

Tests for different types of glycosides were carried out and both plants tested positive for cardiac glycosides. *G. brevis* was consistently positive to Xanthydrol, Baljet, Kedde and Keller-Kiliani tests indicating the possible presence of cardiac glycosides. *S. magnificum* was positive to only the Xanthydrol and Baljet tests and its solution was slightly acidic indicating the probable absence of cardiac glycosides in the extracts.

G. brevis extract was neutral indicating the possible presence of tetracyclic aglycones or aglycones with chemical structures other than the pentacyclic structure (FINAR, 1975; TYLER *et al.*, 1988; EVANS, 1989; FRIEDLI, 2000).

The phytochemical tests on the extracts indicated the presence of secondary metabolic products such as alkaloids, glycosides and tannins in addition to reducing sugars in the extracts tested and outlined in Tables 3.15 - 3.19.

CHAPTER 4

ISOLATION, SEPARATION AND PURIFICATION OF PLANT EXTRACTS

4.1 ISOLATION OF CHEMICAL CONSTITUENTS

Isolation of bioactive compounds is a key procedure and a most important step in studies on natural products. It is fraught with difficulties and every step requires judgement, improvisation and discovery (COLEGATE and MOLYNEUX, 1993). On one hand knowledge and information are gained from isolation and structures obtained from the isolated constituents, and on the other hand isolation may lead to discovery of substance(s) that might have some economic value or can serve as lead substances for synthesis of more complex structures (FARNSWORTH, 1966). As a lead, novel chemical structures isolated from plants have prompted chemists to successfully develop a series of modified synthetic compounds e.g. development of homatropine from atropine (TYLER *et al.*, 1988). Isolation of bioactive agents had helped in providing chemotaxonomic evidence for classification of genera or species especially those where classification based on morphological characters alone was not definitive (SOFOWORA, 1993).

The isolation of bioactive agents from plant material for investigation can be grouped into two fundamental procedures in accordance with two main objectives (SOFOWORA, 1993).

- (i) **Biological Screening:** This is a search for physiological effects which the plant extract produces. There may be a need for the production of sufficient amounts of the known compound so that it can be used for further work or more extensive biological testing (CANNELL, 1998).
- (ii) **Phytochemical Screening:** This is the search for the substance itself. Isolation for phytochemical reasons may involve purification of a sufficient amount of a compound for purposes of partial or full characterization.

Isolation and purification of organic active constituents from natural sources or plant materials can be accomplished by means of chromatographic and a host of other techniques.

4.1.1 Chromatographic Techniques

Chromatographic techniques are described by OLANIYI and OGUNGBAMILA (1991), as the most powerful approach to separate mixtures with an unequalled ability among other separation methods to separate complex mixtures of similar substances.

Chromatography is defined as a technique which basically involves separation due to differences in equilibrium distribution of sample components between two immiscible phases. One of these phases is moving or mobile and the other is stationary (FARNSWORTH, 1966; KIRKLAND, 1971). The sample components migrate through a chromatographic system only when there is a mobile phase and separation results from different velocities of migration as a consequence of differences in equilibrium distribution (BECKETT and STANLAKE, 1986).

There are four basic modes of chromatography that can be applied in the isolation and analysis of organic compounds, partition, ion-exchange, adsorption and size-exclusion or filtration, and these may involve elution or frontal displacement (BECKETT and STANLAKE, 1986). These techniques differ from each other according to the nature of the stationary phase, mobile phase, and the apparatus used and can be classified according to stationary phase and mobile phase selected.

Gas chromatography encompasses those methods in which the mobile phase is gas and liquid chromatography those with liquid mobile phases. The different stationary phases give rise to names such as Liquid-solid chromatography (LSC) and Liquid-liquid chromatography (LLC). Liquid chromatography can be classified according to the mechanism of selection; via ion-exchange, adsorption, partition and exclusion or filtration chromatography respectively. These mechanisms are well known except for the exclusion mechanism which forms the basis of gel permeation chromatography (GPC) or gel filtration chromatography (GFC) (KIRKLAND, 1971).

Liquid chromatographic techniques were employed in this work. Liquid chromatography can be performed in a column, as in column chromatography (CC), on open beds as in thin layer chromatography (TLC), on paper as in paper chromatography (PC) or on a modified column as in high pressure liquid chromatography (HPLC).

4.2 MATERIAL AND METHODS

4.2.1 Materials

The TLC strips of pre-coated plates of silica gel₆₀ with a fluorescent marker (Merck, F₂₅₄) were extensively used for establishing an appropriate solvent system and for monitoring of the Fractions. The column was packed with non-binding silica gel₆₀ (Merck Art 7734, 0.063-0.200 mm i.e 70-230 mesh). All the extracts and fractions were concentrated using a rotary evaporator (Büchi K4R Electronic). Ultraviolet light at wave lengths of 254 and 366 nm were used to detect constituents. All the reagents used were of laboratory or analytical grade.

4.2.2 Methods

Developing and Establishing an Appropriate Solvent System: Thin Layer Chromatographic Technique (TLC)

Thin layer chromatographic (TLC) techniques were introduced by STAHL (1969) and MELOAN (1968) when he added 5 to 10% plaster of paris to silica gel to 'plaster' it to glass. TLC has the following advantages:

- It is quick and very sensitive with low limits of detection around 10^{-9} g ;
- A wide range of samples can be handled ; and
- It is a valuable tool in both qualitative and quantitative analysis and experimental parameters can easily be varied to affect separation (KIRKLAND, 1971).

The TLC plates may provide separation equivalent to thousands of theoretical plates in a relative short distance (HARBORNE, 1998) and molecules occurring as similar as *cis*- and *trans*-isomers can be separated clearly.

The extent of movement is described by R_f values, which is the ratio of the distance traveled by the band maximum to the distance traveled by the leading edge of the mobile phase. The same substances have the same R_f values under the same experimental conditions. This forms the basis of the qualitative process of TLC.

A thin layer chromatographic technique as an instrument of qualitative analysis was initially employed to determine the solvent system that would offer the best resolution for the extracts being analyzed.

The phytochemical tests described earlier (Chapter 3) were aimed at revealing the possible nature of the active constituents. Therefore the results of the tests served as guides in selecting solvent efficient systems.

The following solvent systems were investigated. The calculated quantity of each solvent in the solvent system was measured into a dry and clean beaker and were thoroughly mixed. This was then transferred to a washed and dry chromatographic tank and covered for at least 30 min to equilibrate before introducing the TLC plate that had been loaded with the 5 to 10 μ l samples. The plate was allowed to develop, then removed from the tank and dried before being viewing under UV light at 254 or 366 nm respectively. Merck silica gel₆₀ F₂₅₄ (0.25 mm) TLC plates were used.

The following solvent systems were used :

- (i) Benzene : ethylacetate : diethylamine (90:5:5), (VAN DAN ELCKHOUT *et al.*, 1980). This solvent system could not resolve the extracts, since all remained on the base line. It was therefore discontinued.
- (ii) Acetonitrile : methanol : water (1:1:1). Resolved *S. magnificum* extracts and *G. brevis* extracts into three spots each.
- (iii) Toluene : diethylamine (9:1). Resolved *G. brevis* extracts into two spots but *S. magnificum* extracts were not resolved.
- (iv) Chloroform : methanol (9:1). No resolution in *G. brevis* extracts but *S. magnificum* extracts were resolved into two spots.
- (v) Ethylacetate : acetone : ammonia (55:43:2) (LIKHITWITAYAWUID *et al.*, 1993). Resolved *S. magnificum* extracts into three spots and *G. brevis* extracts yielded only one spot.

- (vi) Acetone : ammonia (90:3). *S. magnificum* extracts were resolved into four spots while *G. brevis* extracts produced only one spot.
- (vii) Ethylacetate : acetone (55:45). *G. brevis* extracts produced only one spot while *S. magnificum* extracts were not resolved.
- (viii) Ethylacetate : methanol : acetic acid (45:54:1). Resolved *S. magnificum* extracts into three spots and *G. brevis* extracts into only one spot.
- (ix) Ethanol : ammonia : dichloromethane (2.50:0.75: 46.75) (SUZUKI *et al.*, 1994). Resolved *S. magnificum* extracts and *G. brevis* extracts into two spots each.

4.3 RESULTS AND DISCUSSION

Many solvent systems were investigated, but ethylacetate : acetone : ammonia (55:43:2) and acetone : ammonia (90:3) proved to have the best resolving capacities for the *S. magnificum* extracts obtained in this study. The first solvent system was successfully employed by LIKHITWITAYAWUID *et al.* (1993) to resolve bisbenzylisoquinoline alkaloids. The phytochemical tests (Chapter 3.4.3) showed *S. magnificum* to contain alkaloids and glycosides as active constituents.

The nature of the alkaloid is yet to be established, but it is most likely to exist as an independent moiety from glycosides. Alkaloids and glycosides have been reported in the Rubiaceae and occur as independent moieties (EVANS, 1989). While the acetone : ammonia (90:3) solvent system did resolve the extracts of *S. magnificum* and *G. brevis* respectively, a better resolution was obtained with the former solvent system.

G. brevis extracts was positive in the cardiac glycoside test, and was well resolved with the acetonitrile : methanol : water (1:1:1) solvent system. This solvent system was successfully employed by FUJI *et al.* (1980) to separate *Digitalis* cardiac glycosides using micro high performance chromatography. De TOMMASIX *et al.* (1998) successfully used a chloroform : methanol (9:1) solvent system to resolve glycosides, but this only partially resolved the extracts of *G. brevis* and *S. magnificum*. On modification of the solvent system to a ratio of 7:3 a better result was obtained. Ethylacetate: methanol: formic acid (45:54:1) being a polar solvent system was expected to give a better resolution but the results showed that *G. brevis* extracts were

resolved into only two spots and *S. magnificum* extracts into three spots. Therefore the methanol : ethylacetate : acetone : ammonia (4.4:3:2.5:0.1) solvent system was adopted for routine monitoring of the isolated extracts and subsequent Fractions.

4.4 INITIAL EXTRACT PURIFICATION AND SEPARATION - COLUMN CHROMATOGRAPHIC TECHNIQUES

4.4.1 *S. magnificum*

A glass column (75 x 3 cm) was packed dry with 60-g silica gel and glass wool placed on top. The sample (10 g) was mixed with enough silica gel (7 - 10 g) to produce a powdery mixture. This was then introduced into the column and the top covered with glass wool. It was then sequentially extracted starting with a very non-polar to a not too polar solvent and then to a very polar solvent as indicated below :

- (i) Toluene (250 ml)
- (ii) Chloroform (350 ml)
- (iii) Ethylacetate (300 ml)
- (iv) Acetone (300 ml)
- (v) Methanol (400 ml)
- (vi) Methanol/H₂O (70%) (300 ml)
- (vii) H₂O (200 ml)

Using methanol : ethylacetate : acetone : ammonia (4.4:3:2.5:0.1) as solvent system the Fractions with the same TLC characteristics were pooled. A total of 50 g of the sample was cleaned and separated and three Fractions were obtained as indicated below :

A =	1.501 g	3.0 %	of the total weight separated
B =	20.780 g	41.6 %	"
C =	<u>4.503 g</u>	<u>9.0 %</u>	"
	<u>26.790 g</u>	<u>53.57 %</u>	"

4.4.2 *G. brevis*

The extract (50 g) was fractionated in batches of 10 g. The process was repeated as for *S. magnificum*, starting with n-hexane. Two hundred and fifty milliliters each of n-hexane, toluene, chloroform and ethylacetate, 350 ml of acetone and 500 ml each of

methanol and 70% methanol were used. The Fractions were fractionated using TLC with chloroform and methanol (7:3) as solvent system. Three fractions were obtained as follows :

A = 3.83 g	7.76 %	of the total weight separated
B = 20.66 g	41.32 %	"
C = <u>10.37 g</u>	<u>20.74 %</u>	"
<u>34.86 g</u>	<u>69.82 %</u>	"

4.4.3 Discussion

Column chromatography is a well tried chromatographic technique and today still remains one of the most widely used methods of separation because of its simplicity in the separation of natural and other organic compounds. It has been employed for separation of triterpenoids (MUTHU-KUMARA and TAN, 2000; KOORBANALLY *et al.*, 2000; GUO and KENNE, 2000) triterpenoid sapogenins (MISRA *et al.*, 1991); triterpenoid glycosides (JIANG *et al.*, 1991) and alkaloids (JEFFERYS, 1970; HOUGHTON and YANG, 1985). In most cases integrated column chromatographic techniques which involve traditional chromatographic methods and a variety of its modified versions e.g. flash chromatography are used (COLEGATE and MOLYNEUX, 1993).

Column chromatographic techniques were employed for the initial purification and separation of the chemical constituents of the root extracts of *S. magnificum* and leaf extracts of *G. brevis*. This technique was successfully used by MARQUINA *et al.* (2001); HISHAM *et al.* (2001) and LAVAUD *et al.* (2001) to isolate chemical constituents of simple mixtures. Using the chloroform-methanol solvent system in the different ratios, 50:1, 25:1, 15:1, 10:1 and 5:1, chemical constituents of *S. magnificum* were isolated (HOUGHTON and YANG, 1985). A sequential method of separation starting with a highly non-polar organic solvent, toluene, to a highly polar solvent, 70% aqueous methanol, was successfully employed to also separate other constituents of *S. magnificum* and *G. brevis* respectively (COLEGATE and MOLYNEUX, 1993). The *S. magnificum* root extract yielded two major Fractions. The third Fraction was not adequately resolved from the second Fraction and was discarded. The *G. brevis* leaf extract yielded three major Fractions.

4.5 FURTHER SEPARATION AND PURIFICATION OF FRACTIONS

4.5.1 *S. magnificum*

Fractions A and B of *S. magnificum* obtained from the initial purification (Section 4.4.1) were subjected to further separation on column chromatography and the resulting Fractions purified by a combination of centrifugal and PTLC methods. A TLC investigation showed that Fraction C overlapped with B. As it was a water soluble Fraction no further work was done on it.

(I) Development of a Solvent System for Further Separation of Fractions

The following solvent systems were investigated on qualitative thin layer chromatographic plates as a prelude to column chromatographic separation.

(i) Cyclohexane : chloroform : diethylamine (50:40:10). All the Fractions were unresolved and remained on the base line.

(ii) Chloroform : acetone : diethylamine (5:4:1). The result was the same as in (i).

(iii) Dichloromethane : methanol : water (87:12:1).

Fraction A showed a single spot with a good R_f value while Fraction B was resolved into five spots.

(iv) Benzene : chloroform (7:3). None of the Fractions were resolved by this solvent system.

(v) Acetonitrile : methanol : water (1:1:1).

Fraction A yielded only one spot with an R_f value at 0.5 while Fraction B was resolved into three spots.

(vi) Solvent system (iii) above (87:12:1) was modified to a (65:33:2) ratio. The resolution obtained was not as good as that obtained with the initial solvent system.

(vii) Chloroform : methanol : water : acetic acid (24:17.5:3:0.1).

Fraction A yielded a spot with an R_f value at 0.5 while fraction B was resolved into two spots.

Solvent systems (iii) and (v) gave the best results and were therefore adopted for further use.

(II) Separation of Fraction A

Using several qualitative TLC investigation steps with different solvent systems Fraction A yielded only a single spot - an indication of having only one chemical component. It was, therefore, not subjected to further purification. The identity of the single component was subjected to NMR analysis. Fraction A was then coded as M₁.

(III) Separation of Fraction B - Use of a Sephadex LH₂₀ Column

Fraction B was resolved in the qualitative TLC experiments earlier by the dichloromethane : methanol : water (87:12:1) solvent system. This solvent system was incompatible with Sephadex LH₂₀ and was discontinued. A more polar solvent system, methanol: acetonitrile: water (1:1:1) that gave a good result on qualitative TLC was modified to 6:3:1 for better resolution and used.

(IV) Fraction B Fractionation

The Sephadex LH₂₀ granules (50 g) with a bed volume of 4 ml g⁻¹ of dry gel in water, methanol or chloroform was pre-soaked with 250 ml distilled methanol and allowed to swell overnight. This was loaded into a glass column (75 cm x 3.0 cm i.d.), covered with distilled methanol, and left overnight to allow the Sephadex to settle to its final volume. Finally the solvent system methanol : acetonitrile : water (6:3:1) was run through the column and left for at least one hour to equilibrate.

The sample (3.5 g) was dissolved with 3 to 5 ml distilled methanol, introduced into the column with a pipette and eluted with the solvent system. The Fractions were monitored with a dichloromethane : methanol : water (87:12:1) solvent system and fractions with TLC similarity pooled together to give three main Fractions. This experiment was repeated two more times with 3.0 g and 3.5-g of material respectively and all the Fractions with similar chromatographic properties pooled.

The total available amount of Fraction B (10.0 g) was separated and the dried weights of the Fractions obtained were as follows :

Fraction I	=	0.2 g (1.45% of B) (M ₂)
Fraction II	=	7.2 g (62.20% of B)
Fraction III	=	0.11 g (0.99% of B) (M ₇)

TLC investigations revealed that Fraction II consisted of more than five spots. It was therefore subjected to further column chromatographic separation on Sephadex LH₂₀. Fractions I and III were coded (M₂) and (M₇) respectively.

(V) Fractionation of Fraction II

The second Fraction (II, 7.2 g) indicated above was subjected to further separation using Sephadex LH₂₀ as stationary phase using the same solvent system as before (methanol : acetonitrile : water (6:3:1)). Three Fractions were obtained, two of the Fractions were crystalline in nature and the third resinous.

The first Fraction was coded M₃ and the second M₄ and the last resin-like component(s) Fraction C. The yield was :

Fraction I	=	0.53 g (M ₃)
Fraction II	=	0.47 g (M ₄)
Fraction III	=	3.67 g (C)

TLC investigation revealed Fraction C to consist of at least five spots and it was therefore subjected to further separation using centrifugal chromatography.

(VI) Separation of Fraction C by Centrifugal Chromatography (Chromatotron) and Development of a Solvent System

The following solvent systems were investigated :

- (i) Ethylacetate : methanol : acetonitrile (5:3:2) ;
- (ii) Chloroform : methanol : water (14:6:1) ; and
- (iii) Chloroform : methanol : water : formic acid (25:20:4:1)

Solvent systems (ii) and (iii) gave better resolution of the sample than solvent system (i). These two solvent systems were therefore used for the analyses.

Five hundred mg of the sample were dissolved with 1.5 ml each of methanol and water and introduced onto a rotating 2 mm silica gel disc with a Pasteur pipette and first eluted with 150 ml of chloroform : methanol : water (14:6:1) and then with 150 ml chloroform : methanol : water : formic acid (25:20:4:1).

The Fractions were monitored using a combination of solvent systems (ii) and (iii) (1:1) and Fractions with TLC similarities pooled to give three main Fractions. The experiment was repeated with the following quantities of the sample 0.51 g, 0.55 g and 0.51 g. The total quantity separated was 2.07 g and the dried weights of the two main Fractions recovered were :

Fraction A (CT₁) = 0.99 g (47.75% of C) M₅

Fraction B (CT₂) = 0.86 g (41.42% of C) M₆

(VII) Physical Characteristics of the Isolated Fractions

M₁ was crystalline in nature, soluble in methanol but very soluble in 70% ethanol.

M₂ resinous, soluble in aqueous ethanol (70%).

M₃ crystalline in nature, soluble in methanol.

M₄ crystalline, sparingly soluble in ethanol and methanol but soluble in aqueous ethanol or methanol (70%).

M₅ resinous in nature, freely soluble in methanol.

M₆ crystalline in nature, soluble only in aqueous ethanol (70%).

M₇ crystalline, soluble in aqueous ethanol (70%).

(VIII) Final Purification

Preparative thin layer chromatography (PTLC) has been extensively used in the final purification of isolated organic chemical constituents (HARBORNE, 1998; LAVAUD, 2001). PTLC consists of glass, aluminum or plastic backed plates with a thicker sorbent coating usually between 0.5 to 4 mm compared to analytical TLC with a sorbent coating of 0.1 to 0.2 mm (GIBBONS and GRAY, 1998).

The organic compound to be separated or purified is streaked as a band 2 to 3 cm from the base of the plate and developed in a chromatographic tank containing an appropriate solvent system. Detection of the separated component band(s) is achieved by the use of a UV detector if the sorbent contains a fluorescent and the chemical constituent contains a chromophore that can absorb at the given UV wavelength, otherwise an appropriate detecting reagent for the chemical constituent(s) in question is used.

(IX) Developing an efficient Solvent System

The following solvent systems were investigated for further separation and purification of the Fractions obtained :

- (i) Ethylacetate : methanol (9:1) ;
- (ii) Ethylacetate : cyclohexane (8:2) ;
- (iii) Chloroform : methanol : formic acid (6.5:3.4:0.1) ;
- (iv) Ethylacetate : acetone : ammonia (55:43:2) ;
- (v) Acetonitrile : methanol : formic acid (40:55:5) ; and
- (vi) Chloroform : methanol : water (87:12:1).

M₁ had an R_f value at 0.5 in all the solvent systems, and yielded only a single spot in all except in system (ii) in which case it did not move off the baseline.

M₂ was only resolved by solvent system (v) (acetonitrile : methanol : formic acid) and into three spots.

M₃ resolved by solvent systems (iii) and (v) and yielded only one spot.

M₄ resolved by solvent system (v) into two spots.

M₅ resolved by solvent system (vi) into five spots.

M₆ resolved by solvent system (vi) but yielded only one spot.

M₇ resolved by solvent system (v) and yielded only one spot.

Solvent systems (ii) and (vi) were further investigated by varying their solvent combinations. Two more systems were also employed :

- (a) Dichloromethane : methanol : water (16.2:3.6:2) ; and
- (b) Acetonitrile : methanol : formic acid (60:35:5).

M₁ again yielded a single spot with these solvent systems at R_f 0.5 and 0.6 respectively.

M₂ had a better resolution with acetonitrile : methanol : water (1:1:1).

M₃ yielded one spot with all solvent systems.

M₄ yielded a single spot on TLC, with acetonitrile : methanol : formic acid (60:35:5) (b), an indication of consisting of only one constituent.

M₅ separated into five spots with dichloromethane : methanol : water (87:12:1).

M₆ showed one spot with dichloromethane : methanol : water (87:12:1).

M₇ was resolved with acetonitrile : methanol : water (1:1:1) showing one spot with an R_f value at 0.6.

(X) Purification of M₁

TLC investigations on Fraction M₁ revealed a single spot, an indication that it contains a single chemical constituent. It was therefore purified by crystallization (SHANKLAND *et al.*, 1998).

An excess quantity of M₁ (180 mg) was dissolved with 3.5 ml of warm methanol (35-40°C) in a test tube and the test tube immersed in ice cold water (0-4°C). M₁ crystallized out, was filtered and washed twice with pure methanol and then left in a stream of air to dry. The pure sample (30 mg) was recovered and subjected to structure elucidation by NMR.

(XI) Purification of M₂ and M₅

M₂ (0.063 g) and M₅ (0.055 g) were respectively dissolved with 2 ml aqueous methanol (70%) and streaked on preparative silica gel₆₀ plates (2 mm thick) containing a fluorescent maker. The plates were developed in a tank containing 200 ml dichloromethane : methanol : water (87:12:1). The migration was carried out twice and the separated bands scraped off under UV light at 254 nm. The UV-active constituents were recovered by eluting the scraped silica gel with 15 ml purified methanol, stirred continuously for 15 min and filtered through a No. 3 sintered glass funnel under vacuum. The silica gel was further washed with 5 ml portions each of purified methanol. The filtrates were combined and dried to yield the following dry weights for M₂ and M₅ respectively.

$M_{2.1}$	=	0.020 g
$M_{2.2}$	=	0.017 g
$M_{5.2}$	=	0.016 g
$M_{5.3}$	=	0.012 g
$M_{5.6}$	=	0.002 g
$M_{5.7}$	=	0.006 g

These fractions were used for anti-convulsant tests and structure elucidation of the active components then attempted.

(XII) Purification of M_3 , M_4 and M_7

Fractions M_3 , M_4 and M_8 were very soluble in hydrophilic solvents. The solvent system, acetonitrile : methanol : water (3:6:1) is polar and did not give good results with PTLC. Another solvent system ethylacetate : methanol : water (18:7:10) was tried. This solvent system gave good compound separation with PTLC and was used.

The following samples M_3 (0.05 g), M_4 (0.10 g), and M_7 (0.062 g) were dissolved with 1 to 2 ml aqueous methanol (50%) respectively and the procedure completed as earlier, to yield the following Fractions :

$M_{3.1}$	=	0.006 g
$M_{3.2}$	=	0.041 g
M_4	=	0.033 g
$M_{7.1}$	=	0.019 g
$M_{7.2}$	=	0.004 g

These Fractions were subjected to anti-convulsant tests and structure elucidation of active components then attempted.

4.5.2 *G. brevis*

G. brevis was shown by phytochemical tests to contain cardiac glycosides among other organic chemical compounds. The solvent systems used for *S. magnificum* were utilised. *G. brevis* at this point had been separated into three major Fractions A, B and C. The first Fraction A called G_1 , which was a non-polar Fraction was subjected to

further CC separation using silica gel while Fractions B and C were subjected to further separation using Sephadex LH₂₀ as stationary phase.

(I) Separation of Fraction A (G₁)

The sample (3.0 g) was separated on 30 g silica gel 60 (Merck 0.04 - 0.63 mm) CC using the following solvent systems: dichloromethane : methanol : water (87:12:1), (300 ml); dichloromethane : methanol : acetonitrile (5:3:2), (100 ml) and methanol : acetonitrile (4:1), (100 ml). The Fractions were monitored with dichloromethane : methanol : acetonitrile (87:12:1) and Fractions with TLC similarity pooled to give three major Fractions with their dry weights as follows :

I	=	2.784 g	G _{1A}
II	=	0.270 g	G _{1B}
III	=	0.091 g	G _{1C}

The TLC examination of the fractions showed that Fraction II contained three or more compounds. It was therefore subjected to further centrifugal chromatographic separation.

(II) G_{1A} Separation: Use of Centrifugal Thin Layer Chromatography (CTLC)

In this method, the circular preparative plate of silica gel containing a fluorescent marker was rotated at 800 rpm (COLEGATE and MOLYNEUX, 1993). G₁ (0.5 g) was dissolved with 3 ml purified methanol and introduced in the center of the rotating 2 mm silica gel disc with a Pasteur pipette and eluted with dichloromethane : methanol : water (87:12:1) at 1 to 2 ml s⁻¹. The Fractions were monitored with the same solvent system using analytical TLC. Fractions with TLC similarity were pooled to yield three major Fractions. The experiment was repeated three more times using 0.5 g of G_{1A} each time, to yield the following dry weights :

A	=	0.339 g
B	=	0.092 g
C	=	0.061 g

These were coded G_{1A1} , G_{1A2} , and G_{1A3} , respectively. They were subjected to further separation and final purification using preparative TLC.

(III) Separation of Fraction B using a Sephadex LH₂₀ Stationary Phase

The total quantity of Fraction B separated was 6.603 g and acetonitrile : methanol : water (3:2:1) used as solvent system. The Fractions with the same TLC profiles were pooled to give the following :

I	=	4.06 g G_2
II	=	2.05 g G_3
III	=	0.42 g G_4

These were coded G_2 , G_3 and G_4 respectively.

Fraction G_2 was subjected to further separation on 30 g silica gel₆₀ (Merck 0.04 - 0.63 mm) and the following solvent systems; dichloromethane : methanol : water (87:12:1) (150 ml); chloroform : methanol : formamide (90:60:1) (100 ml); and acetonitrile : methanol : water (2:4:0.5) (120 ml) used for fractionation. Fractions with the same TLC characteristics were pooled to yield the following dry weights :

(i)	$G_{2,1}$	=	0.555 g
(ii)	$G_{2,3}$	=	0.923 g

These Fractions were purified using preparative TLC.

(IV) G_3 : Further Separation

G_3 precipitated with alcohol, but was freely soluble in aqueous methanol (70%). The initial separation was carried out with centrifugal chromatography. The sample was loaded and eluted with dichloromethane : methanol : water (87:12:1) (300 ml) first and then with methanol : water (7:3) (250 ml). Two Fractions with similar TLC characteristics were obtained with the following dry weights :

A	=	0.065 g
B	=	0.347 g

These were then subjected to further purification with preparative TLC.

(V) Fraction G₄

This was not subjected to further CC separations since TLC investigation revealed it to contain only two components. These were purified using PTLC.

(VI) Separation of Fraction C using a Sephadex LH₂₀ Column

Quantity of material separated = 5.4020 g.

Acetonitrile : methanol : water (3:2:1) and water : methanol (1:1 to 7:3) were used and the similarity of the Fractions produced monitored with chloroform : methanol : water : formic acid (20:25:4:1) to yield two Fractions with similar characteristics with the following dry weights :

G5	=	1.23 g
G6	=	1.079 g

(VII) G₅ Further Separation

G₅, (604 mg) was subjected to a further CC separation on silica gel (30 g) 0.040 - 0.063 mm (Merck) and eluted with methanol : acetonitrile : water (6:3:1) (100 ml). The Fractions obtained were monitored with the same solvent system (1:1:1) to yield three major TLC Fractions with the following dry weights :

Fraction I	=	0.178 g
Fraction II	=	0.065 g
Fraction III	=	0.267 g

Fraction I was shown to contain only one component, Fractions II and III yielded two components each. They were subjected to further separation and purification using PTLC.

(VIII) G₆ Further Separation

This sample (307 mg) was subjected to CC separation on silica gel (30 g) 0.040 - 0.063 mm (Merck) and eluted with methanol : acetonitrile : water (6:3:1) to give two major Fractions of similar characteristics, with the following dry weights :

Fraction I	=	0.128 g
Fraction II	=	0.063 g

They were shown to have one component each on TLC, and were subjected to further purification using PTLC.

(IX) Physical Properties of *G. brevis* Fractions

G₁ (A, B, C) was a dark greenish resinous substance, freely soluble in ethanol, methanol and other non-polar organic solvents.

G₂ was a dark brownish resinous substance, also freely soluble in ethanol, methanol and organic solvents but insoluble in water.

G₃ was a solid substance, partially soluble in methanol but freely soluble in ethanol (70%) or (70%) methanol and freely soluble in water.

G₄ was greenish non-powdery solid and soluble in 70% ethanol or methanol.

G₅ was dark brown and soluble in 70% ethanol or methanol.

G₆ was also brown in colour and soluble in 70% ethanol or methanol.

These Fractions were used for anti-convulsant tests and the active Fraction(s) further purified and structure elucidation attempted as the main purpose of the project was to identify anti-convulsant compounds.

CHAPTER 5

BIOLOGICAL ACTIVITIES OF THE FRACTIONS AND PURIFIED COMPONENTS FROM *S. MAGNIFICUM* AND *G. BREVIS* : *IN VITRO* METHODS

5.1 INTRODUCTION

In vitro animal studies concerning epilepsy are physiochemical methods which estimate the binding capacity of a test agent with the neurochemical transmitter receptors in the central nervous system. Binding of an agent with the receptors alters neuronal activity of transmitters, such as GABA and glycine, and produces some physiological changes in the body. GABA and glycine are important neurochemical transmitters in the central nervous system. GABA is a major inhibitory neurotransmitter in the mammalian brain and is implicated in the mechanism of drug action in the central nervous system (MESSER, 1998; KUBOVA, 1999). GABAergic neuronal activity plays important roles in the body's neurophysiology and in moderating a number of neurological diseases especially epilepsy, anxiety and pain (KUBOVA, 1999; PARADES and ÁGMO, 1992). The anti-seizure actions of anti-convulsants and GABA-mimic agents are brought about by their abilities to increase the circulatory GABA content by inhibiting GABA uptake at the GABA receptor sites.

GABA is synthesized in the nerve endings by the transamination of α -keto-glutaraldehyde to glutamic acid, which is in turn decarboxylated by glutamic acid decarboxylase (GAD) to GABA. The classic presynaptic release of GABA is vesicular and occurs through a Ca^{2+} dependent mechanism. This can be blocked by tetanus toxin (KUBOVA, 1999). There is also Ca^{2+} independent GABA release that is secondary to the depolarization of the postsynaptic membrane and Na^+ influx. This release depends on the reverse of the function of GABA transporter (GAT) and it is blocked by GABA uptake inhibitors such as nipecolic acid whereas Ca^{2+} -dependent GABA release is regulated by auto receptors (KUBOVA, 1999).

There is evidence that GABA acts through receptors and there are two major types - subtype A and subtype B. GABA_A is a Cl⁻-coupled channel complex which belongs to a ligand-gated family. Activation of GABA_A receptor by GABA leads to an increased influx of Cl⁻ resulting in membrane hyper-polarization and neuronal inhibition (MENNINI *et al.*, 1993). The effects of GABA on GABA_A are mimicked by muscimol, a mushroom toxin and are blocked by bicuculline, while the effects of GABA on GABA_B receptor can be activated by an anti-spasmodic amino acid, baclofen.

The ability of some GABA analogs to block ³H-GABA uptake either in the neuronal or in glial cell cultures has led to the classification of the GABA transporter system as either neuronal or glial and may be used as a basis for the experimental evaluation of GABAergic activities of chemical compounds exhibiting anti-convulsant effects. A number of GABA uptake inhibitors have been introduced. Among them, RS-nipecolic acid, *cis*-4 - hydroxy nipecolic acid and guvacine were found to be equipotent with GABA in inhibiting GAT-1- mediated uptake (KUBOVA, 1999). These compounds are useful as tools for the exploration of GABAergic transmissions and in the determination, evaluation and estimation of GABA-like activity of other compounds and they can serve as standards. The anti-epileptic activity of a compound is determined by its ability to block the uptake of GABA at the receptor site. The capacity of a compound to do this is compared to that of a standard compound in an anti-convulsant assay.

Radiolabeled GABA binds to the synaptic membrane preparation of the mammalian brain. The most prominent of which is the sodium independent binding of ³H-GABA to brain membranes, which has characteristics consistent with labeling of GABA receptors. The relative potencies of several amino acids and some secondary metabolic products in competing for the binding sites parallel their abilities to mimic GABA neurophysiology. Therefore, the sodium-independent binding of ³H-GABA provides a simple and sensitive method to evaluate compounds for GABA-mimetic properties.

5.2 MATERIALS

- (i) Partially purified Fractions and purified compounds from *S. magnificum* and *G. brevis*.
- (ii) Male Wistar rats (100 -150 g) obtained from The Biomedical Resource Center, University of Durban-Westville.

5.2.1 Preparation of Reagents

- (i) Gamma amino butyric acid (^3H -GABA), $\text{H}_2\text{NCH}_2(\text{CH}_2)_2\text{COOH}$ (2,3,4 - ^3H)
Molecular mass = 103.1 g.
The ^3H -GABA solution had activity of 2.18TBq / mmol or 59Ci/mmol.
The test dose was 5 μl , prepared by diluting 15 μl of ^3H -GABA solution to 1 ml with Tris-maleate buffer (0.05 M, pH 7.1).
- (ii) Muscimol (3 -Hydroxy -5 -aminomethylisoxazole) MW = 114.1.
0.1 mM solution contain 11.41 mg in 1000 ml.
- (iii) Tris-maleate 0.05 M buffer (pH 7.1).
Tris-base (6.05 g) was dissolved with 100 ml distilled water in a one litre volumetric flask and the volume made up to 1000 ml with distilled water. Tris-maleate (5.93 g) was dissolved with 100 ml distilled water in a 500 ml volumetric flask and made to volume with distilled water.
The 0.05 M Tris-maleate buffer (pH 7.1) was prepared by slowly adding the Tris-maleate solution to the Tris- base solution until the pH 7.1 was reached.
- (iv) Sucrose (0.32 M) was prepared by dissolving 109.50 g of sucrose in 250 ml distilled water and the volume made to 1000 ml and stored at 4°C.

5.2.2 Instruments

- (i) Scintillation counter, model Beckman LS 6000LL, USA.
- (ii) Centrifuge, model J-TB - 0045 from Beckman, USA.

5.2.3 Removal of the Rat Brain

The brains (Figures 5.2 and 5.3) were removed according to the procedure described by ROWETT (1952).



Figure 5.1: Wistar rat



Figure 5.2: Dissection of the rat brain



Figure 5.3: Removal of the rat brain

5.2.4 Preparation of brain homogenate

The methods as described by VOGEL and VOGEL (1997) were modified and used.

- (i) Whole rat brains were rapidly removed and homogenized in 30 ml of ice-cold 0.32 M sucrose.
- (ii) The homogenate was centrifuged at 1000 g for 10 min. All centrifugation was done at 2 to 4°C.
- (iii) The pellet (nuclear fraction) was discarded and the supernatant fluid then centrifuged at 20,000 g for 20 min.
- (iv) The supernatant was discarded and the crude mitochondrial pellet resuspended in 15 ml distilled water using a Teflon homogeniser. The suspension was centrifuged at 8000 g for 20 min.
- (v) The supernatant and the pellet's soft, upper, buff layer was collected and used. It was carefully re-suspended, using a gentle squirting motion. This suspension was then centrifuged at 48,000 g for 20 min.

- (vi) The final crude synaptic membrane pellet was re-suspended (without homogenization) in 15 ml of distilled water and centrifuged at 48000 g for 20 min.
- (vii) The supernatant was discarded and the centrifuge tubes containing the pelleted residue (membrane pellet) capped with parafilm and stored frozen at -70°C.

5.2.5 Assay Procedure

The frozen membrane pellet from one whole rat brain was resuspended in 40 ml of 0.05 M Tris-maleate buffer (pH 7.1) by homogenization at 4°C. Triton X-100 was added to a final concentration of 0.05%. This suspension was incubated at 37°C for 30 min followed by centrifugation at 48000 g for 10 min. The supernatant was discarded and the pellet resuspended by homogenization in the same volume of 0.05 Tris-maleate buffer (pH 7.1) at 4°C.

Na⁺ - Independent ³H -GABA Binding Evaluation

Aliquots of the previously frozen, Triton-treated crude synaptic membranes were incubated in triplicate at 4°C for 30 min in 0.05 M Tris-maleate buffer (pH 7.1) containing 15 nM ³H-GABA alone or in the presence of 0.1 mM isoguvacine or muscimol or test drug as follows :

Standards

- (i) 0.5ml 0.05M Tris-maleate homogenate and 5 µl ³H-GABA, or
- (ii) 0.5ml 0.05M Tris-maleate homogenate and 5 µl ³H-GABA and 5µl 0.1 mM isoguvacine or muscimol.

Test Drug

Three concentrations 0.02, 0.04 and 0.1 mg ml⁻¹ were used and prepared as follows: The sample (5 mg) was weighed into a 10 ml vial, and dissolved with 5 ml distilled water or methanol (Solution A).

Solution A (100 µl) was transferred to an Eppendorf tube and the volume made up to 1 ml (1000 µl) with degassed purified water (Solution B). Solution B therefore contained 0.1mg ml⁻¹ and from this solution 400 µl were made up to 1000 µl to give Solution C which contained 0.04 mg of the sample per ml. This solution was diluted twice to obtain Solution D which contained 0.02 mg of the sample per ml.

Methods

^3H -GABA (5 μl) and 5 μl of the test drug or 5 μl muscimol or isoguvacine were added to 500 μl of 0.05 M Tris-maleate homogenate and incubated at 4°C for 30 min. The reaction was terminated by filtering 400 μl of the incubated homogenate through a GF/C fiber filter and the fiber filter washed twice with 1 ml of the Tris-maleate buffer. It was then transferred to scintillation vials, and 8 ml of scintillating fluid added and the radioactivity measured in a liquid scintillation counter.

5.2.6 Preparation of Standard Curve for Muscimol and Isoguvacine

Muscimol (2.8525 mg) or isoguvacine (4.09 mg) were carefully and accurately weighed and transferred to clean dry 10 ml volumetric flasks. The compounds were dissolved with 5 ml distilled water and the volume made to 10 ml with distilled water to give a stock solution containing 2.5 mmol of muscimol and isoguvacine respectively. The stock solution (4.0 ml) was placed in a clean and dry 100 ml volumetric flask and made to volume with distilled water to give a 0.1 mM solution. The following dilutions were used 0.08, 0.06, 0.04 and 0.02 mM.

The experiment was then completed as described above. Scintillating readings for different concentrations of muscimol and isoguvacine using 15 nmol ^3H -GABA solution per test are given below.

Results

Table 5.1: ^3H -GABA radioactivity recorded for Muscimol binding to GABA receptors

Muscimol (mM)	No of Readings	Average ^3H -GABA (Bq)
0.1	2	129
0.08	2	76
0.06	2	103
0.04	2	120
0.02	2	136

Bq = Disintegration per second

Table 5.2: ^3H -GABA radioactivity recorded for Isoguvacine binding to GABA receptors

Isoguvacine (mM)	No of Readings	Average ^3H -GABA (Bq)
0.1	2	93
0.08	2	108
0.06	2	122
0.04	2	156
0.02	2	111

Bq = Disintegration per second

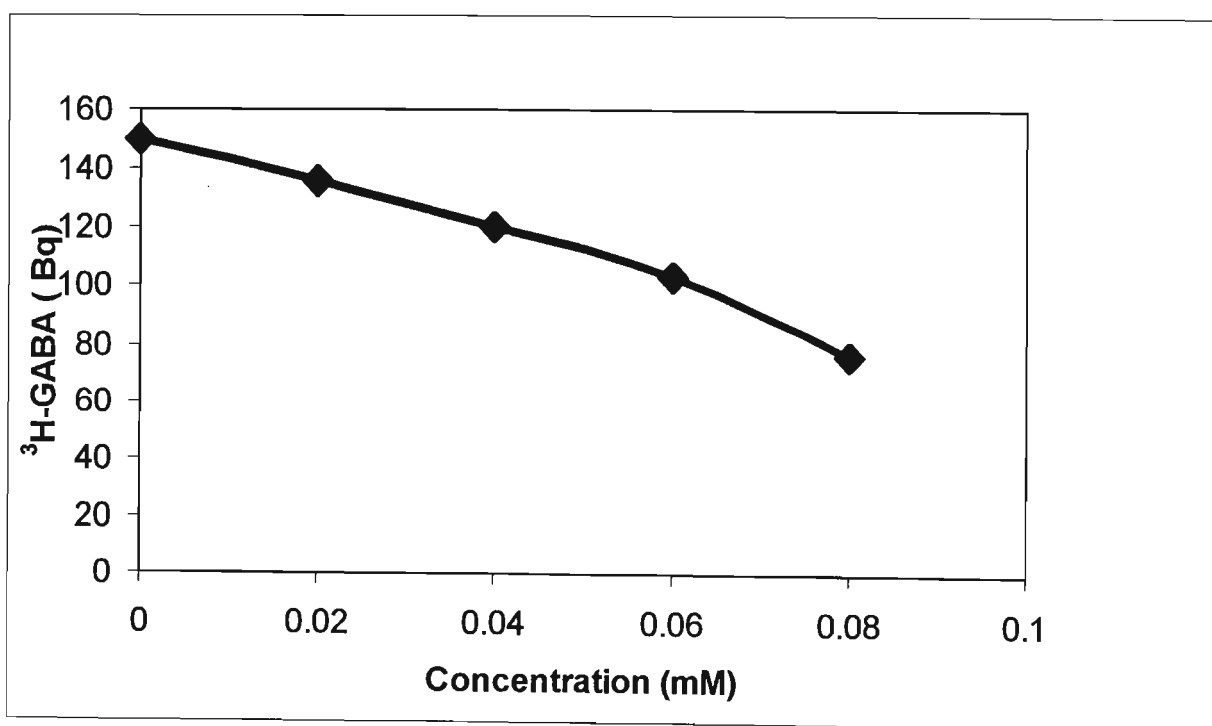


Figure 5.4: Standard Curve for Muscimol binding to GABA receptors

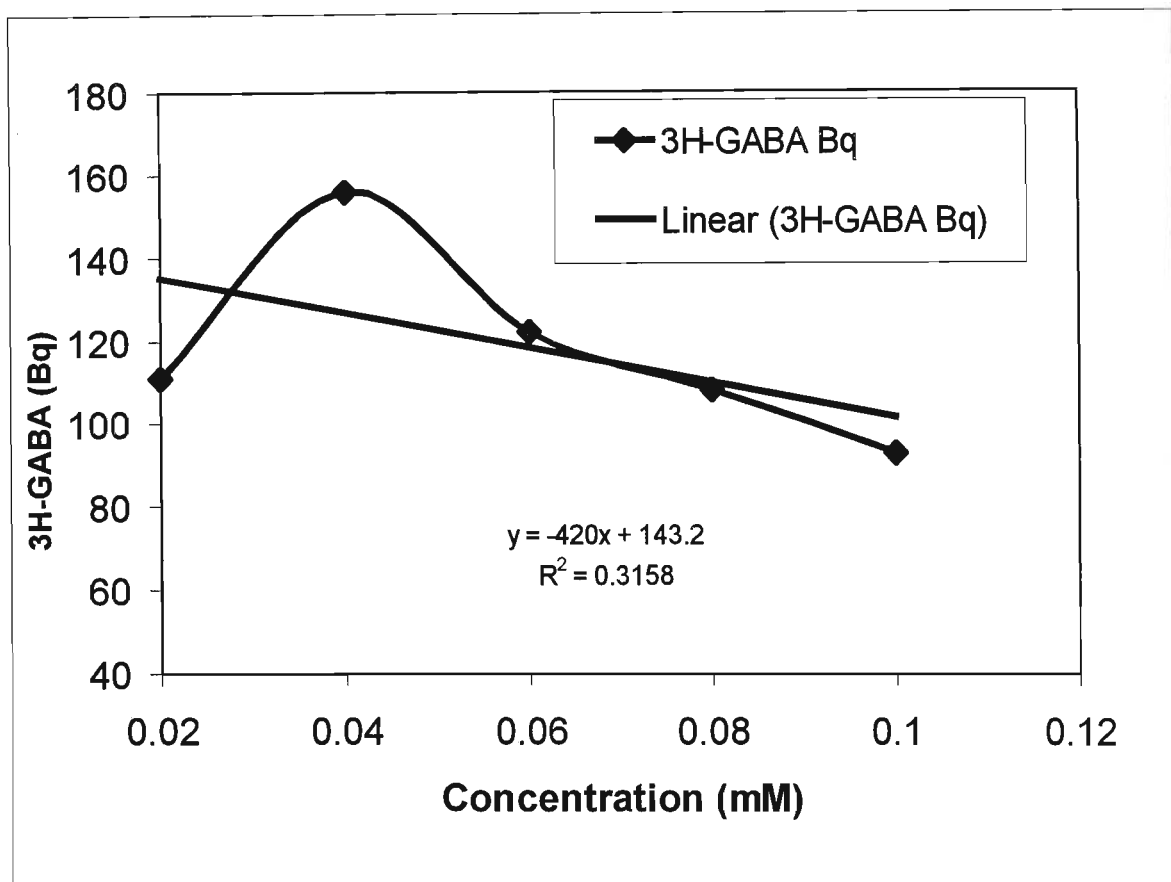


Figure 5.5: Standard Curve of Isoguvacine binding to GABA receptors

5.2.7 Determination of Time Effects on Binding of ^3H -GABA in the Presence of Muscimol or Isoguvacine

The binding capacity of ^3H -GABA in the presence of the GABA_A receptor antagonists, muscimol (0.1mM) or isoguvacine (0.25mM) were determined at 10, 20, 30, 40 and 60 min respectively. The method used was as described earlier. The results are presented in Table 5.3.

Table 5.3: Time Effect on Binding of ^3H -GABA in the Presence of Muscimol or Isoguvacine

Time (min)	No. of Readings	Average Radioactivity (Bq)		
		Muscimol	Isoguvacine	GABA ONLY
0	3	203	173	323
10	3	31	75	70
20	3	31	49	131
30	3	14	18	151
50	3	15	12	23
60	3	23	24	26

^3H -GABA average disintegration s^{-1} (Bq) after 10 min incubation period

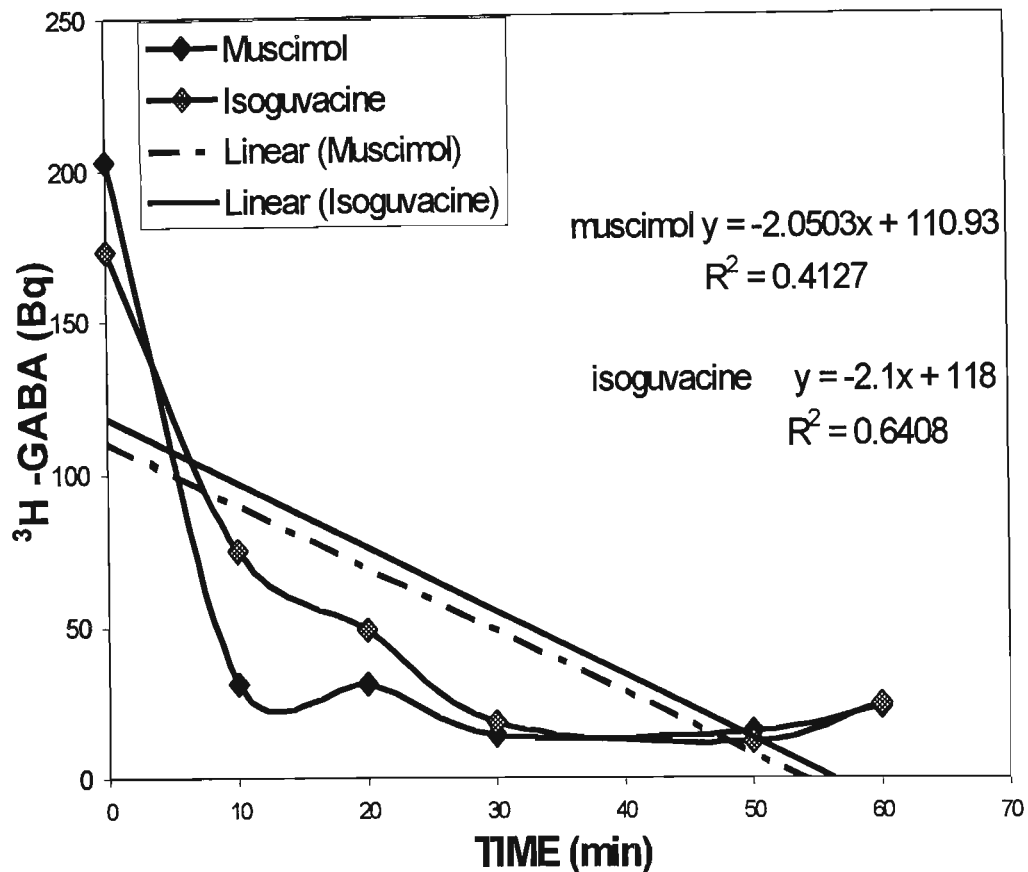


Figure 5.6: Time Effect on Binding of $^3\text{H-GABA}$ in the Presence of Muscimol or Isoguvacine

Discussion

In vitro studies showed that incubation time has an effect on the binding of muscimol and isoguvacine to the receptor site. The increase in extracellular GABA caused by these two agents is dose dependent like other drugs of this class (REDDY, 1998). Muscimol gave the best increase in the extracellular GABA concentration at 30 min incubation hence the minimum $^3\text{H-GABA}$ binding concentration to the receptor sites was obtained at this time period (Figure 5.3). With longer incubation periods there was an increase in $^3\text{H-GABA}$ binding signifying a decrease in the extracellular concentration of GABA. Isoguvacine had the best extracellular concentration between 30 - 40 min incubation (Figure 5.3).

5.2.8 Evaluation of Fractions

S. magnificum and *G. brevis* Fractions were evaluated in order to determine if a particular Fraction of the extracts' were responsible for the observed anti-convulsant activity or if the activity was a synergism resulting from activity of more than one chemical component.

S. magnificum Fraction M₁ obtained from Fraction A and M₂, M₃, M₄, M₅, M₆ and M₇ obtained from Fraction B (Chapter 4) were evaluated for anti-convulsant activity as described above to establish which, if any, of the Fractions are responsible for the biological activity observed with the extracts. The results are presented in Table 5.4.

Table 5.4: Evaluation of *S. magnificum* Fractions for ³H-GABA binding

Fraction	Concentration (mg ml ⁻¹)	No. of Readings	Average Radioactivity (Bq)
Reference			
Muscimol	0.1 mM	4	13
Isoguvacine	0.125 mM	4	24
M ₁	0.04	4	24
	0.02	4	34
M ₂	0.04	4	52
	0.02	4	53
M ₃	0.04	4	51
	0.02	4	56
M ₄	0.04	4	63
	0.02	4	39
M ₅	0.04	4	24
	0.02	4	44
M ₆	0.04	4	42
	0.02	4	45
M ₇	0.04	4	42
	0.02	4	43

The results showed that the activity appeared to reside strongly in Fractions M_5 and M_1 . At a concentration of 0.04 mg ml^{-1} Fraction M_5 was almost as active as isoguvacine (0.1 mM) and 57% as effective as muscimol in the stimulation of GABA receptors resulting in an increase in circulatory GABA content, an important mechanism in relieving epileptic seizures. M_1 at a concentration of 0.02 mg ml^{-1} was 86% as active as isoguvacine. Other fractions exhibited weak or no anti-convulsant activities. The components of Fraction M_5 were therefore separately evaluated in an attempt to identify the compound that is responsible for the activity or if the activity is as a result of synergism of the activities from all the components. No further work was done on those Fractions showing little or no activity.

5.2.9 Evaluation of Components of Fraction M_5 of *S. magnificum*

Fraction M_5 yielded the following components $M_{5.2}$, $M_{5.3}$, $M_{5.6}$ and $M_{5.7}$ (Chapter 4, Section 4.4.3). These components were investigated to determine which one of them had anti-convulsant activity or if they, when combined, contribute to the biological activity. The results are presented in Table 5.5.

Table 5.5: Evaluation of Fraction M_5 Components for ^3H -GABA binding

Subfraction	Concentration (mg ml^{-1})	No. of Readings	Average Radioactivity (Bq)
Standards			
Muscimol	0.1 mM	4	15
Isoguvacine	0.125 mM	4	27
Subfractions			
$M_{5.2}$	0.04	4	24
	0.02	4	27
$M_{5.3}$	0.04	4	40
	0.02	4	36
$M_{5.6}$	0.04	4	23
	0.02	4	39
$M_{5.7}$	0.04	4	34
Combined	0.02	4	35

The 0.04 mg ml⁻¹ dosage of components M_{5,2} and M_{5,6} exhibited almost the same degree of anti-convulsant activity as the standard, isoguvacine. At this concentration they had a better displacement capacity for ³H-GABA than isoguvacine (0.125 mM) which means that they had more capacity to increase circulatory GABA content than isoguvacine but they were less active than muscimol (Table 5.5). Fraction M₁ appeared to be affective at both 0.02 and 0.04 mg ml⁻¹ concentrations. It seems to possess better potential as an anti-convulsant than other components screened. It showed positive and constant anti-convulsant activity in all the investigations carried out.

5.2.10 Confirmation of the Anti-convulsant Activities of Components M₁, M_{5,2} and M_{5,6}

The anti-convulsant activities of Fractions M₁, M_{5,2} and M_{5,6} were further investigated in order to confirm the results obtained above.

Table 5.6: Anti-convulsant Activity of Components M₁, M_{5,2} and M_{5,6}

Subfraction	Concentration (mg ml ⁻¹)	No. of Readings	Average Radioactivity (Bq)
Standards			
Muscimol	0.1 mM	4	30
Isoguvacine	0.125 mM	4	32
Fractions			
M ₁	0.04	4	28
	0.02	4	27
M _{5,2}	0.04	4	38
	0.02	4	43
M _{5,6}	0.04	4	34
	0.02	4	33

Component M₁ consistently showed activity in inhibiting GABA uptake more than other chemical components isolated from *S. magnificum*. The anti-seizure actions of anti-convulsants and GABA-mimic agents are brought about by their abilities to increase the circulatory GABA content by inhibiting GABA uptake at the GABA receptor sites. The tests revealed that M₁ was as active as muscimol and isoguvacine at both concentrations used and it consistently demonstrated some activity at concentrations of 0.02 and 0.04 mg ml⁻¹. It appears that the extract owes most of its anti-convulsant activity to component M₁. Therefore the minimum inhibitory concentration of M₁ was determined.

5.2.11 Anti-convulsant Activity of *G. brevis* Fractions

The evaluation of *G. brevis* was done using the methods described above. The results are presented in Table 5.7.

Table 5.7: Evaluation of Anti-convulsant activity *G. brevis* Fractions

Fraction	Concentration (mg ml ⁻¹)	No. of Readings	Average Radioactivity (Bq)
Standards			
GABA only		4	52
Muscimol	0.1 mM	4	18
Isoguvacine	0.125 mM	4	16
Fraction			
Fraction 1	0.04	4	31
	0.02	4	34
Fraction 2	0.04	4	22
	0.02	4	34
Fraction 3	0.04	4	28
	0.02	4	38
Fraction 4	0.04	4	37
	0.02	4	34

The results of the experiment showed that the anti-convulsant activity is not pronounced in any particular Fraction from *G. brevis* leaf extracts. All the Fractions exhibited activity ranging between 17% to 32% compared with isoguvacine activity and 38% to 50% for muscimol activity. There was no particular Fraction that could be said to be solely responsible for the anti-convulsant activity observed in the whole extract. For this reason no Fraction was further evaluated for biological activity.

5.2.12 Determination of Inhibitory Concentration at 50% (IC₅₀) for Component M₁ from *S. magnificum*

The determination of inhibitory concentration was done using the methods previously described. The results are presented in Table 5.8.

Table 5.8: Inhibitory Concentration at 50% (IC₅₀) for Fraction M₁ from *S. magnificum* roots

Fraction	Concentration (mg ml ⁻¹)	No. of Readings	Average Radioactivity (Bq)
Standards			
Muscimol	0.1 mM	4	40
Isoguvacine	0.125 mM	4	36
Fraction M ₁			
	0.001	4	142
	0.002	4	99
	0.004	4	98
	0.006	4	66
	0.008	4	74
	0.01	4	59
	0.02	4	38
	0.04	4	31

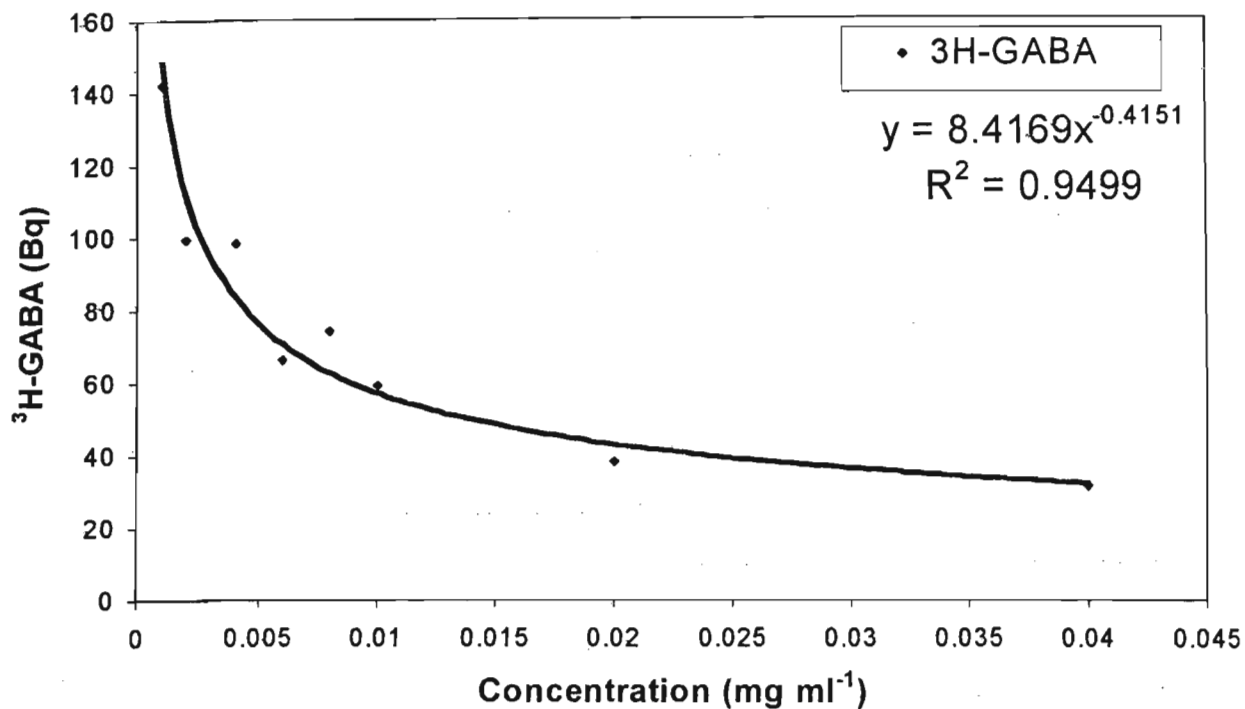


Figure 5.7: Inhibitory Concentration at 50% (IC₅₀) of Fraction M₁

The general equation can be written as $Y = 8.4169 X^{-0.4159}$ and the inhibitory concentration at 50% (IC₅₀) calculated at the median when Y is equal to 75.

$$\text{Log } 75 = \text{log } 8.4169 - 0.4159 \text{log}$$

$$1.8751 = 0.9252 - 0.4159 \text{log} X$$

$$\text{log } X = -2.2840$$

$X = 0.005 \text{ mg ml}^{-1}$ which is the IC₅₀.

5.3 IDENTIFICATION OF BIOLOGICALLY ACTIVE COMPOUNDS FROM *S. MAGNIFICUM*

The identity of the major active compound was established by elucidation of its structure using nuclear magnetic resonance (NMR) techniques. NMR and mass spectral instruments make structure elucidation for pure isolated compounds less of a problem if available. Two dimensional (2D) NMR techniques such as Long-range ^1H - ^{13}C correlation spectroscopy (COSY) spectra, also known as Heteronuclear multiply bond connectivity (HMBC) and NOESY spectra, two-dimensional spectra which records all proton to proton binding were employed for complete structure determination (WILLIAMS and FLEMING, 1995). NOESY effects are particularly useful in providing connectivity patterns of hydrogen and carbons. Where the obtained spectra were not clear enough to allow for clear identification a confirmatory test was necessary by determining the mass of the compound. If the atomic mass was found to be the same as the molecular mass the identity of the compound is confirmed. NMR spectra were obtained by Mr Watson and Mr Craig. Deuterated methanol was used as solvent and tetramethylsilane (TMS) as internal reference.

- (i) Fraction M_1 : This compound was shown in all the tests above to be consistent and potent in inhibiting GABA uptake at all the concentrations tested. It proved to be responsible for the most anti-convulsant activity of *S. magnificum*. The chemical structure was therefore elucidated. By comparing its carbon and proton NMR spectra to the previous study by TANE *et al.* (1990) and KASHIWADA *et al.* (1990) it was found that the compound had the structure shown below (Figure 5.8) as determined from the data in Figures 5.9 and 5.10 and Table 5.9.

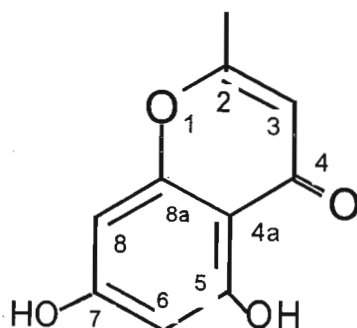


Figure 5.8: Structure for Noreugenin ($\text{C}_{10}\text{H}_8\text{O}_4$, Mw = 192 g) (2-methyl- 5,7-dihydroxybenzopyran- 4 - one)

Table 5.9: ¹³C and ¹H NMR data for Noreugenin

C	δppm	H	δppm
2	49.34	2	-
3	94.91	3	3.33
4	183.99	4	-
4a	48.66	4a	-
5	169.30	5	6.34
6	108.89	6	6.33
7	166.02	7	4.89
8	100.02	8	6.08
8a	48.82	8a	-
Me	20	Me	2.39

The proton C₃ absorbed at 3.33 ppm. This is an ethylenic proton and normally absorbs at between 4.6 - 5.9 ppm (MORRISON and BOYD, 1992), but because of an electron donor attached at the beta position it suffers shielding that made it more basic, hence reduction in shift.

The hydroxyl groups at C₅ and C₇ are phenolic in nature and absorb at between 4 - 12 ppm. H of C₇ OH absorbed at 4.89 ppm. H of C₅ OH absorbed at 6.34 ppm. The influence of an oxygen atom at position C₄ makes it to be very acidic, hence the down field absorption experienced. C₆ and C₈ are aromatic, C₆ being more acidic than the C₈ hydrogen.

13C NMR SPECTRUM OF NOREUGENIN (FRACTION M₁)

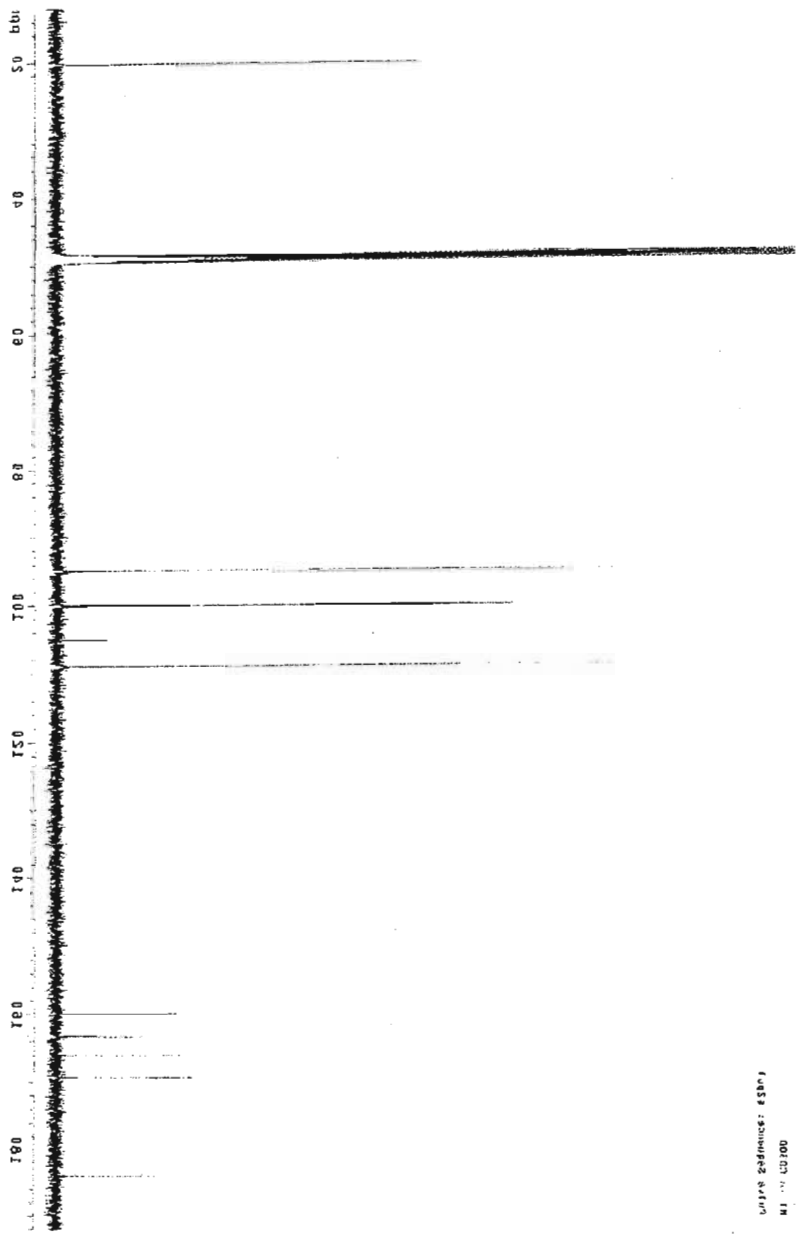


Figure 5.9: ¹³C NMR SPECTRUM OF NOREUGENIN (FRACTION M₁)

M1 in CD3OH
Pulse Sequence: zgpg30

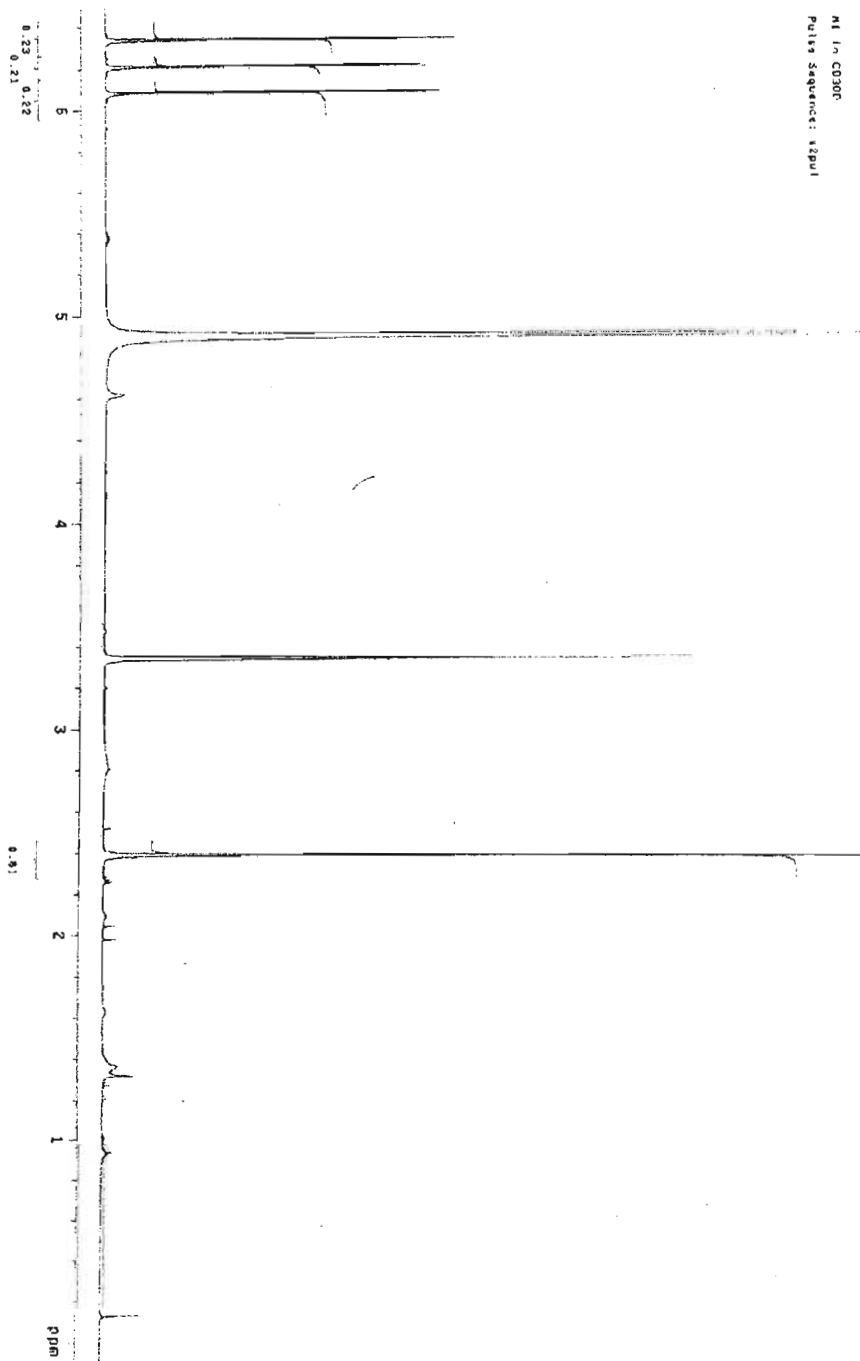
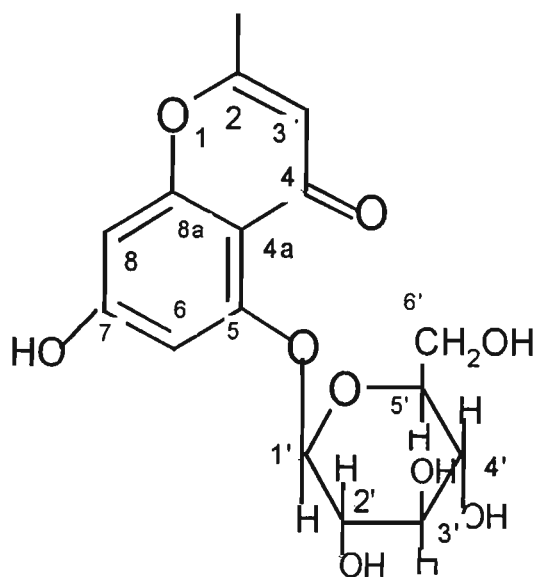


Figure 5.10: ¹H NMR SPECTRUM OF NOREUGENIN (FRACTION M₁)

- (ii) Fraction M_{5.2}: This compound was shown in the tests above to inhibit GABA uptake at all the concentrations tested. It had some anti-convulsant activity and might be contributing to some extent to the overall anti-convulsant activities of *S. magnificum* root extracts. The chemical structure was therefore elucidated. By comparing its carbon and proton NMR spectra to the previous study by TANE *et al.* (1990) and KASHIWADA *et al.* (1990) it was found that the compound had the structure shown below.



2-methyl -5,7-dihydroxychromone-5-O- β -D-glucopyranose

Figure 5.11: Structure for Schumannioside A (C₁₆H₁₉O₉; Mw = 355 g)

The ¹H and ¹³C NMR spectra of the compound are shown in Figures 5.12 and 5.13.

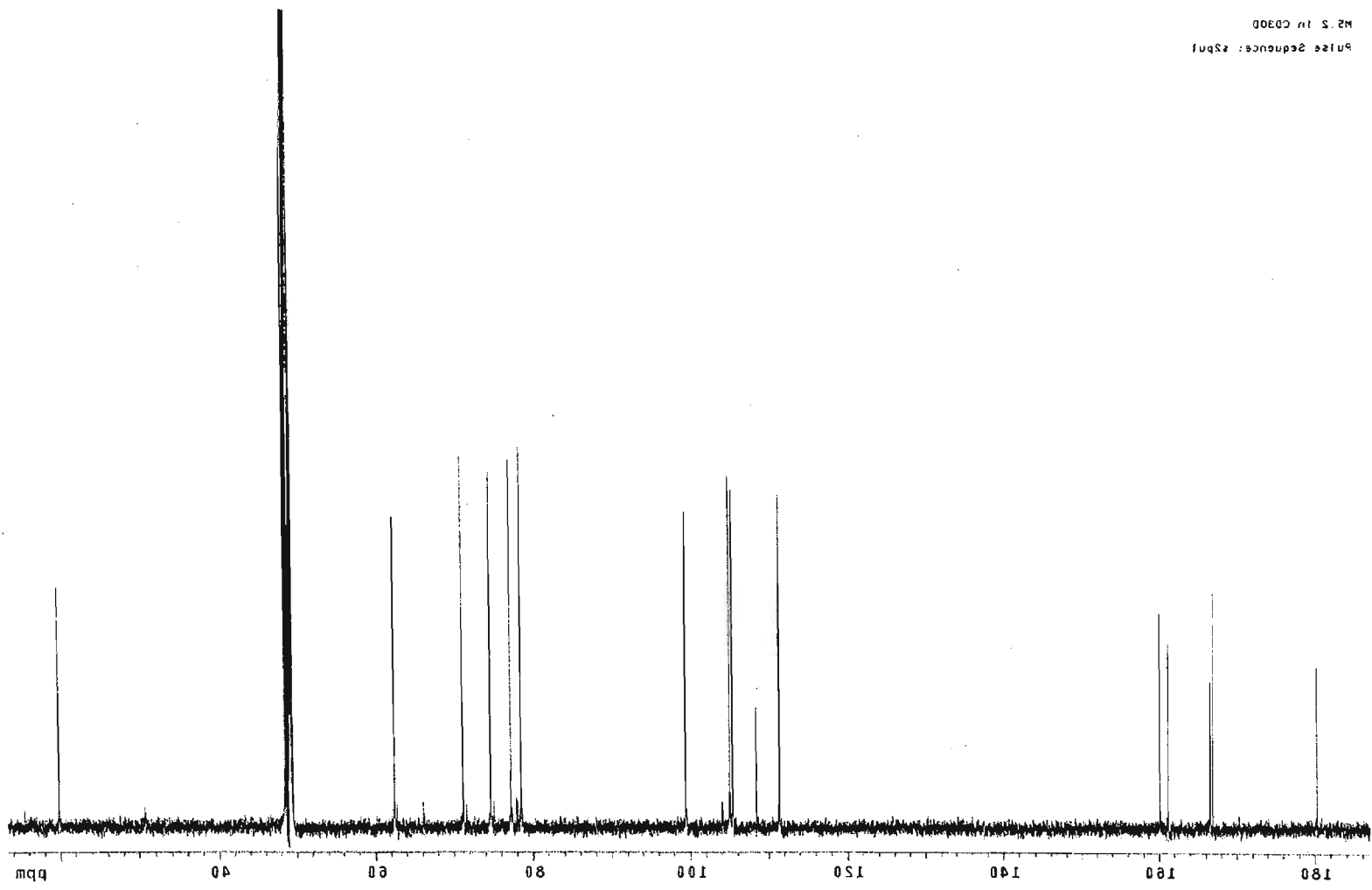


Figure 5.12: ¹³C SPECTRUM OF SCHUMANNIOFOSIDE A (FRACTION M 5.2)

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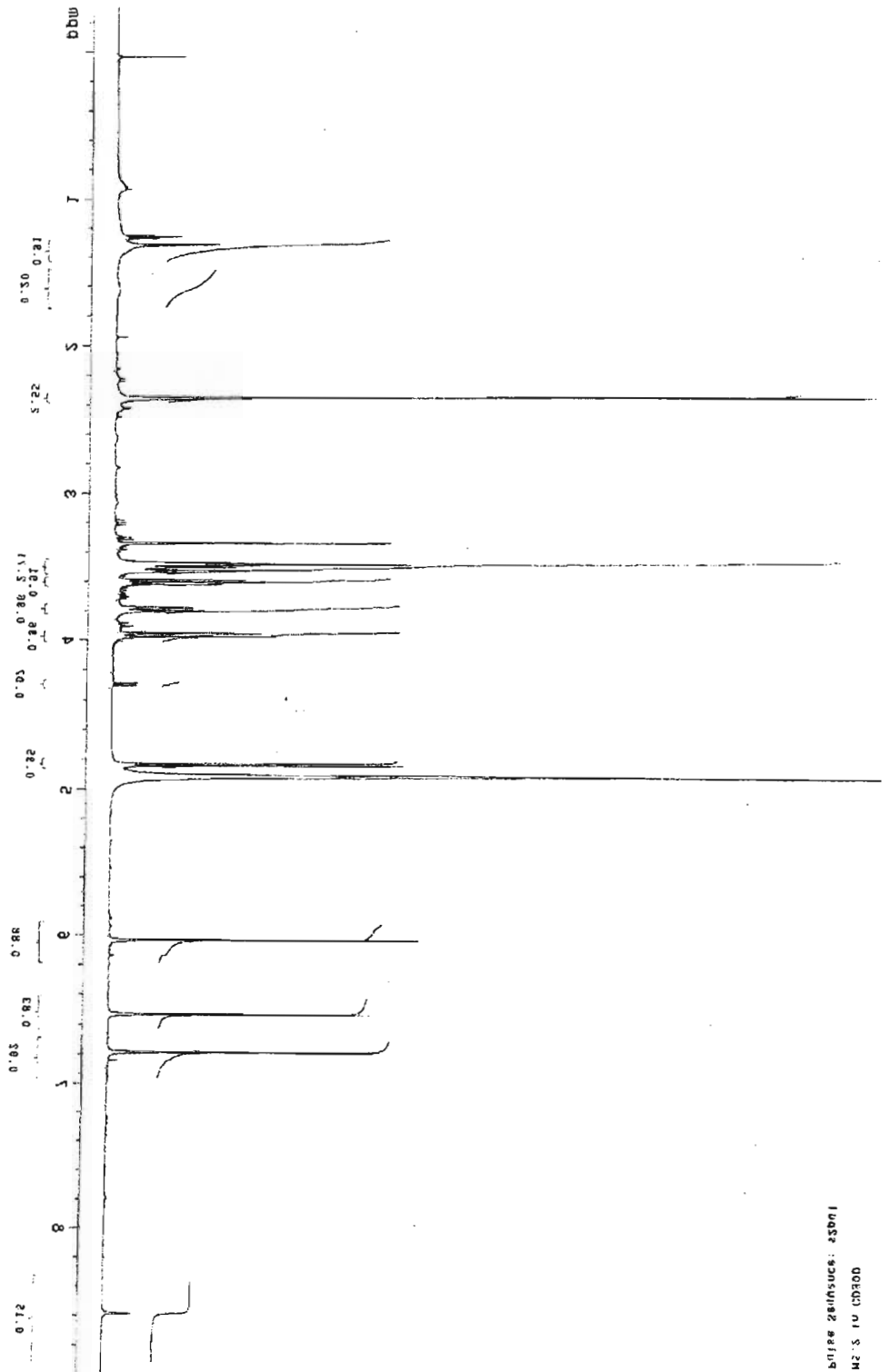


Figure 5.13: ¹H NMR SPECTRUM OF SCHUMANNIOFOSIDE A (FRACTION M 5.2)

CHAPTER 6

GENERAL DISCUSSION

There is a renewal and growing interest in the use of plant-derived biological active compounds as drugs for the treatment of various ailments (HOUGHTON and RAMAN, 1998). Consequently drugs obtained from plants/herbs are now highly valued and in demand in modern medicine. Biological active compounds or active principles of a drug form part of organic compounds known as secondary metabolic products or natural products. Many of these compounds are known to exert some useful physiological activities and plants/herbs or their parts containing them have been used as drugs for the treatment of various ailments since ancient times. Plants have an extensive history of use as therapeutic agents (COLEGATE and MOLYNEUX, 1993; SILVA *et al.*, 1998). This study was on the biological and chemical evaluations of anti-convulsant effects of such biological active compounds obtained from the medicinal plants, *Schumanniphyton magnificum* Harms and *Glyphea brevis* (Speng) Moraches, which are used in Igbo traditional medicine in the treatment of epilepsy and convulsion. They are used either singly or in combination or with other herbs.

The results obtained from both *in vivo* and *in vitro* studies showed extracts from these plants to possess some anti-convulsant activities in the investigated animal models, thus supporting aspects of folkloric use. The plant parts such as leaves or roots are used in traditional or alternative 'herbal' medicine as decoctions or crude extracts made with water or dilute alcohol and they have little or no history of scientific testing for efficacy or safety (IWU *et al.*, 1992). The use of such preparations in the treatment of epilepsy is based on belief. People who use them justify their action based on grounds of tradition. There is no evidence of clinical trials, therefore efficacy and safety of the preparations is based on the faith, popularity, adherents' enthusiasm and anecdotal accounts rather than on the results of organised research (HOUGHTON and RAMAN, 1998; TANG and LEUNG, 2001).

Identification of medicinal plants/herbs whose secondary metabolic products exert useful physiological activity against some diseases, or a specific disease to be investigated, is very important and is the starting point in the scientific study of a natural product. This is usually accomplished with the help of a traditional medical practitioner who has at a time employed the plant in the treatment of the disease(s) in question. A detailed scientific study on the plant usually starts with the extraction of the chemical compounds present, be it for the purposes of biological assays in order to investigate its physiological activities, or for the purposes of phytochemical analysis to identify the chemical nature of the constituents that are to be investigated.

Extraction processes constitute a very crucial stage in the study of natural products and is one of the most intensive areas of secondary metabolite research today (SOFOWORA, 1993). This is because the effective extraction of chemical compounds from natural sources may be marred by several factors which in turn may adversely affect the physiological activity. Of paramount importance is the extraction method used since wrong extraction methods may not only extract some unwanted compounds, but may lead to some chemical reactions such as decomposition of the compounds owing to hydrolysis and auto-oxidation, rearrangement of the compounds, and/or isomeric changes occurring during the extraction process (HARBORNE, 1998). Consequently the resulting chemical changes of the constituents will affect the physiological and pharmacological activities of the extracts giving rise to false negative results.

The chemical constituents of the studied plants were extracted with aqueous ethanol (70%) (KINGHORN, 1985). Ethanol is an organic hydrophilic solvent. Organic hydrophilic solvents possess the capability to penetrate both exo- and endo-cellular compartments of plants and other biological materials better than any other solvents and therefore extract a wider range of organic compounds from natural sources (KINGHORN, 1985). Addition of water to alcohols potentiates its hydrophilic character and at the same time attenuates its hydrophobic or organic characters thereby minimizing the tendency to undergo some undesired chemical reactions such as ether or ester formation with alcoholic or acidic functional groups present in secondary metabolites (KINGHORN, 1985; EVANS, 1989). These reactions are usually experienced with absolute alcohols when used for extraction of natural products containing glycosides. Extraction of tetracyclic saponin with absolute alcohol or 90% ethanol did result in ether formation (FUJIMOTO *et al.*, 1986).

Natural products can be effectively extracted sequentially from powdered plant material starting with a highly non-polar solvent with gradual increase to a highly polar solvent (COLEGATE and MOLYNEUX, 1993). This principle was utilised in this work in the isolation of different organic components of the extracts. Chemical reactions that occur between absolute alcohols and natural products containing glycosides during extraction may be attributed to the presence of reactive functional groups especially alcoholic and carbonyl groups (FINAR, 1975; PINE, 1987). These reactions are usually not common with natural products containing alkaloids. Thus the alkaloids of *S. magnificum* were extracted with absolute ethanol (HOUGHTON and YANG, 1985). In the present work *S. magnificum* chemical constituents were also extracted with absolute ethanol. Extraction of alkaloids from their natural sources can be accomplished with acidic or basic solvents which extract alkaloids in the form of salts or as precipitated basic alkaloids respectively (FINAR, 1975; CORDELL, 1981; TYLER *et al.*, 1988; EVANS, 1989). The suitability of the extraction method is usually verified by the use of a standard bioassay to confirm if the extract had the anticipated or expected physiological activity and with standard phytochemical tests to ascertain chemical identities of the compounds extracted (SOFOWORA, 1993).

The development of sensitive chromatographic and spectroscopic techniques for the isolation and structure determination of natural products have facilitated phytochemical investigation (PHILLIPSON, 1995). These techniques were employed in the phytochemical investigations especially in the isolation and structure elucidation of the isolated chemical constituents. Using phytochemical methods of identification as outlined by FARNSWORTH (1966); ODEBIYI and SOFOWORA (1978 and 1979) and SILVA *et al.* (1998) the preliminary phytochemical tests were carried out. The so-called alkaloid reagents were used for the detection of alkaloids and their derivatives (GILMAN *et al.*, 1950; FINAR, 1975; PINE, 1987).

These reagents basically are heavy metal salts such as mercuric salts found in Mayer's reagent, and they act as chelating agents reacting with a lone pair of electrons in the nitrogen to form an insoluble precipitate, a positive indication for alkaloids. Unfortunately false positive reactions may occur since other chemical compounds notably coumarins and quinones containing a lone pair of electrons can react with the

so called alkaloid reagents to form precipitates. False negative reactions have also been reported, especially with alkaloids occurring as N-oxides or quaternary ammonium compounds, which owing to a lack of a lone pair of electrons, because of the oxidation state of their nitrogen, failed to react with chelating agents to give positive results that might be expected of the alkaloids (FARNSWORTH, 1966; EVANS, 1989).

The results of the phytochemical tests indicated the presence of alkaloids in *S. magnificum* and their absence in *G. brevis*. Confirmatory tests were carried out to confirm the positive result for the alkaloids (WALL *et al.*, 1952; FARNSWORTH, 1966).

There is no general test for glycosides but the presence of reducing sugars may indicate the possible presence of glycosides (FINAR, 1975; EVANS, 1989). Tests for reducing sugars were carried out using Fehling's solutions (I and II) which contain Cu^{2+} salts as an active reactant. Reducing sugars reduce Cu^{2+} oxide to Cu^+ oxide with formation of brick red precipitates, which is a positive sign of the probable presence of glycosides. Confirmatory tests for glycosides involve subjecting the sample to hydrolysis and repeating the above tests. A more intense brick red precipitate compared with the former confirms the presence of glycosides. These tests were performed under alkaline conditions in order to obtain better results (EVANS, 1989).

The results showed that both plant extracts contained glycosides. Therefore, tests for different types of glycosides were carried out and cardiac glycosides were found to be present in both extracts (EVANS, 1989).

The importance of cardiac glycosides as therapeutic agents can never be over emphasized. Most frequently utilized tests for the qualitative, as well as the quantitative evaluation of cardiac glycosides, have been the Baljet (2,4,6 trinitrophenol), Kedde (3,5-dinitrobenzoic acid), Raymond (m - dinitrobenzene), or the Legal (sodium nitroprusside) reagents which react with the methylene group as found in the C_{17} unsaturated lactone moiety were employed to ascertain the nature of the glycosides (FARNSWORTH, 1966). The extracts were positive to the above tests for cardiac glycosides.

Also utilized to a lesser extent, for the detection of cardiac glycosides, were the reagents which react with the deoxy sugar moiety of the cardiac glycosides. These

include the Keller - Kiliani test (ferric chloride - glacial acetic acid - sulphuric acid) and Pesez (xanthydrol) reagent. *G. brevis* gave positive results with the reagents and reacted with xanthydrol to give a red colouration, a positive indication of the possible presence of deoxy-sugar and hence the presence of a cardiac glycoside.

Column chromatographic techniques were employed for the initial cleaning and separation of the chemical constituents (HISHAM *et al.*, 2001; LAVAUD *et al.*, 2001; MARQUINA *et al.*, 2001). Chromatographic techniques in general were described by OLANIYI and OGUNGBAMILA (1991), as the most powerful approach to separate mixtures with an unequalled ability among other separation methods to separate complex mixtures of similar substances. Using a chloroform-methanol solvent system in the following ratios 50:1,25:1, 15:1,10:1 and 5:1, the extracts of *S. magnificum* were separated into different fractions with different chemical constituents (HOUGHTON *et al.*, 1992). Sequential methods starting with toluene a highly non-polar organic solvent to a highly polar solvent, 70% aqueous methanol, were successfully employed to separate the constituents of *S. magnificum* and *G. brevis* (COLEGATE and MOLYNEUX, 1993). *S. magnificum* afforded two major Fractions and *G. brevis* gave three major Fractions.

The final isolation and purification of the chemical constituents was achieved using a combination of different planar chromatographic methods. Centrifugal chromatography and preparative thin layer chromatography were employed for initial and final purification respectively (MacGILLIVRAY *et al.*, 1978; HUCK *et al.*, 2000; LAVAUD *et al.*, 2001). The crude extracts, partially and purified chemical constituents were investigated for anti-convulsant activity.

Recent research on epilepsy has focused on the cellular biology of seizures. Epileptiform neurons undergo paroxysmal depolarization shifts (PDS) which results in excessive sustained neuronal firing seen in epilepsy (YERBY, 2001). These shifts are due to either an impairment of GABA-mediated inhibition or an enhancement of aspartate or glutamate-mediated excitatory transmission (PARADES and ÁGMO, 1992; YERBY, 2001). The anti-convulsant activities of the crude extracts were evaluated using *in vivo* methods in mice (NWAIWU and AKAH, 1986; AMABEOKU *et al.*, 1998), while the fractions and the isolated chemical constituents were evaluated using *in vitro*

methods (VOGEL and VOGEL, 1997). The *in vitro* tests primarily aimed at possible identification of the specific chemical constituent(s) responsible for the anti-convulsant activities of the extracts and/or to establish if the anti-convulsant activities were as a result of synergistic effects resulting from the actions of more than one component. In the *in vivo* evaluation, chemical convulsant agents (analeptics), namely strychnine, pentylenetetrazole (PTZ) and picrotoxin were used to induce different types of epileptic seizures or to elevate the seizure threshold. Threshold or sub-threshold doses of the analeptics were subcutaneously administered in mice.

The extracts had significant anti-convulsant activities in different animal models investigated. These findings therefore verified the claimed traditional uses of the plants which are employed for their anti-convulsant and sedative effects (UGBOAJA, 1996). The mere fact that the extracts were active against different types of epileptic seizures suggests that they may be acting through some mechanisms other than inhibition of GABA to elicit their anti-convulsant actions. Sedation in the central nervous system is mediated by the inhibitory neurotransmitter GABA through GABA_A receptors (MENNINI *et al.*, 1993). GABA is implicated in the mechanisms of drugs acting in the central nervous system (MESSER, 1998; KUBOVA, 1999). It acts through receptors, with subtype A and subtype B as two major types. GABAergic neuronal activity plays an important role in body neurophysiology and is implicated in a number of neurological diseases including epilepsy, anxiety and pain (KUBOVA, 1999; PARADES and ÁGMO, 1999). The anti-convulsant actions are brought about by the ability of the agent to inhibit GABA uptake at the receptor sites. The effects of GABA on GABA_A are mimicked by muscimol, a mushroom toxin and are blocked by bicuculline while the effects of GABA on GABA_B receptor can be activated by an anti-spasmodic amino acid, baclofen.

Compounds that potentiate GABAergic inhibition have been found to have a seizure - protective effect in a wide range of animal models of epileptic seizures, the only exception being in the model of absence seizures, where an increase of both GABA_A and GABA_B - mediated inhibition exhibits proconvulsant action (KUBOVA, 1999). The crude extracts were found to be effective in suppressing, in mice, various epileptic seizure types. The tonic convulsions induced by strychnine were greatly reduced in intensity with a prolongation in the latency time. Strychnine administered subcutaneously acts by blocking postsynaptic inhibition mediated by glycine especially

in the spinal cord and induces maximal threshold tonic extensor seizure. Pentylentetrazole (PTZ) administered subcutaneously block GABA mediating neuronal activities in the brain and induce a model of generalised clonic - tonic type of convulsion in the animal which is similar and/or equivalent to grandmal convulsion in human subjects. Subcutaneously administered pentylentetrazole (PTZ) acts through direct stimulation of the neuronal membrane and induces minimal-threshold clonic seizure (FOYE, 1990). In mice generalized tonic - clonic type of epileptic seizures, is formed by three phases: wild running phase, tonic phase and clonic-tonic phase.

The first and third phases are characterized by rapid movement of all four limbs, while in the clonic phase there is also absence of righting reflexes in addition to limb movement (AMABEOKU *et al.*, 1998). The extracts were effective in PTZ-induced convulsions, reducing the severity of seizures and the time of death compared with the controls. The agents effective in PTZ tests in experimental animals were effective in suppressing or even inhibiting petitmal seizures in human subjects (THONGPRADITCHOTE *et al.*, 1996; HUSSEINZADEH and MADANIFORD, 2001). The generalized tonic - clonic seizures induced by picrotoxin were suppressed with remarkable increase in the seizure latency period. Picrotoxin administered subcutaneously has been found to act by blocking presynaptic inhibition mediated by GABA and induces minimal threshold clonic seizure (FOYE, 1990). Picrotoxin interacts with the GABA receptor complex and blocks the Cl⁻ ionophore. The drugs which act by inhibiting or suppressing picrotoxin-induced convulsions are therefore likely to produce their anti-convulsant effects by enhancing chloride currents through picrotoxin - sensitive chloride channels and by weak inhibition of sodium (Na⁺) and N-methyl-D-aspartate (NMDA) channels (SMITH *et al.*, 2001).

S. magnificum at a dosage of 1000 mg kg⁻¹ body weight greatly increased the threshold for tonic seizures and offered 100% protection against picrotoxin-induced convulsion. At a dose of 800 mg kg⁻¹ body weight it offered 80% protection to strychnine and PTZ-induced convulsions respectively.

G. brevis in the dosage of 800 mg kg⁻¹ body weight offered 60% protection in PTZ-induced convulsion and a 400 mg kg⁻¹ body weight dose offered 60% protection against strychnine- induced convulsion.

The wisdom of the combination of the drugs in the treatment was investigated. A dose of 200 mg kg⁻¹ of the extracts, mixed in equal proportion, greatly reduced the severity of convulsion and also prolonged the latency period. When combined the extracts were found to be effective in a relatively low dosage, thus justifying the combination of the drugs for treatment.

Toxicity tests were carried out and these suggested the extracts to be non-toxic. The effective dosages at which 50% of the animals were killed by the extract (LD₅₀) is 4096 and 3979 mg kg⁻¹ body weight for *S. magnificum* and *G. brevis* respectively. The LD₅₀ of 4096 and 3979 mg kg⁻¹ body weight are far above the effective protective dosages of both extracts in all the animal models investigated. The distribution was binomial and chi probability are <0.001 and < 0.00003616 respectively which is very significant. Regression coefficients of 0.001176 and 0.01256 were obtained which are also the same as the gradients of the slope. The general regression equations can be written as $Y = 0.001176 x - 4.82$ and $Y = 0.01256 x + 4$ respectively. Y is the number of animals that died and x is the dose in milligram (mg).

A number of GABA uptake inhibitors have been introduced which act as GABA receptor antagonists. Amongst them guvacine or isoguvacine and muscimol were found to be equipotent with GABA in inhibiting gamma amino butyric acid transporter one (GAT -1)-mediated uptake (KUBOVA, 1999). These compounds have been found useful as tools for the exploration of GABAergic transmission. Muscimol, a natural compound, is isolated from *Amanita muscaria* and is active as GABA_A receptor (RANG and DALE, 1987). It inhibits GABA uptake in synaptosomes as well as neuronal and glial cultures, and systemic administration has led to an increase of brain GABA levels.

The anti-convulsant activities of the isolated compounds were, therefore, evaluated on the basis of their ability to act as GABA receptor antagonists and their capacity to do this was compared with muscimol or isoguvacine as known GABA receptor antagonists as standards.

The results showed that all the Fractions from the extract of *G. brevis* had some activity while the activity was found in Fractions M₁ and M₅ in *S. magnificum*. Fractions M₁ yielded only one component. The component M_{5,2} of Fraction M₅ also demonstrated anti-convulsant activity.

The structure of component M₁ was elucidated and found to be 5,7-dihydroxy-2-methyl-benzopyran-4-one, a Noreugenin (Figure 5.4) with molecular formula C₁₀H₈O₄. It was surprising that this compound possessed a better anti-convulsant activity than other components of this fraction for a simple reason. Chemically noreugenin can be described as an oxygenated monoterpenoid. Monoterpenoids in some cases with sesquiterpenes/sesquiterpenoids are known to be the chemical constituents of essential oils. Essential oils have been employed since ancient times in the treatment of epilepsy and convulsions (NWAIWU and AKAH, 1986). Fraction M_{5,2} was identified as schumanniofoside A (7-hydroxy-2-methyl-5-glucobenzopyran-4-one) (Figure 5.5). Schumanniofoside A is a glycoside and was expected to be more active physiologically.

Noreugenin is a benzo- γ -pyrone, a chromone, with the chemical formula, 2-methyl-5,7-dihydroxylchromone and can exist as a free substance as well as a combined sugar product, a chromone glycoside, known as schumanniofoside A. Noreugenin has been isolated before (HOUGHTON and HAIRONG, 1986; TANE *et al.*, 1990). Schumanniofoside A was isolated by AKUNYIULI and AKUBUE (1986), TANE *et al.* (1990) and KASHIWADA *et al.* (1990). These compounds have not before been screened for any biological activity and therefore their toxicity is yet to be evaluated.

Noreugenin has a benzo- γ -pyrone, basic nucleus. The presence of a benzene ring with two reactive phenolic hydroxyl groups at the meta position to each other attached to an O-cyclic ethylenic ketone may be responsible for the observed anti-convulsant activity. One of the reactive hydroxyl groups combines with a sugar to form the glycoside known as schumanniofoside A. Noreugenin is therefore an aglycone of schumanniofoside A. They do not belong to, nor do they have structural resemblance to, any class of the drugs currently being used in the treatment of epilepsy.

Future clinical studies will hopefully confirm which one of the drugs is more effective as an anti-convulsant. Such studies were however, beyond the scope of this investigation.

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