

VITEX DONIANA SWEET (VERBANACEAE):

**EVALUATION OF PHARMACOLOGICAL BASIS OF
USE IN TRADITIONAL MEDICINE**

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A thesis submitted in partial fulfillment for the Degree of Master of Science in
Pharmacognosy and Complementary Medicine of the University of Nairobi.

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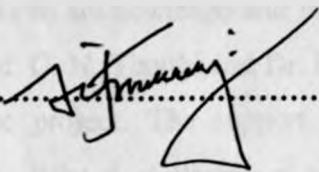
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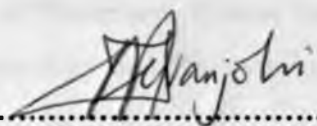
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ACKNOWLEDGEMENT

First of all, I would like to acknowledge and thank most sincerely, my Supervisors Prof J. W Mwangi, Prof. G. N. Thoithi and Dr. F. A. Okalebo for their guidance and support throughout the project. The support and assistance of other academic members of staff at the School of Pharmacy namely; Dr. N. Mungai, Dr. K .O . Abuga, Dr B. K. Amugune, Dr. S. N. Ndwigah and many others whose guidance and inspiration kept me on track, cannot go without mention.

I would like to thank Dr. H. K. Chepkwony, Director NQCL for being very kind to allow me to use the equipment in his institution. I cannot forget to thank the technical staff at the School of Pharmacy. Special thanks go to Mr. J. Mwalukumbi of Department of Pharmacology and Pharmacology who assisted in the extraction and isolation process.

Special thanks go to the following that helped in one way or another: Mr. K. Maloba, Mr. A. Mwaniki, Mr. D. Karume and W. Masengo all of the Department of Pharmacology and Pharmacognosy. I cannot forget to thank Mr. A. King'onde, of the Department of Pharmaceutical Chemistry for his invaluable assistance especially freeze drying the extracts. I would like to thank Mr. P. Mutiso of the Herbarium, Department of Botany, University of Nairobi for identifying and authenticating the plant samples.

I would like to thank my immediate family for their moral support and patience which gave me peace of mind to concentrate on my work. Finally, I thank the Almighty God for everything in my life.

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LIST OF ABBREVIATIONS AND SYMBOLS

AEDs	Anti-epileptic drugs
AMU	Atomic Mass Units
ANS	Autonomic nervous system
CAM	Complementary alternative medicine
CC	Column chromatography
CHCl ₃	Chloroform
CNS	Central nervous system
DARU	Drugs analysis and research unit
DMSO	Dimethyl Sulfoxide
EA	Elementary Analysis
EEG	Electroencephalography
EtoAc	Ethyl acetate
EtoH	Ethanol
GABA	Gamma amino butyric Acid
GAD	Gamma amino butyric Acid decarboxylase
GABA –T	Gamma amino butyric Acid Transaminase
GPR	General Purpose Reagent
HOAc	Acetic acid
ILAE	International League Against Epilepsy
H ₂ SO ₄	Sulphuric Acid
I.P	Intraperitoneal
IV	Intravenous
LC ₅₀	Concentration killing 50% of larvae
LD ₅₀	Dose killing 50% of experimental animals
Me ₂ CO	Acetone
MeoH	Methanol
NaCl	Sodium Chloride
NH ₃	Ammonia
Nm	Nanometers
NQCL	National quality control laboratories
PMS	Premenstrual syndrome

TLC	Thin layer chromatography
Tm	Traditional medicine
UV	Ultraviolet
UV/VIS	Ultra violet / visible spectroscopy
V/v	Volume by volume
WHO	World Health Organization
W/v	Weight by Volume

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Abstract

Vitex doniana is a medium sized deciduous tree, 8 to 18 m high, with a heavy rounded crown and a clear pole up to 5 m in height. It is quite wide spread occurring in Savanna regions, Coastal woodlands, riverine and low land forests. It also occurs in deciduous woodlands extending as high as upland grasslands. It is native to Kenya, Angola, Botswana, Lesotho, Namibia, Niger, Senegal, Somalia, South Africa, Sudan, Tanzania, Uganda, Zambia and many others.

Ethno botanical uses include: treatment of epilepsy, infertility, eye problems and pain management. It is also used to treat anemia, jaundice, leprosy and dysentery. The root is used to treat gonorrhoea and women drink a decoction to treat backache. The stem bark is dried and ground and used in form of tea infusion to treat epilepsy. The young tender leaves are bounded and juice squeezed into eyes to treat eye infections. A leaf decoction is applied externally as a galactagogue and against headache, stiffness, measles, rash, fever, chickenpox and hemiplegia and internally as a tonic, anodyne and febrifuge, and to treat respiratory diseases. Pastes of bounded leaves and the bark are applied to wounds and burns. Root decoction is administered orally to treat ankylostomias, rachitis, gastroenteritis and jaundice. Powdered bark is added to water and taken to treat colic. Bark extract is used to treat liver diseases and control bleeding after child birth. Dried and fresh fruits are used to treat diarrhea and vitamin A and B deficiencies. The twigs are used as chewing sticks for tooth cleaning.

The main objective of this study was to evaluate *Vitex doniana* extract for anti convulsant activity. This is important because the present anti-epileptic drugs (AEDs) are only used to control seizures and in some cases, control of seizures becomes refractory with time. In addition current AEDs have hypnotic activities. Furthermore the study was also carried out to evaluate *Vitex doniana* extract for antinociceptive and spasmolytic activities.

The leaves, fruits, stem and root bark of *Vitex doniana* were collected from outskirts of Bungoma Town. They were air dried for 14 days and ground into powder using a grinding mill. The powder was then extracted using 85% ethanol by cold maceration. Another sample was subjected to sequential maceration extraction using petroleum

ether (60-80°C), chloroform, ethyl acetate and ethanol respectively for 48 hours. The extracts were subjected to phytochemical investigations for the presence of different chemical compounds.

The anticonvulsant activity was investigated using 4-aminopyridine and strychnine chemoconvulsant models in mice. The anti-nociceptive activity was evaluated using hot plate method and acetic acid writhing test. The spasmolytic activity was evaluated by determination of its effects on the isolated rabbit ileum.

The plant extracts were found to have alkaloids, glycosides, flavonoids, saponins, tannins, steroids and terpenoids. In the general central nervous system (CNS) observation screen, the plant extract was found to be a mild CNS depressant. It did not cause significant reduction in spontaneous motor activity. The mice had blunted response to manipulation such as touch indicating that it may have anxiolytic activity. From general observation screen it was concluded that the extract may be a CNS depressant and this was further evaluated by determining its effects on pentobarbitone sleeping time. It was concluded that crude ethanolic extract of *Vitex doniana* has anticonvulsant activity especially against 4-aminopyridine induced convulsions. In both models of chemoconvulsion, ethyl acetate fraction of crude extract was more active. The ethylacetate fraction had the highest activity and delayed the onset of convulsions by 36.5minutes.

The crude extract was found to have peripheral anti-nociceptive activity in the acetic acid induced writhing test. It showed no central analgesic effect using hot plate assay. The extract showed contractile activity on the rabbit ileum. It had a positive inotropic effect on the isolated rabbit myocardium.

The extract was also subjected to toxicity test using brine shrimp lethality assay and acute toxicity in mice. The study showed that the ethyl acetate fraction of extract had LD₅₀ of 81.48 µg/ml. The extract did not cause death in mice in doses as high as 4g/kg. This indicated that it is safe for use in rodents.

The investigations suggest that *Vitex doniana* extract has mild CNS depressant activity and are active against chemo convulsion in mice. The study further revealed that the extract has peripheral analgesic and contractile activity on smooth muscles. Therefore the use of *Vitex doniana* for treatment of epilepsy, as analgesic and control of postpartum bleeding and diarrhea treatment may be justified.

Two compounds were isolated, VD1 and VD2. No pharmacological activity was carried out on the isolated compounds and a partial structural elucidation undertaken using spectroscopic data.

CHAPTER 1

1.0 INTRODUCTION AND LITERATURE REVIEW

Traditional / herbal medicine has been used all over the world since pre-historic times. It is increasingly becoming popular and it is quite widespread in developing countries while on the other hand it is increasing rapidly in developed countries. Methods of folk healing throughout the world commonly used herbs as part of their tradition. Traditional African medicine is a socio-economic and cultural heritage serving over 80 % of the population in Africa. Today traditional medicine has been brought into focus for meeting goals of a wider coverage of primary health care delivery, not only in Africa but also, to various extents, in all countries of the world (1). From the critical view point of primary health care (PHC), it is easy to assess the orthodox alongside traditional medicine in the African context, specifically in areas of social acceptability, cost affordability, self-reliance, cultural compatibility, relevance and community participation. The orthodox medicine has not been adequate for majority of African populations and there is urgent need to fully embrace traditional/herbal medicine.

The only health care providers close to populations are traditional / herbal practitioners, living with them and providing health care services in the same communities. The Western type of health institutions are out of reach of most people in terms of distance and costs, especially in the village setting. On the other hand, the orthodox medicine, as currently made available today in most African countries, cannot provide basic health care needs including full time resident medical personnel and readily available and affordable drugs especially for rural populations.

In Asia and Latin America, people continue to use traditional medicine (TM) as a result of cultural beliefs. In China, TM accounts for about 40 % of all health care delivered (1). In many developed countries alternative medicine (CAM) is becoming more and more popular. The percentage of population which has used CAM at least once is 48 % in Australia, 70 % in Canada, 42 % in USA, 38 % in Belgium and 75 % in France. In many parts of the world,

expenditure on TM/CAM is not only significant, but growing rapidly. In Malaysia, an estimated USD 500 million is spent annually on TM compared to about USD 300 million on allopathic medicine. In the USA, total 1997 out of pocket CAM expenditure was estimated at USD 2700 million. In Australia, Canada and UK, annual CAM expenditure is estimated at USD 80 million, USD 2400 million and USD 2300 million respectively. In developing countries, widespread use of TM is attributed to accessibility and affordability. In Uganda, for example, the ratio of TM practitioners to population is between 1:200 to 1:400 which contrasts with availability of allopathic practitioners for which ratio is typically 1:20,000 or even less. In Kenya the ratio of doctor: patient is 1:7142 while TM: patient ratio is 1: 987 (Mathare –Urban area and 1:378 Kikuyu –rural area (1 -2). These figures are not very different for other developing countries.

In many developed countries, popular use of TM is fuelled by concern about adverse effects of chemical drugs, questioning of approaches and assumptions of allopathic medicine and greater public access to health information. At the same time longer life expectancy has brought about with it increased risk of developing chronic debilitating diseases such as cancer, diabetes, mental disorders and many others. For many patients, TM appears to offer a gentle means of managing such diseases than does allopathic medicine (1). In addition to direct use as templates for development of allopathic medicine, it is estimated that 33% of drugs produced in the developed countries are derivatives of compounds originally isolated from higher plants and 25% of these owe their origin to the tropical rain forests of Africa, Asia and Latin America. Examples of drugs developed in this manner a bound. Medicinal uses of foxglove (*Digitalis purpurea*) gave rise to digoxin as an indispensable cardiac drug. Ergot of Rye (*Claviceps purpurea*) was discovered as a foremost natural uterine stimulant while the analgesic morphine was produced from opium poppy flower (*Papaver somniferum*) by a French scientist. Strychnine from *Strychnos nux-vomica* was isolated as a CNS stimulant in the 19th century. Quinine for malaria fever from *Cinchona* bark was reported during the same period. Ephedrine (from *Ephedra sinica*) was discovered for asthma from traditional Chinese medicine in the 20th century.

The first British Pharmacopeia of 1863 contained descriptions of 187 crude drugs including *Digitalis*, *Datura*, *Belladonna* and *Hyoscyamus*. There is therefore little or no doubt that ethnopharmacognosy research can provide important clues leading to new drugs for allopathic medicinal use.

In Africa, basic information as a lead to scientific probing of medicinal plants has been obtained from herbalists, native herb sellers and indigenous people (3). By early nineteenth century screening work on African medicinal plants had advanced with publications in following research areas: Antimicrobial (16 %), molluscidal (1 %) antimalarial (7 %), antitumor (4 %) and others (54%) (4-6). For molluscidal activity: *Phytolacca dodecandra*, *Tetrapleura* and *Swarizia madagascariensis* have become an international research interest for the control of schistosomiasis (7). Gedunin and nimbolide, two of the several limonoids in *Azadirachta indica* were identified as the anti-malarial constituents (8). The root of *Cryptolepis sanguinolenta*, used for treating urinary infections in Traditional Medicine is strongly antimicrobial with cryptolepine as the active principle. Its extract has been formulated by the Center for Research into Plant Medicine in Ghana. The common chewing sticks used by the Africans in various communities for traditional dental care have been reported to possess actions against oral microbial flora and to contain various minerals which can hinder plaque formation (6). The most outstanding of the chewing sticks is *Zanthoxylum zanthoxyloides* (Lam.) Waterm (Rutaceae). An ant sickling and anticancer plant, was found to contain the alkaloids: berberine, fagaronine, canthin-6-one and benzoic acid derivatives as the main active ingredients. *Ancistrocladus abbreviatus* (Ancistrocladaceae), a Cameroonian plant species, showed a strong anti-HIV activity in the laboratory. The antiviral component has been pinned down to michelamine B, which is being developed for people living with HIV/AIDS.

1.1. Local Drug formulation and Production from African Plants

The need has since been expressed for industrial drug production from medicinal and aromatic plants in Africa in order to increase the economic and health potentials as well as the social benefits from our natural resources. To

date, over 30 % of the pharmaceutical products manufactured in Egypt are plant-derived such as *Ammi visnaga*, *Glycyrrhiza glabra*, *Aloe vera* and many others. Rwanda and Zimbabwe also produce pharmaceuticals from plants' essential oils. Dr Fumba's Centre in Burundi provides and makes available both orthodox and traditional drugs for the hospital dispensaries.

In the Centre for Scientific Research into plant medicine established in Ghana since 1973, pilot drug production is carried out to provide well-formulated, stable, standardized and safe preparations from plants for clinical evaluation, utilization and monitoring in a clinical setting. Similarly, in the Centre for Research on Pharmacopoeia and Traditional Medicine in Rwanda, *Datura stramonium*, *Eucalyptus globulus*, *Capsicum frutescens* and *Plantago lanceolata* are prepared in the Dispensary of Traditional Medicine where they are administered for antispasmodic, pulmonary disinfectant, counter-irritant and anti-tussive activities respectively. In Mali, several herbal products have been formulated as tea bags for use. This include: Dysenteral (*Euphorbia hirta* for dysentery), Laxa cassia (*Cassia italica* for constipation) and Hepatisane (*Combretum micranthum* for constipation). The "Village Chemist" outfit in the Department of Pharmacognosy of Obafemi Awolowo University, Ile-Ife, in Nigeria has embarked on herbal drug manufacture of many standardized and efficacious herbal preparations, for use in the management of different opportunistic infections in HIV/AIDS in people living with HIV/AIDS (PLWHA) such as antithrush, antifever, anticough and various skin pathogens (4).

Medicinal plants have been used for tooth ache and tooth extraction. The immature pericarp of *Ganipa americana* (Family Rubiaceae) is used for tooth extraction by placing the pulp onto the aching tooth, left in place for several weeks to promote disintegration of the tooth which is then removed in pieces, with little or no trauma (9). The stem-sap of *Stigmaphyllon* species (Malpighiaceae) is placed on the carious tooth for about 4 hours followed by other repeated applications throughout the day. After one week, the tooth can be removed without bleeding or pain. The aggressive stinging ants, found inside the stems of *Triplaris* species (Family Polygonaceae) are crushed and placed on the aching tooth for one week. The tooth is then pulled out with the

fingers. It is believed that formic acid (among other substances) in the stinging ants is responsible for the activity. One application, followed by repeated contact, using cotton swab of the latex of *Chlorophora tinctora* (Family Moraceae), is also used for tooth extraction. No pain, trauma or bleeding is involved (10). Carelessness resulting in spillage or damage to other teeth, adjacent to carious teeth, may lead to unintended extraction of unaffected teeth. The use of the juice of *Ficus* species (Moraceae) for toothache has revealed analgesic and anti-irritant properties. Other plant species recorded for tooth extraction include *Conssapoa glaberima* (Moraceae) and *Curcuma domestica*, *Piper guineense* and *Syzygium aromaticum*.

Vitex doniana has been used by Bukusu traditional medicine men to treat epilepsy (11). Like many cases of traditional medicine, this claim needs to be investigated to establish the pharmacological basis of this treatment. Furthermore the conventional treatment of epilepsy is an issue as drugs are only used to prevent occurrence of episodes of convulsions and in some cases seizures become refractory to drugs with time (12).

1.2 Aetiology, classification and epidemiology of epilepsy

1.2.1 Aetiology

The causes of epilepsy are divided into two broad categories. Epilepsy is a single disease entity and all its forms have a common cause. Different types of epilepsy result from different chemical, anatomic or functional disorders. Epilepsy is an asymptomatic complex disease characterized by recurrent paroxysmal aberration of brain functions usually "brief and self-limited". All forms of epilepsy originate in the brain and appear to result from changes in neuronal activity. The changes such as excessive neuronal discharge may in turn be brought about by disturbance of physico-chemical function of electrical activity of the brain (12). All normal neurons may become epileptic if subjected to excessive excitation. Two possible mechanisms for convulsive disorders are first a loss of normal inhibitory control mechanism and secondly chemical super sensitivity that increases excitability of neurons. The origin of seizures was established as early as 19th century. An intense discharge of gray matter in various regions of the brain initiates seizures.

Discharge of excessive electrical (neurons) energy has indeed been substantiated by brain-wave studies using electroencephalography (EEG).

1.2.2. Classification of epilepsy

1.2.2.1 Partial seizures

Partial seizures respond fairly well to antiseizures drugs (AEDs). They respond well to carbamazepine, hydantoins and barbiturates. Unfortunately these have substantial sedative effect and become refractory after long use. The specific symptoms displayed during simple partial seizure depend on area of the brain which is affected and will occur in opposite side of the body from lesion. Combinations of symptoms are frequent hence accurate diagnosis is a big challenge. Temporal lobes are the most common origin of partial seizures. Symptoms include fear, panic, hallucinations, autonomic signs such as flashing and sweating, unpleasant smell or taste.

Focal motor attacks most commonly start in one hand, foot or side of the face. The onset of seizures is not specific. Should focal motor seizures spread to contiguous critical areas, there may be orderly sequence of events such as repeated movement of hands, face and legs. This is called epileptic march which is also referred to as Jacksonian seizure. If it spreads to the other half of the body, a generalized seizure may follow. In contrast, seizures originating from parietal lobes are termed sensory seizures and present with altered sensations, tingling, numbness and pain. Foci in occipital lobes produce nystagmus, blinking and visual disturbance such as flashing lights or appearance of strange colours. In complex partial seizure, consciousness is impaired which manifests as staring, irresponsiveness, amnesia and automatism. If partial seizures evolve to secondary generalized seizures, the patient experiences an "aura" prior to generalize tonic-clonic phase. An aura manifests as seeing blinking lights and unusual sounds and is usually an important warning to patients.

1.2.2.2 Generalized seizures

Generalized seizures involve both cerebral hemispheres and loss of consciousness. It involves widespread neuronal discharge. It is thought to be caused by diffuse lesions, toxic and metabolic disturbances and constitutional genetic factors. They are classified as typical or atypical generalized seizures. Atypical absence seizures have a long duration of onset and last longer. Differential diagnosis is made on the basis of the EEG patterns. Typical absence seizures respond fairly well to ethosuccimide, sodium valproate and clonazepam. Tolerance to these drugs is the main problem. Antiepileptic drug treatment of typical absence seizures is less successful. Lennox-Gastaut syndrome is a mixed seizure disorder consisting of a typical absence seizures, tonic, tonic-clonic or myoclonic patterns. It starts in childhood and includes mental retardation and adequate control of seizures is rarely achieved.

1.2.2.3 Tonic seizures

These occur mostly in children. It is characterized by increased tone in extensor muscles leading to falling on the ground.

1.2.2.4 Atonic seizures

These are characterized by reduced tone of muscles leading to head drop, loss of muscle tone resulting into falling. This exposes victims to high risk of injury. It is difficult to attain control. Some individuals respond to valproate, felbamate, lamotrigine and topiramate.

1.2.2.5 Clonic seizures

These occur in babies/young children. Loss of consciousness may occur together with tonic contractions followed by brief period of asymmetric jerking motions.

1.2.2.6 Status epilepticus

This is a single prolonged seizure lasting more than five minutes. It may be tonic-clonic, simple partial or complex partial. Tonic-clonic seizure is the commonest and life threatening. Treatment includes

intravenous diazepam, lorazepam, fosphenytoin and phenobarbitone. Absence status epilepticus is characterized by impaired consciousness lasting from 30 minutes to 12 hours. Treatment involves diazepam/lorazepam, followed by ethosuximide.

1.2.3 Epidemiology of epilepsy

Epilepsy is one of the most common neurological disorders. It is estimated that approximately 7 per 1000 of the world's population have epilepsy, which is about 40 million people worldwide. It affects men and women of all ages. It knows no geographical, racial or social boundaries. It is frequently diagnosed in infancy, childhood, adolescence and old age. Studies in developing countries such as Colombia, Ecuador, India, Liberia, Nigeria, Panama, Tanzania and Venezuela indicate that its prevalence is 10 per 1000, which is higher than most developing countries (21).

The common causes of epilepsy include brain damage at birth, congenital or metabolic disorders, drug or alcohol abuse, severe head injury, stroke, brain infection and tumors. The higher incidence of epilepsy in developing countries could be attributed to the higher risk of acute and chronic brain infections as well as pre/post -natal complications at birth. People with a family history of seizure disorders are also at risk of suffering from epilepsy.

Studies in developed countries suggest an annual incidence of epilepsy of approximately 50 per 100,000 of the general population. However, studies in developing countries suggest that this figure is nearly double at 100 per 100000 (21-22). Epilepsy is associated with an increased risk of mortality. Death may be related to an underlying brain disease, such as a tumor or infection, seizures in dangerous circumstances leading to drowning, burns or head injury. Other causes of death in epilepsy include status epilepticus, sudden and unexplained causes of possible respiratory or cardio-respiratory arrest, seizures and sometimes suicide (21-22).

1.3. Treatment of Epilepsy

1.3.1 Historical background

Attempts to treat epileptic seizures and the disease epilepsy date back to pre-historic times. In every historical epoch, the beliefs which people held about the disease dictated the types of therapy which were used. In the time before Hippocrates, when the "sacred disease" was thought to be an illness sent by the gods, people would offer sacrifices, seek expiation and take part in religious acts under the instructions of doctor priests (preferably in the temple) in attempt to be cured.

The supporters of Hippocratic medicine, who believed that epilepsy had a natural cause, tried to treat the disease using natural means (humoral pathology, the ancient physiological theory of fluids or humors). The treatment was based on dietetics, or a structured, sensible life style. These dietetic therapies were based on three pillars: dietary regulations, regulation of excretions and physiotherapy. In addition to dietetics, medicines which were mainly of a herbal nature, played only a secondary role. In the middle ages, epilepsy was no longer considered to have natural causes but was rather thought to be the work of devils, evil spirits and demons (*morbus daemonicus*).

As a result, "therapeutic" methods also changed and took the form of prayer, fasting, offering sacrifices, making pilgrimages or undergoing exorcisms. People turned to several saints for direct help or prayed to them to intercede with God on their behalf. Many secret devotional objects were used to combat epilepsy (treatment using the saints and sacred objects ("hagiotherapy").

After the plague, epilepsy was the disease with the most saints who were responsible for providing a cure and the most important one in Germany was Valentine (probably because of the similarity of his name with a Germany word "to fall" (fallen), "falling sickness." Alongside the medieval Christian attempts to find a cure, various superstitious methods of treatment also developed such as witchcraft and fetishism spells the use of emulates. These actually continue to be

used in some countries today. "Phytotherapy", treatment using plants and parts of plants, was also widely used in the middle ages. During this period there was hardly a plant which was not used to treat the falling sickness, as epilepsy was called. The most important plants were valerian, peony, mugwort, thorn apple, common henbane, mistletoe, belladonna, foxglove, bitter orange and Peruvian bark (23).

Mugwort (*Artemisia vulgaris*), in earlier centuries this was the magic cure -all. Even in orthodox medicine mugwort was believed to be an effective remedy for epilepsy. Absinth, which has amaroids also present in mugwort flowers, was also used to treat epilepsy. During the renaissance, chemical substances were increasingly used to treat the "falling sickness." The most important of these were copper (which had been used by the ancient Greeks), zinc oxide, silver nitrate, mercury, bismuth and tin. It was not until the second half of the ninth century when people began to learn more about epilepsy and medicine that drugs were finally found which did have an effect on epileptic seizures.

The first two substances which were proven to have an anti-epileptic effect and which are still used today were bromine (first used in 1857) and phenobarbitone (first used in 1912). Today they are about 20 chemical substances which can be used successfully to treat epileptic seizures, either singly or in combination therapy. Just under 60 % of all people with epilepsy can become seizure free under modern drug therapy. In another 20 % the seizures can drastically be reduced, and only in one fifth of all epileptic patients do modern anti- epileptic drugs fail to have any effect whatsoever. Modern surgery can help a number of these "therapy resistant" patients, however. Therefore more efforts have been made to find new AEDs effective in refractory seizures and other modes of treatment. Traditional medicine using plants is one such mode of treatment.

1.3.2. Mechanisms of action of AEDs.

Causes of abnormal neuron discharge involve defective neuron ion channels and imbalance between excitatory and inhibitory synaptic function. Various AEDs exhibit different combinations of actions on neuronal functions hence selectivity of seizure type controlled.

1.3.2.1 Ion Channels

After depolarization, sodium channels remain refractory for a period of time during which repetitive firing is inhibited. Some AEDs, act on the sodium ion channels such as phenytoin, carbamazepine, lamotrigine, phenobarbitone, oxcarbazepine Sodium valproate, topiramate.

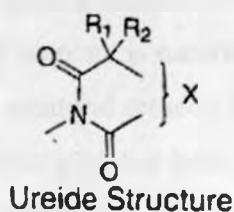
1.3.2.2. Synaptic inhibition and excitation

Neuronal firing depends on balance of excitatory and inhibitory stimulation. Gamma Amino Butyric Acid (GABA) is predominantly inhibitory neurotransmitter in the brain. It is synthesized by Gamma Amino Butyric Acid Decarboxylase (GAD) and inactivated by GABA Transaminase (GABA -T). GABA binds to 2 receptors –GABA_A and GABA_B. GABA_A occurs in chloride ion channels hence leads to influx of chloride ions. GABA_B are linked to a G-protein and potassium and calcium ion channels. These mediate inhibition in the CNS. A number of AEDs augment GABA –mediated inhibition or affect GABA concentration. Benzodiazepines, barbiturates and topiramate enhance action of GABA on GABA_A chloride channels. Tiagabine decreases reuptake of GABA while vigabatrin and gabapentin decreases GABA metabolism.

1.3.3 Anti-epileptic drugs

The primary use of anti-epileptic drugs is to prevent and control epileptic seizures. An ideal AED should have a number of the following properties: It should completely suppress seizures in doses devoid of sedation and CNS toxicity. They should be well tolerated, highly effective and have a rapid onset and a long duration of action after oral administration. It should not have side effects(S/E) on vital

organs and functions. It should have a rapid onset of action after I.V administration. Carbamazepine was introduced in 1974. Newer AEDs which include the following: felbamate, carbapentin, lamotrigine, levotiracetam, oxycabacepine and tiagabin have the advantage of lack of hypnotic activity and less drug interactions. They act by either enhancing brain GABA activity or inhibiting metabolism of GABA (17-20). Many of standard AEDs contain ureide moiety and structural changes can cause significant changes in type of seizure control (15-16) (Figure 1).



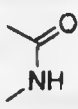



Class of Compounds	X
Barbiturates	
Hydantoins	
Oxazolidinediones	
Succinimides	

Figure 1. Structure of anticonvulsant drugs containing the ureide moiety.

About 60 % of patients with epilepsy become seizure free with frontline therapeutic drugs and another 20 % have epilepsy controlled with more than one AED. The remaining 20 % do not respond to the current frontline therapeutic drugs and are always prescribed with no obvious benefit. Hence much effort has been made to find new AEDs

effective in refractory seizures and other modes of treatment. TM is one such a mode of treatment.

1.3.4. Plants used in the treatment of epilepsy

Phytotherapy, treatment of epilepsy using plants date back in the middle ages. During this time many plants were used to treat epilepsy such as Valerian, Peony, Mugwort, Thorn Apple, Mistletoe and many others. This practice has continued and today many plants are used in traditional medicine all over the world to treat epilepsy with very encouraging out comes.

In traditional medicine, *Negilla sativa* L (black cumin seed) has been used to treat epilepsy. This plant is naturally distributed in Iran and it has been widely used as a natural remedy for paediatric seizures for a long time (24). No scientific work has been carried out and compounds responsible for this activity have not been isolated.

In Tanzania many plants have been cited by traditional healers for the treatment of epilepsy .This includes the following:-

Abrus precatorius L (Papilionaceae), *Clausena anisata* (Wild olive), (Rutaceae) and *Hoslundia opposita* Vahl (Lamiaceae) which have been shown to have anti convulsant activity (25). Neither compounds have been isolated nor has scientific work been done on these claims. In Brazil, several essential oil producing species are used as anticonvulsants in traditional medical systems. *Acolanthus suaveolens* (Lamiaceae) is used by Caboclos in the Brazilian Amazon in homemade antiepileptic formulations, which has led to its ethno pharmacological exploration. Linalool and γ -decanolactone have been identified as the active compounds (26).

The leaves of *Pyrecanthia staundtii* has been used in traditional medicine in Nigeria. This has led to ethno-pharmacological exploration in which it has been shown that aqueous leaf extract of the plant (100-400 mg/ kg I.P) demonstrated a protective effect against strychnine-

induced convulsions (27). *Passiflora incarnata* (Passion flower) is used in traditional medicine of Europe and South America to manage seizures .The anti-convulsant effects of hydro –alcoholic extracts has been studied and it has been demonstrated that a dose of 0.4 mg/kg I.P prolonged onset of time of seizures and decreased duration of seizures with a mortality protection of 100% (28).The compounds responsible for this activity have not been isolated, however, antiepileptic activity has been attributed to flavonoids.

Passiflora edulis Sims is used in traditional medicine in Cameroon to manage epilepsy. Pharmacological studies/exploration in mice has demonstrated that dried leaves decoction has anti convulsant activity It protected mice against strychnine induced seizures with LD₅₀ of 320 mg /kg I.P (29). Phytochemical evaluation for compounds responsible for the activity has not been done.

Diaspyros fischeri Gurke (Ebanaceae) is used in traditional medicine in Tanzania / Zanzibar to treat epilepsy. The anti-convulsant activity has been investigated and it has been shown that 80% ethanol extract of stem bark caused dose–dependent suppression of convulsions induced by 10 mg/kg picrotoxin at doses between 100-3200 mg/kg body weight (30). No compounds have been isolated so far.

Caesalpinia decapetala (Caesalpinaceae), *Carrissa edulis* (Apocynaceae), *Gardenia ternifolia* (Rubiaceae), *Vitex doniana* (Verbanaceae), are used to treat epilepsy in Western Kenya (31).Neither isolation of compounds nor Pharmacological activity has been carried out on this antiepileptic claims.

Vernonia jugalis is claimed to treat epilepsy in which a decoction of whole plant is drunk (32). The roots of the plant are used to promote after birth and the leaves are used to treat stomach problems. *Bersama abyssinica* decoction of the root is drunk three times daily for treatment of epilepsy (Arusha people in Tanzania). The root decoction is also used to treat haemorrhoids and wash wounds. *Clerodendrum*

capitatum infusion of the leaves is applied to the head of a child when the child is recovering from fits, it is also given as a broth made from roots and a flesh of young chicken (32). *Dissotis senegambiensis* leaves are soaked in water and infusion is taken to treat epilepsy. *Oliverella hildebrandtii* infusion of the leaves is poured over the head of the patient suffering from epilepsy. Some of the liquid is also blown into their ears and the nostrils (32). Neither isolation of compounds nor has scientific work been done to evaluate these claims.

1.3.5 Compounds isolated from plants with antiepileptic activity

Linalool and γ -decanolactone isolated from *Aeollanthu suaveolens* (Lamiaceae), used by Cabocles in Brazilian Amazon in homemade antiepileptic formulations have been shown to have anticonvulsant activity (33). Gossypin, a bioflavonoid (gossypin-8-o glucoside. 3, 5,7,3,4-pentahydroxy-8-o-glucosylflavone), is naturally occurring in various plants belonging to family *Malvaceae*. It has demonstrated anticonvulsant activity in the pentylenetetrazole, strychnine and maximal electroshock convulsive methods in mice (34). Extracts of *Coriandrum sativum* L. seed have demonstrated anticonvulsant activity in the pentylenetetrazole (PTZ) and maximal electroshock tests. Phytochemical investigations have shown that the plant seed contains among other components the following: quarcetin 3-glucoronide, Linalool, geraniol and coumarins.

Other than compounds with antiepileptic activity, compounds with other biological activities have been isolated from vitex. Five South Africa vitex have been investigated and found to contain labdane diterpene (epimeric mixture of 12s,16s/R-dihydroxy-ent-labda-7,13-diene-15,16-olide) and flavonoid- Cirsimaritin. The labdane diterpene was demonstrated to be antimicrobial and antimalarial.

1.4. Literature Review

1.4.1 The Genus *Vitex*

Vitex is a genus of about 250 species of shrubs and trees, 3.5 to 35 meters tall. The genus is native to tropical, sub-tropical and warm temperate regions throughout the world. It is included in the family Verbanaceae. Plants in this genus have leaves which are opposite, three to five foliate in number, mostly with long petioles. The leaflets are unequal. Flowers have five lobes which are unequal. Fruit is a drupe. Species in the genus include: *Vitex agnus-castus* (chaste tree, chaste berry, monks pepper is indigenous to the Mediterranean region. *Vitex cuneata*, known in West Africa for its sweet black fruit. *Vitex lignum-vitae* is known as lignum-vitae in Australia, *Vitex lucens* is known as Puriri, is endemic to New Zealand. *Vitex keniensis* is known as Meru oak and endemic in Kenya. *Vitex negundo* is a five-leaf chaste tree found in Tropical areas of Asia and Africa. *Vitex trifolia* is known as simple leaf chaste tree. *Vitex doniana* is ubiquitous throughout the world, is the subject of this study.

Vitex agnus-castus is the most widely studied. It is native to Mediterranean region and western Asia. It is a shrub/small deciduous tree that bears slender spikes of violet-blue 8-10 cm flowers. The medicinal part of the plant is its pepper corn-sized fruit. The berries are aromatic and have a peppery taste. It has several other names including hemp tree, wild pepper, chaste berry (chaste tree), and monk's pepper (35). The ingredients which have been isolated from the fruit berries include: iridoid glycosides (agnoside, aucubin) (used in combination with female hormones to manage PMS), flavonoids (casticin kampferol, quarcertagetin, vitexin) (have activities such as anti-convulsant anti-oxidant, anti-tumour), progestins (progesterone hydroxyprogesterone, testosterone, epitestosterone, androstenedione), which are used in modulating hormone levels of the female reproductive system, alkaloids (viticin), volatile oils containing 1, 8-cineol, linalool (has been shown to be anti-convulsant), terpinyl acetate, alpha pinenes, beta pinenes and essential fatty acids (palmitic acid, oleic acid, linoleic acid, stearic acid (36).

1.4.2 Medicinal uses of genus *Vitex*

Vitex agnus-castus is reputed to have a hormonal effect and is often used for diseases of the female reproductive system. It has been approved in Germany for normalizing irregular menstrual periods and relieving symptoms of Pre-menstrual syndrome (PMS) such as bloating, breast tenderness and moodiness. Chaste berry works by making the pituitary glands to raise progesterone levels. It induces the pituitary gland to free lutenizing hormone (LH) and stop follicle-stimulating hormone (FSH). *Vitex agnus* contains an active ingredient that binds to dopamine (D2) receptors in hypothalamus/ anterior pituitary which action inhibits the release of prolactin. This leads to increased secretion of progesterone during luteal phase of menstrual cycle. *Vitex* can modulate levels of hormones required for normal menstrual function and fertility. It lowers levels of prolactin and aids normal functioning of ovaries hence provide opportunity for conception (37). *Vitex* may stimulate flow of milk in lactating mothers. It can stabilize hormone levels and can be beneficial in controlling symptoms of declining hormone levels such as hot flashes, sweating, vaginal dryness, nervousness; anxiety/depression (37). *Vitex* was also considered a mild sedative in Spain and France. It has been used in Kurdistan and Iraq as cold and stomachache remedy. *Vitex* may also be used as a mild diuretic to reduce water retention before menstruation. It may also help to treat fibroids, fibrocystic breast disease and acne during puberty. Recently, Japanese scientists have isolated four new flavonoids with potential ant-tumor properties from the root bark of *Vitex negundo*. They have also isolated a potent mosquito repellent in the leaf (38-39) (Table 1). Flavonoids isolated from passion flower in Iran have demonstrated anticonvulsant activity. Linalool has been evaluated and shown to have antiepileptic activity. The flavonoids 5,4-dihydroxy-3,6,7,3''-tetramethoxyflavone, luteolin, artemetin and isorhamnetin together with new ones-luteolin 6-C-(4''-methyl-6''-o-trans-caffeoylglucoside), luteolin 6-C-(6''-O-trans-caffeoyl glucoside), luteolin 6-C-(2''-O-trans-caffeoylglucoside) and luteolin 7-

O-(6"-p-benzoylglucoside) isolated from the root bark of *Vitex agnus castus* have shown cytotoxic activity.

Table 1: Medicinal uses of genus *Vitex* (40)

Vitex species and part used	Country	Uses
<i>Vitex agnus castus</i> (entire plant)	England	Anaphrodisiac, expel after birth, emmanagogue ,ovarian stimulant
<i>Vitex agnus –castus</i> (Flowers and leaves)	France	Sedative ,Galactagogue Antispasmodic
<i>Viex agnus – castus</i> (fruit)	Germany	Menorrhagia Anti-estrogenic
<i>Vitex agnus –castus</i> (entire plant)	IRAN	Narcotic Tonic Anti-flatulent Diuretic
<i>Vitex agnus – castus</i> (Root)	Turkey	Fistula treatment
<i>Vitex agnus –castus</i> (Seed)	Morocco	Calefacient
<i>V. buchananii</i> (Root)	Tanzania	Asthma, venereal diseases, malaria hiccups giddiness allergies
<i>V. cannabifolia</i> (fruit)	China	Anti-inflammatory
<i>V. cannabifolia</i> (dried fruit and root)	S. Korea	Emmanagogue Induces Menstruation
<i>V. doniana</i> (Root)	Senegal	Epilepsy
<i>V. cienkowskii</i> (Bark)	Ivory Coast	Infertility
<i>V. cuneata</i> (Fruit)	Guinea – Bisau	Help Conception
<i>V. doniana</i> (Bark)	Guinea	Leprosy Infertility

<i>V. doniana</i> (Leaf, Stem bark)	Nigeria	Dizziness Tonic, antihypertensive Diarrhea / Dysentery
Trunk	“	Chewing stick
<i>V. ferruginia</i> (entire plant, Root bark, trunk)	Ivory coast	Purgative, diuretic emmanagogue sleeping sickness
<i>V. fischeri</i> (leaf)	Tanzania	Epilepsy, sedative, skin infections, venereal diseases.
<i>V. glabrata</i> (stem bark)	Thailand	Antihelmintic astringent
<i>V. grandifolia</i> (entire plant)	Ivory coast	Purgative, diuretic emmanagogue
<i>V. iringensis</i> (Leaf root)	Tanzania	Stop vomiting Dysentery
<i>V. mombassae</i> (Root)	Kenya Tanzania	Infertility
<i>V. negundo</i> (stem bark, entire plant)	Iran	Snake bite
<i>V. negundo</i> (stem bark, entire plant)	India	Antispasmodic Anti-inflammatory Antiarthritic
<i>V. negundo</i> (entire plant)	Fiji	Insect repellent
<i>V. negundo</i> (leaf)	Nepal	Antipyretic Swollen testis Joints
<i>V. negundo</i> (leaf)	Phillipines	Anticancer Galactagogue Headache, Rheumatism Insect repellent Diabetes
<i>V. negundo</i> (root)	Taiwan	Liver diseases
<i>V. Peduncularis</i>	India	Malaria, fever
<i>V. trifolia</i> leaf	Bangladesh	T.B.

		Cancer Sprains, mining fever rheumatism
<i>V. Peduncularis</i> (leaf)	Bangladesh	Stop excess menstrual bleeding
<i>V. doniana</i> (stem bark leaves)	Kenya	Epilepsy, Analgesic, Antipyretic,

1.4.3 *Vitex doniana*: botanical characteristics

Vitex doniana Sweet is a medium deciduous tree measuring 8-18 m high, with a heavy crown and a clear pole up to 5 m high (Figure 1).



Figure 2:

A mature *Vitex doniana* taken on the outskirts of Bungoma Town (September, 2008).

The bark is rough, pale brown or greyish-white, rather smooth with narrow vertical fissures. The bases of old trees have oblong scales (Figure 3).



Figure 3:

Vitex doniana stem bark, showing greyish white bark with narrow vertical fissures.

The leaves are opposite, glabrous, 14-34 cm long, usually with 5 leaflets on the stalk. The leaflets are distinctly stalked, ovate/obovate-elliptic or oblong and entire. Leaf tips are rounded or emerginate and bases are cuneate (Figure 4).



Figure 4. *Vitex doniana* branch showing five leaflets, the long Petiole and unripe fruit.

The flower petals are white except on largest lobe which is purple in dense opposite and auxiliary cymes. Flowers are small, blue or violet in color and only a few being open at a time. The fruit is oblong measuring about 3 cm long. It is green when young and turns purplish-black on ripening. Each fruit contains one hard, conical seed (41) (Figure 5)



Figure5. Flowers, the leaves, unripe and ripe fruits of Vitex doniana.

Vitex doniana is known by various names- which are presented in Table 2

Table 2: Common names of *Vitex doniana*

Local Name	Language
<i>Black plum</i>	English
<i>Mfundu, Mfuru, Mfuu, Mfudu, Matuhuru, Mfulu</i>	Swahili.
<i>Plem</i>	Amharic
<i>Mufutu</i>	Bemba
<i>Galbihi</i>	Fula
<i>Dinya</i>	Hausa
<i>Uchakoro</i>	Igbo
<i>Munyamazi</i>	Luganda
<i>Mfifya, Mfimyfa, mfutu. Msimsya</i>	Nyanja
<i>Kashilumbulu</i>	Lunda
<i>Oori -nla</i>	Yoruba
<i>Muhuru</i>	Kikuyu
<i>Muekelwet</i>	Kipsigis
<i>Jwelu, kalemba</i>	Luo
<i>Tirkirwa</i>	Pokot
<i>Mufutu, Muhutu</i>	Luhya

The fruit is edible and its wood is used for carpentry, making charcoal and firewood. The pulp of the fruit is used for making jam and fruit juice is used to make beverage and alcoholic liquor and wine (42). Other species in the genera in Kenya include *Vitex ferruginea* Schum, *Vitex payos* Merr, *Vitex strickeri* Vatke, *Vitex fischeri* Gurke, *Vitex keniensis* turill (*Meru Oak*), *Vitex mambassae* Vatke (42).

1.4.4. Ethno-botanical uses of *Vitex doniana*

In Nigeria, a decoction of chopped stem bark of *Vitex doniana* is taken orally to treat gastroenteritis. It is also used to improve fertility and juice may be squeezed into eyes to treat conjunctivitis. Hot aqueous extracts of the leaves are used for treatment of stomach and rheumatic pains, inflammatory disorders, diarrhea and dysentery (42-43).

This shows that the plant leaves may possess antibacterial, anti-inflammatory and analgesic properties. The roots and leaves are used for management of nausea, colic and epilepsy (43). In Western Kenya, the stem bark is used for the treatment of epilepsy. *Vitex doniana* roots are cooked and warm decoction is taken to treat backache (in women). Pounded leaves are used as remedy for eye problems (Acholi people). Hot water extract of stem bark is used to treat leprosy and sterility in Guinea. In Nigeria, dried leaves are used to treat dizziness and hot water extract of dried stem bark is used as stimulant, antihypertensive and in treatment of dysentery, diarrhea and control of bleeding after parturition. The dried drunk is used as chewing stick (44, 45 & 47).

1.4.5. Reported Pharmacological activity of *Vitex doniana*;

Stem bark extract of *Vitex doniana* has demonstrated antihypertensive effect (42-44). The extract exhibited a marked dose-related hypotensive effect in both normotensive and hypertensive rats. The extracts of stem bark have also demonstrated some level of *in vitro* trypanocidal activity against *Trypanosoma brucei* (43). In Tanzania, fruits of *Vitex doniana* are used to treat anemia. The root is used to treat jaundice, leprosy, dysentery, gonorrhoea and to improve fertility (45).

Extracts of fruits have shown transient reduction in reproductive functioning in female olive baboons (*Papio hamadryas anubis*). The presence of progestogen-like compounds in the fruit has been suggested as probable cause of fertility reduction (46). Tests on aqueous extract on rats suggest that stem bark is hepato-protective. The aqueous extract of chewing sticks made of *Vitex doniana* have been shown to have a strong activity against a wide spectrum of bacteria including medically and dentally relevant bacteria. This supports the traditional use of the chewing sticks with reported anti-carries effect (47). From tests with hot water extracts of the stem bark on uterine muscle strip preparations, it was concluded that the use of the bark to control postpartum bleeding may be justified. The traditional use of *Vitex doniana* against diarrhea was supported in tests with aqueous methanol extracts of the stem bark on perfused isolated

rabbit jejunum and on castor oil-induced diarrhea in mice. Stem bark extracts were able to inhibit the growth of clinical isolates of *Salmonella typhi*, *Shigella dysenteriae* and *Escherichia coli*, suggesting that they may be valuable in the treatment of dysentery and other gastro-enteric infections (45).

In Benin some species of *Vitex doniana* have been used in combination with other plant species for treatment of epilepsy and other neurological disorders. It has been used in combination with *Burkea africana*, *Azelia africana* and *Cyanotis lanata* to treat epilepsy. It has been used in combination with *Azelia africana*, *Securidaca longipedunculata* and *Smilax kraussiana* to treat insanity. *Vitex simplifolia* has been used in combination with *Longhocarpus laxiflorus*, *Cymbopogon schoenanthus* and *Hymenocardia acida* to treat mental illness. The extract of leaves of *Vitex doniana* have been investigated for anti-inflammatory and analgesic effects and was found that the extract significantly ($P < 0.05$) inhibited the formation of paw edema induced by agar in rats and increased reaction latency to thermal pain in a dose-dependent manner. This suggests that leaves possess anti-inflammatory and analgesic activities (48)

1.4.6. Uses of other *Vitex* species in East Africa:

In East Africa, different species of *Vitex* are used by different communities for different ailments. In Tanzania, *Vitex buchananii* roots eaten fresh/dried are said to be an effective cure for venereal diseases (Luguru people - Tanzania). The root decoction is also used to treat malaria, hiccups, giddiness and allergic conditions. The hot water extract of dried leaf of *Vitex fischeri* has been used to treat epilepsy and anxiety (as sedative) in Tanzania. Hot water extract of *Vitex iringensis* leaves is used to stop vomiting and the root is used for treating venereal diseases. A decoction of *Vitex payos* root (in Tanzania) is used to treat leprosy and infertility. *Vitex ferruginea* leaves are used to treat stomachache by the Boni people, Kenyan coast. *Vitex mombassae* root decoction is used to treat infertility and stop vomiting by the Zigua people. *Vitex strikkeri* leaves are boiled and

applied as a hot fermentation to reduce inflammation in cases of snake bites. The roots are boiled and the decoction is taken orally to treat influenza and colds (44).

1.4.7. Study problem

Universal accessibility to affordable health care is one of Millenium Development Goals set by the United Nations at the turn of this century to be achieved by the year 2015. In Kenya, efforts towards provision of affordable health care have been hindered by lack of drugs in the public health care facilities which cater for the health needs of the majority of Kenyans. There is therefore a need to develop cost-effective, accessible and quality efficacious medicines to improve delivery of health care services. One solution is the integration of traditional medicine into the conventional health care system. Whereas many of the herbs used medicinally have traditional claims for their uses, there is little scientific documentation of their pharmacological actions and active constituents (28). Yet for successful integration of phytomedicines in the conventional health care system, scientific evidence of their pharmacological activities is required.

The medicinal properties of plants are normally dependent upon the presence of certain active principles. Isolations of these compounds may provide potential therapeutic agents or provide lead compounds for chemical derivatization of better analogs. On the other hand, activities of herbal medicine have been known to be synergistic of the components. Once pharmacological activity has been demonstrated, the medicine can be standardized and integrated into conventional health care system.

Despite the recent advances in neurobiology and molecular dysfunction of epilepsy, 20% of patients do not adequately respond to current frontline therapeutic drugs. More often, more than two AEDs are prescribed without any obvious benefit. These points to a need for new AEDs and other modes of treatment of refractory seizures. Though many plants are used traditionally for management of epilepsy

their use has not been validated. *Vitex doniana* has been reported in the treatment of epilepsy by Bukusu Traditional medicine men. This Traditional Treatment has not been validated. Although the stem bark has been demonstrated to have anti-bacterial and other activities no scientific work on anti-epileptic activity is available. *Vitex doniana* has been reported in the traditional treatment of bacterial diarrhea, management of pain, as an anti-inflammatory, anti-spasmodic, such as management of post-partum bleeding. In the proposed study, Pharmacological evaluation of use of *Vitex doniana* in Traditional Medicine was carried out.

1.4.8. Justification

Vitex doniana bark has been used in traditional medicine for the treatment of epilepsy and other ailments. Literature review shows neither pharmacological activity studies nor isolated active compounds have been reported from this plant for the treatment of epilepsy. It is important to carry out the study to establish pharmacological basis of use of this medicinal plant in the management of epilepsy. Furthermore use of allopathic medicine is not curative and in some cases, after long use drugs become refractory. It is therefore important to carry out the study so that if anti-convulsant activity is demonstrated, further studies can be done with a view to isolating compounds that may serve as leads for new compounds for management of epilepsy. Furthermore demonstration of anti-convulsant activity may be the basis of standardization therefore use as a herbal cure of epilepsy. This justifies research on this plant.

1.4.9. Hypothesis

Vitex doniana stem bark extracts have anticonvulsant activity against chemo convulsions in animal models.

1.4.10. Objectives

1.4.10.1. General Objective

The main aim of the study was to demonstrate the potential usefulness of *Vitex doniana* stem bark for the treatment of epilepsy.

1.4.10.2. Specific objectives

The specific objectives of the study were to:

1. Isolate phytochemicals present in *Vitex doniana* stem bark
2. Evaluate sedative and anticonvulsant activity of stem bark extract
3. Evaluate stem bark extract for anti-nociceptive and spasmolytic activity.
4. Determine the safety of the bark extract by determining their acute toxicity.

CHAPTER 2

2.0. MATERIALS AND METHODS

2.1 Materials, reagents and equipment

Filtrations was carried out using Whatman filter paper No.1 (Whatman International Ltd, Madstone England). Column chromatography was carried out on normal phase silica gel powder porosity 32-63 μm (Sigma-Aldrich GmbH & Co. Seelze, Germany). Thin layer chromatography on aluminium plates precoated with 0.2 mm normal phase silica 60 GF₂₅₄ (Sigma-Aldrich GmbH & Co., Seelze, Germany) was used for qualitative analysis of the extracts, fractions and isolated compounds. Potassium bromide (Merck, Darmstadt, Germany) was used to suspend samples for Infrared spectroscopy.

The solvents for extraction, fractionation, isolation and crystallization were all general purpose reagent grades. (Sigma-Aldrich GmbH, Seelze, Germany) and were distilled before use. Dimethylsulfoxide (Fischer Scientific, Loughborough, United Kingdom) was used to prepare suspensions of extracts for pharmacological screening. Vanillin for visualization was prepared using vanillin powder (BDH Chemicals Ltd, Poole, England) in concentrated sulphuric acid (Loba Chemie PVT Ltd, Mumbai, India). Iodine vapor was prepared using Iodine resublimed general reagent (Merck, Damastadt, and Germancy). Ammonia solution, ferric chloride, lead subacetate and sodium hydroxide used in phytochemical tests were all obtained from Kobian Ltd, Nairobi. Ethanol (Prolabo, UK), 3,5-dinitrobenzoic acid (May and Baker Ltd, Dagenham England), carbon tetrachloride (BDH Chemicals Ltd), glacial acetic acid (Pancreac Quimica Barcelona Spain), potassium iodide (Loba Chemie), and mercuric chloride (Loba Chemie) were also used for phytochemical tests.

Instrumentation

A hammer grinding machine was used to mill the plant material into a powder. Soxhlet extraction was carried out on an extractor (Quickfit-Birmingham, England) on an electrotherminal isomantle. A rotary vacuum evaporator (Heidolph Electro GmbH & Co. KG, Kelheim, Germany), connected to a cooler (PolyScience, Niles, USA), a Heidolph WB2000 water bath (Heidolph

Electro GmbH & Co. KG, Kelheim, Germany) and a diaphragm vacuum pump (KNF Neuberger, GmbH, Freiburg, Germany) was used to reduce extracts to dryness. A PB 3002 Delta range top loading balance (Mettler Toledo AG Greifensee, Switzerland) was used for sample weighing more than 500 mg, and an electronic analytical balance (Shimadzu Au W 220D Kyoto, Japan) was used for weighing materials upto 500 mg.

The extracts were fractionated in a glass column (2 cm internal diameter, 50 cm long) and the chromatographic fractions were collected manually. Thin layer chromatography plates were visualized using a Min UV/VS ultraviolet light lamp (Desaga GmbH, Heidelberg, Germany), The melting points of the isolated compounds were determined using a melting point apparatus (A. Gallenkamp & Co. Ltd, London, England). All glassware used was dried in a universal oven (Mettler GmbH & Co. KG, Schwabach, Germany).

A fourier transform infrared spectrophotometer (IRPrestige-21, Shimadzu Corporation, Kyoto, Japan) was used to run infrared spectroscopy. A hydraulic press machine (Perkin-Elmer GmbH Germany) was used to prepare the IR discs Resolution software was used in the analysis and recording. Ultraviolet/visible spectrophotometer was carried out using a UV/Vis Lambda 12 spectrophotometer. A Varian Mercury 200 MHz using a spectrometer (Varian Inc. Palo Alto, California, USA) with a magnet from Oxford Instruments (Oxford, UK) and an online computer (Sun Microsystems, California, USA) was used for NMR spectroscopy, Mass spectroscopy was obtained by Electron Impact mass spectroscopy (EIMS) using a JEOL GC Matell mass spectrometer using 70eV ionizing energy.

Langendorff set-up

Transducer UGO and two channel recorder Gemini 7070 from Basile Italy. Student Kymograph CAT Number 1020 from Scientific and Research Institute Limited, for recording the movements of the ileum. Isolated tissues for Pharmacological work were amounted on organ baths. The heart was set up on the langendorff apparatus. All tissue movements were recorded on a student Kymograph.

2.2 Methodology

2.2.1 Phytochemical investigations

Plant collection, Identification and preservation

The root bark, leaves fruits and stem bark of *V. doniana* were collected from outskirts of Bungoma town in the larger Bungoma District, Western Province of Kenya, approximately 500 Km west of Nairobi. The plant material was collected in September 2008. The pressed voucher specimen (Voucher No. SS 2008/09) was identified and stored at the Herbarium, Department of Botany, University of Nairobi. A sample of pressed voucher specimen was deposited at the department of Pharmacology and Pharmacognosy, School of Pharmacy, University of Nairobi.

2.2.2 Extraction of plant material

About 200 g of powdered stem bark plant material was separately extracted by cold maceration with 1000 ml of 85 % v/v ethanol at room temperature for 72 hours with occasional shaking. The extract was filtered and filtrate was reduced under vacuum using rotary evaporator and dried in an oven at 40 °C. The dried stem bark material was also extracted by sequential maceration with 1500 ml petroleum ether (60-80 °C), chloroform and ethyl acetate for 48 hours each. Extracts were separately filtered using filter paper and reduced using a rotary evaporator. They were stored in refrigerator at about 4°C. Water extraction was done by boiling 50 g of powdered stem bark material in 500 ml of distilled water for 25 minutes. The extract was decanted and filtered through filter paper. The filtrate was reduced to minimum volume and freeze dried (Figure 6). The process was repeated with powdered root bark material to compare the yields.

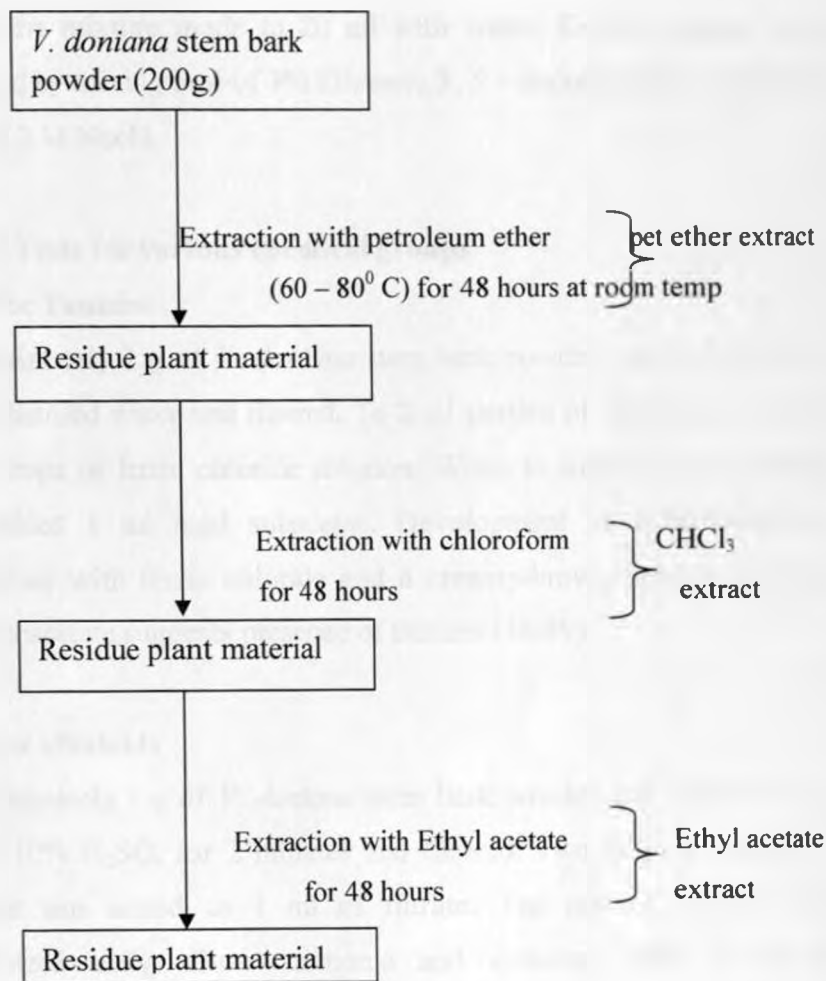


Figure 6: Sequential maceration of stem bark of *V. doniana*

2.2.3 Phytochemical tests

2.2.3.1 Reagents for phytochemical tests

Mayer's reagent was prepared by dissolving 27 g ferric chloride in 12ml water and 1 g of potassium iodide in 2 ml water. The two solutions were mixed and made to volume of 20 ml with distilled water.

Dragendorff's reagent was prepared by dissolving about 0.20 g of basic bismuth nitrate in a mixture of 2.9 ml acetic acid and 10 ml water to produce solution A. About 2 g of potassium iodide was diluted in 5 ml water to produce solution B. About 4 ml of acetic acid was measured and 1 ml of each of the two solutions A and B were added to

it and the mixture made to 20 ml with water. Keddes reagent was prepared by mixing 5ml of 3% Ethanoic 3, 5 – dinitrobenzoic acid with 5 ml of 2 M NaOH.

2.2.3.2 Tests for various chemical groups

Test for Tannins

Approximately 1 g of *V. doniana* stem bark powder was boiled in 10 ml of distilled water and filtered. To 2 ml portion of filtrate was added three drops of ferric chloride solution. While to another 2 ml portion was added 1 ml lead subacetate. Development of a brown-green precipitate with ferric chloride and a creamy-brown precipitate with lead subacetate suggests presence of tannins (48-49).

Test for alkaloids

Approximately 1 g of *V. doniana* stem bark powder was warmed in 5 ml of 10% H₂SO₄ for 2 minutes and filtered. Two drops of Mayer's reagent was added to 1 ml of filtrate. The rest of filtrate was alkalinized using dilute ammonia and extracted with 2 ml of chloroform. Chloroform was evaporated off to leave a solid residue which was dissolved in 0.2 ml of 10% H₂SO₄ and divided into two portions. A drop of Mayer's reagent was added to one portion while to the other a drop of Dragendorff's reagent. A white to buffy precipitate with Mayer's reagent and an orange red precipitate with Dragendorff's reagent suggested the presence of alkaloids (49-50).

Test for glycosides

About 1 g of *V. doniana* stem bark powder was suspended in 10 ml of 70 % alcohol and heated in water bath at 70⁰C for 20 minutes and then filtered. To the filtrate, 10 ml of water distilled water was added, followed by a strong solution of lead subacetate. The solution was filtered and 10 % H₂SO₄ was added dropwise until no further precipitation occurred. The resulting solution was filtered and extracted with two successful 5 ml portions of chloroform. The two chloroform extracts were combined, washed with 1 ml of distilled water, filtered, divided into two portions and evaporated to dryness.

The dry extracts were subjected to Keller-Killian and Kedde test (49-50).

Kedde test

Two drops of Kedde reagent were added to one of the dry extracts. Purple colour indicated the presence of cardiac glycosides whose aglycone moiety has unsaturated lactone ring (49).

Keller-Killian test

To the second extract, 0.4 ml of acetic acid containing trace ferric chloride was added, shaken gently to dissolve and 0.5 ml of concentrated H_2SO_4 carefully added. A reddish brown colour at the inter phase, which gradually turned blue –green, indicated the presence of deoxy sugars.

Test for anthracene glycosides

Borntrager's test

Approximately 0.5 g of *V. doniana* stem bark powder was boiled with 5 ml of dilute H_2SO_4 for 5 minutes. The extract was filtered whilst hot, cooled and the filtrate shaken with an equal volume of carbon tetrachloride (CCl_4). The organic layer was separated and shaken with a few drops of dilute ammonia. A rose-pink to red colour in ammoniacal layer indicated the presence of anthracene aglycones in the oxidized state.

Modified bornatrager's test

The powder was treated as in Bornatragers test except that a few drops of 5% $FeCl_3$ were added during extraction. A rose-pink to red colour in the ammoniacal layer indicated the presence of anthracene a glycones in reduced state.

Test for saponins

Frothing test

A small amount of *V. doniana* stem bark powder was placed in a test tube and distilled water added. The mixture was shaken and left to stand. Persistent frothing suggested the presence of saponins.

Hemolysis test

About 0.5 g of *V. doniana* stem bark powder was extracted with 20 ml of distilled water in a water bath at 70°C for 5 minutes and filtered. About 2 ml of 1.8 % sodium chloride solution was added to each of the two test tubes. To one test tube was added 2 ml of distilled water while to another was added 2 ml of extract. The tubes were labeled control and test respectively. To each tube was added a drop of blood and the tubes inverted gently to mix. Haemolysis in the tube containing the extract and not in the tube containing water indicated the presence of saponins.

Test for steroids (Liebermann's burchourd test)

Approximately 1 ml of the extract was dissolved in 0.5 ml of acetic anhydride and cooled. This was mixed with 0.5 ml of chloroform then 1 ml of concentrated H₂SO₄ was carefully added by means of a pipette. At the interface of the two liquids a reddish-brown ring was formed which was an indication of the presence of a steroid.

Test for flavonoids (Shibata's reaction)

Approximately 3 ml of extract was warmed with three pieces of magnesium turnings and mixed with 3 drops of concentrated HCl. An orange pink colouration was an indication of presence of flavonoids (49-50)

2.2.4. Isolation and characterization of compounds present in the stem

Bark of *Vitex doniana*

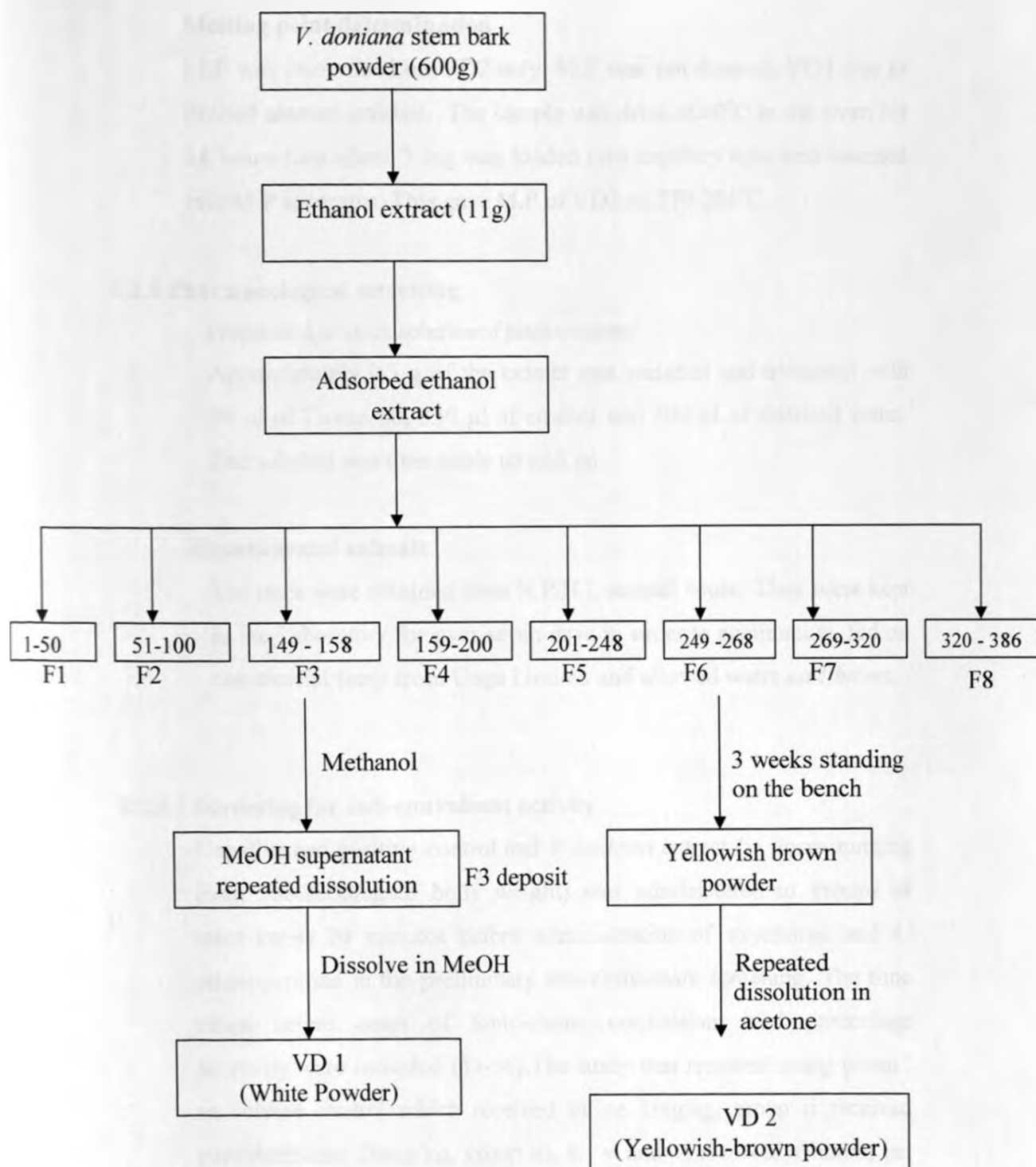
2.2.4.1. Isolation of compounds

Preliminary TLC:

Crude ethanolic extract was subjected to TLC using a variety of mobile systems in order to select an appropriate mobile phase. The eluent that gave the largest number of spots which were well separated was selected. Detection of the separated spots on TLC was done using short UV (254 nm), long UV (366 nm) and visualizing reagents (Iodine vapour and 1% vanillin sulphuric acid) (49-50).

Column Chromatography

About 11 g of crude ethanolic extract was adsorbed on 20 g of silica gel. This was subjected to normal phase silica gel column chromatography with eluent gradient of CHCl_3 - CH_3OH (9.8:0.2 to 6:4) to afford fractions 1-386. Fractions 149 -158 gave similar TLC profile and were pooled together. This on standing for 72 hours formed whitish powdered compound. The white powder was purified by repeated treatment with methanol. This gave on drying 5.9 mg of white powder. This powder was subjected to TLC, and gave a single spot. This was coded VD1. Fractions 249-268 had similar TLC profile hence were pooled together. This on standing for 3 weeks on the bench formed yellowish brown powder. The brown powder was purified by repeated treatment with acetone and on drying yielded 109 mg of yellowish brown powder. This on running TLC gave a single spot. This was coded VD2 (Figure 7).



**Figure 7: Extraction and isolation of compounds from stem bark
Of *V. doniana***

Melting point determination

M.P was determined on VD2 only. M.P was not done on VD1 due to limited amount isolated. The sample was dried at 40°C in the oven for 24 hours then about 3 mg was loaded into capillary tube and inserted into M.P apparatus. This gave M.P of VD2 as 279-284°C.

2.2.5 Pharmacological screening

Preparation of stock solution of plant extracts

Approximately 0.5 g of the extract was weighed and triturated with 70 µl of Tween 80, 330 µl of ethanol and 600 µl of distilled water. The solution was then made up to 5 ml.

Experimental animals

The mice were obtained from N.P.H.L animal house. They were kept in the Laboratory for over seven days in order to acclimatize, fed on commercial feeds from Unga Limited and allowed water *ad libitum*.

2.2.5.1 Screening for anti-convulsant activity

Negative and positive control and *V.doniana* extract (in doses ranging from 500-2000mg/kg body weight) was administered to groups of mice (n=4) 20 minutes before administration of strychnine and 4-aminopyridine in the preliminary anti-convulsant screening. The time taken before onset of tonic-clonic convulsions and percentage mortality were recorded (53-56). The study was repeated using group i as solvent control which received saline 1mg/kg, group ii received pentobarbitone 20mg/kg, group iii, iv, v and vi received ethanol, pet ether, chloroform and ethyl acetate fractions 2000mg/kg respectively. The extracts were administered orally 30 minutes prior to the administration of I.P chemoconvulsant 3microgram/kg and 13.2mg/kg strychnine and 4-aminopyridine respectively. The onset, duration of convulsions and percentage mortality were computed.

2.2.5.2 General CNS observation assessment

Swiss albino mice weighing from 20-30 g of either sex were injected I.P (n=4) 4000 mg/kg body weight then observation made at 30 minutes, 1 hour, and then 2 hours.

2.2.5.3 Motor activity by inclined plane method

The method involves measuring the angle at which the mouse slips downwards when placed on a sloping surface. This is done before and 15 minutes later after drug administration.

The dose of the extract used was 2 g/kg while the dose of tubocurarine was 200 µg/kg body weight. The vehicle was used as negative control.

2.2.5.4 Potentiation of Pentobarbitone sleeping time

The mice were divided into three groups (n=4) of four each. Group I received normal saline as negative control. Group II received extracts (2 g/kg) and group III received diazepam (5 mg/kg) as positive control. The animals were administered with 50 mg/kg IP of sodium pentobarbitone 20 minutes later. The time lapse between administrations of pentobarbitone and loss of righting reflex was recorded as onset of sleep. Duration of sleep was taken as the time between the loss and recovery of righting reflex (56).

2.2.5.5 Analgesic activity

Acetic acid writhing test

Vitex doniana extract (62.5 mg/ml) was prepared by dissolving 312.5 mg of extract in 5 ml of distilled water. Sodium salicylate was prepared by dissolving 15 mg of the compound in water to give a 5 mg/ml solution. The negative control was prepared as described above. Acetic acid 0.6% was prepared by dissolving 0.06 ml acetic acid in water. The test is an initial screening for peripheral analgesic activity. Writhing movement is described as stretching of the abdomen, accompanied by extension of at least one of the hind limbs. For this test, the mice were divided into three groups of six mice each. The groups were first pretreated with the test drug, control or standard for 30 minutes before intra-peritoneal injection of 0.6 % v/v acetic acid.

The number of writhes occurring during the 30 minutes period after injection was observed and recorded (57-59)

Hot plate assay

The mice to be used in this experiment were initially screened by placing on a hot plate maintained at a constant temperature of 55⁰C only those that did not react within 10 seconds were used. The selected mice were divided into four groups of six mice each. The first group was injected with sodium salicylate 50 mg/kg. The second with 1 g/kg of the extract, the third 2 g/kg of the extract and the fourth group with the control (57-58). The assay was done by placing the mice on the hot plate and recording their reaction time. The end point was the time taken for the mouse to stand on its hind legs and lick its front paws. Mice that did not respond in 40 seconds were removed from the hotplate to avoid tissue damage.

Tail pressure method

The drugs were administered to mice of both sexes and after one hour the tail of the mouse 2-3 cm from the tip was placed under the Teflon tip of the analgesy-meter and the pressure gradually increased. The point at which the animal struggled was taken to be the reaction threshold. To avoid bruising the animal the pressure exerted was restricted to a maximum of 250 g. The pressure was read on a scale reading from 0-25.

2.2.5.6 Spasmolytic activity

Effect of drug on isolated rabbit ileum

The aim of this test was to test the effect of the extract on motility and tone of the small intestine. Male rabbit bred under standard laboratory conditions (room temperature, 12 – hour light and dark cycle, feed and water *ad libitum*) were obtained from the National Republic Health Laboratory. A rabbit was killed by a blow at the back of its head. The neck was cut and the animal was let to bleed.

The abdomen was cut open and a 4 to 5 cm piece of the jejunum was cut out and mounted in a 25ml double walled organ bath containing Tyrode's solution. The tissue was aerated with 95 % oxygen and 5% carbon dioxide and thermostatically maintained at 37°C.

It was allowed to stabilize for 20 minutes so that the required tone would develop. The movements of the intestine were recorded on a kymograph.

Drug solution was added to the organ bath and it was allowed to act till the maximum response was obtained. The organ bath was drained and the tissue rinsed twice. It was let to rest for 2 to 3 minutes and rinsed again before adding the next drug.

Adrenaline 10 mg/ml, acetylcholine 10 mg/ml and extract 10 mg/ml in 0.1 ml, 0.2 ml and 0.4 ml were injected into the solution after each effect had been observed and the drug washed out.

2.2.5.7. Anti-inflammatory activity

The anti-inflammatory activity was assessed using carrageenan rat paw edema test (Carrageenan from Sigma-Aldrich GmbH & Co. Seelze, Germany). Adult wistar rats in groups of 4 weighing 250-430 g were deprived of food for 24 hours. Each group was administered with either *V. doniana* extract (2 g/kg), indomethacin (10 mg/kg) or vehicle orally by gavage. One hour later the volume of the right hind foot was measured by mercury displacement in Plethysmometer. The foot was immersed in the mercury in the plastic tube up to the level of lateral malleolus and the volume of the displaced mercury measured in the capillary tube. Immediately after measuring the foot volume, 0.1 ml of 1 % v/v of carrageenan solution in a sterile normal saline was injected into the plantar surface of the paw and the animal returned to the cage. One hour later the volume of the same foot was again measured. The difference between the two volumes was designated the edema volume (59).

2.2.5.8 Brine shrimp lethality assay

Hatching of shrimps

A plastic container was divided into two chambers and filled with artificial sea water (made up of 33 g of marine salt and 6 mg of baker's yeast in 1 litre of distilled water). The chambers were separated by a plastic wall having several holes of 2 mm diameter. One chamber was darkened while the other was illuminated by an electric bulb. About 50 mg of brine shrimp eggs were sprinkled in the darkened side. After 48 hours the larvae migrated to the illuminated side. The larvae were harvested using a pipette.

Preparation of solution

About 50 mg of the extract was dissolved in 5 ml brine solution to obtain a concentration 10,000 $\mu\text{g}/\text{ml}$. To enhance solubility, the extract was solubilized using 0.1 ml DMSO then topped up to 5 ml using brine solution.

Procedure

Serial dilution were made using artificial sea water to obtain concentration of 1,000 $\mu\text{g}/\text{ml}$, 100 $\mu\text{g}/\text{ml}$ and 10 $\mu\text{g}/\text{ml}$. About 5 ml of each dilution were transferred to five different test tubes. Each test tube was inoculated with 10 larvae. A set of five test tubes (containing a total of 50 larvae) were grown without exposure to ethyl acetate fraction of extract. This served as a negative control. About 24 hours later all the larvae in the container were counted and the percentage death was computed. The above was repeated at a concentration of 20, 30 and 80 $\mu\text{g}/\text{ml}$. The above procedure was repeated using crude ethanol extract, pet ether fraction and chloroform fraction (62).

2.2.5.9 Acute toxicity test

The mice were divided into five groups of five mice each. The first group was given a dose of 500 mg/kg body weight. The second and third groups were given a dose 1000 mg/kg and 2000 mg/kg respectively and the fourth group were given a dose of 4000 mg/kg. The fifth group was given vehicle as negative control.

CHAPTER 3

3.0. RESULTS AND DISCUSSION

3.1 Phytochemical Investigations

3.1.1 Yields on extraction

The stem and root barks were subjected to cold maceration (at room temperature) with ethanol. For the stem bark the yields of the chloroform and ethanol extracts were 0.20 and 2.6 % respectively. For the root bark the yields relative to ground dry weight of the chloroform and ethanol extracts were 0.2 and 4.9 % respectively. For both the stem and root barks the yields of the ethanol extracts were substantially greater than the yields of chloroform extracts.

In addition, the stem bark was subjected to sequential maceration with petroleum ether, chloroform, ethyl acetate, ethanol and water. The yields are presented in Table 3. The water extract gave the highest yield.

Table 3. Yields of sequential maceration of stem bark of *Vitex doniana*

Extract	%yield of stem bark	% yield of root bark
Petroleum ether	0.04	0.09
Chloroform	0.16	0.2
Ethyl acetate	0.36	0.83
Ethanol	1.66	4.94
Water	9.36	7.79

3.1.2 Phytochemical groups present in *Vitex doniana*

Alkaloids, glycosides, saponins, steroids and flavonoids were detected in the stem bark of *V. doniana* (Table 4). These phytochemical groups have been reported before in this genus. Literature review shows that *Vitex agnus castus*, a widely used and studied member of this genus, contains steroids, flavonoids and alkaloids (35).

Table 4: Phytochemical groups present in *Vitex doniana* stem bark

Chemical Class	Phytochemical test	Relative abundance
Alkaloids	Mayer's	+
	Dragendorff	+
Glycosides	Kedde	++
	Keller-Killian	++
Saponins	Frothing test	+++
	Gelatin test	+++
Steroids	Buchard test	++
Flavonoids	Shibata's reaction	+++

+ Trace amounts, ++ Moderate, +++ Abundant

3.1.3. Structure elucidation of isolated compounds.

Compounds VD1 and VD2 were subjected to ultraviolet (UV), mass spectrometry (MS) and infrared (IR) spectral analysis. Nuclear magnetic resonance (NMR) spectroscopy and melting point (mp) determination was done on VD2. No further investigations were carried out on VD1 due to the small quantity isolated.

Compound VD1

This was a white compound with the following spectral data:

UV λ_{\max} (MeOH) nm (log ϵ): 207.6 (1.37), 280nm (isolated/unconjugated C=O) (Appendix 1)

IR ν_{\max} (KBr) cm^{-1} : 3427.5 (br. OH stretch), 2937.6 (aliphatic C-H stretch), 2870, 2378.2, 1699.3 (C=O stretch), 1460.1 (CH_3 bend), 1383, 1232.5, 1037.7, 960.6(fingerprint region-single bond stretch/bend of polyatomic substituent) (Appendix 2).

MS m/z (relative intensity %): 414.0 (100%), 381.1 (22), 354.0 (5%), 329.1 (25%), 303.1 (35%), 255.1 (29%), 231.0 (18%), 213 .0 (28%), 159.0 (29%), 145.0 (38%), 109.8 (39%), 86.3 (40%) (Appendix 3).

Table 5: fragmentation pattern of VD1

VD1 (m/z)
468
428
414.0 (base peak)
399
398
396
381.1
354.0
329.1
303.1
255.1
231.0
213.00
159.0
145.0
109.8
86.3

Table 5 gives the key peaks in VD1 MS spectrum. The fragment ion with m/z of 399 arises from loss of a methyl group ($M^+ - 15$) from the base ion. The fragment at m/z 396 is due to loss of elements of water ($M^+ - 18$) (63-64). Evidence of a carbonyl group was provided by presence of minor peak at 280 nm ($C=O$) in the UV spectrum.

From the IR spectrum, it was deduced that the compound lacks aromatic or olefinic groups due to absence of unsaturated C-H stretch at slightly above 3000 cm^{-1} . The IR spectrum has prominent peak at 1699, an indication of presence of a carbonyl group. The compound gave positive result with Liebermann–Burchard test indicating it is a

sterol. Further spectral information such as DEPT, HMBC/HMQC, NOE and COSY NMR are required to conclusively identify compound VD1.

Compound VD2

This was a yellowish brown powder, with the following spectral data:

UV λ_{max} (MeOH) nm (Log ϵ): 248.4 (max) (2.23), 292.0 (min), 321.6 (max) (0.94) (Appendix 4).

IR ν_{max} (KBr) cm^{-1} : 3483-3329 (br, H-bonded OH), 2930 (CH_3 C-H stretch), 2859 (CH_2 C-H stretch), 1653 (conjugated C=O stretch), 1460.1, 1383, 1292, 1163, 1057, 812(fingerprint region-single bond stretch/bend of polyatomic substituent) (Appendix 5).

MS (relative intensity % m/z: 327.9 (5.3), 310.8 (6.0), 273.8 (78.6), 259.7 (100), 227.9 (20.3), 202.9 (11.5), 186.9 (5.5), 151.9 (11.7), 136.9 (8.1), 116.1 (12.2), 83.6 (7.4), 68.9 (13.4) (Appendix 6).

^1H NMR: δ 13.15 (2H, s, 2xOH), 7.61 (2H, d, $J=8.2$ Hz, 2x=C-H), 7.01 (1H, s, 1x=C-H) 6.97 (2H, d, $J=8.8$ Hz, 2x=C-H), 6.42 (2H, d, $J=2.4$ Hz, H-1,8), 6.23 (1H, s, 1x=C=H), 3.70 (s, CH_3O -) (Appendix 7).

^{13}C NMR: δ 180.4 (C-4), 165.3 (C-2), 164.0 (C-7), 163.8 (C-5), 158.1 (C-9), 151.5 (C-4), 146.2 (C-3'), 132.6 (C-1'), 124.3 (C-6'), 116.7 (C-5'), 116.2 (C-2'), 115.6(C-10), 114.1/113/112.9^a (C-3), 98.2/96.9 (C-6), 94.1/92.6^a(C-8), 55.822/55.715^a (Appendix 8).

The IR spectrum gave a broad peak at 3427 cm^{-1} which was attributed to hydroxyl group stretch. A peak at 2900 cm^{-1} (C-H stretch) was attributed to C-H stretch of CH_3 . A C=O stretch at 1653 cm^{-1} low frequency observed indicates conjugation (63-69).

The ^1H NMR Spectrum gave 5 peaks between $\delta 6.0$ to 7.8 . This indicates presence of aromatic or olefinic protons. The shift at $\delta 6.23$ appeared as a doublet with coupling constant of 1.8 Hz. This indicated that these two protons were undergoing Meta coupling. A doublet at $\delta 6.97$ had coupling constant of 8.8Hz, which was an indication

of ortho-coupling. A double doublet with a chemical shift had a coupling constant of 8.2 Hz. The peaks at δ 6.97 and 7.61 each represented two protons.

A singlet representing one proton appeared at δ 7.01 indicating that this proton was not coupled to other aromatic protons. The presence of a shift at 13.16 confirmed the presence of 2 hydroxyl- protons. While the presence of three protons at δ 3.9 confirmed the presence of methoxy protons.

In the HNMR spectrum additional peaks were seen at δ 2.0 and δ 1.2. These are solvent peaks attributed to methyl groups in acetone and methanol. Some peaks in the Carbon-13 NMR spectrum of VD2 were replicated (δ 114.1/113.1^b 98.2/96.9)^b.

In the mass spectrum, fragment peak at m/z 260 arose from loss of CH_2 group from fragment peak at m/z 274. A fragment at m/z 273 represents loss of 12 amu (atomic mass units) from the molecular ion. This represents loss of CH -group ($M^+ - 13$). On the other hand the fragment whose m/z value is 260 represents loss of 14 amu from fragment peak at m/z 274. These represent loss of CH_2 group to give the base ion peak. The presence of carbonyl groups was confirmed from the carbon-13 NMR spectrum. Two shifts attributed to unconjugated carbonyl (δ 180) and a conjugated carbonyl (δ 166) was noted. The unconjugated carbonyl was attributed to the acetyl substituent. The carbon 13 shift at δ 98.2 displayed peak replication with a second peak at δ 96.9. This phenomenon is attributed to tautomerism.

Further spectral data such as DEPT, HMQC/HMBC, NOE and COSY NMR is required to conclusively determine and authenticate the structure of compound VD2.

3.2 Pharmacological evaluation of stem bark extract of *Vitex doniana*

3.2.1 Effects on the Central nervous system

4.2.1.1 General Observational screen for CNS activity

Mice were treated with an intra-peritoneal injection of 85% ethanol extract of stem bark (4 g/kg bwt) of *V. doniana*. The mice were observed for 2 hours and the observations made are presented in Table 10.

Table 6. General observational screen for the CNS effects of the ethanol extract *Vitex doniana*.

Type of observation	Observation
Spontaneous motor activity	
Grooming	Present
Squatting	Present
Staggering	Slight
Effect on reflexes	
Pineal reflex	Absent
Corneal reflex	Absent
Righting reflex	Present
Effects on the autonomic nervous system	
(piloerection, salivation, sweating, mydriasis, miosis, heart rate, cyanosis, defecation, urination)	No change. All normal
Responses after manipulation	
Auditory stimuli	Responded
Touch	No response
Pain stimulation	No response

From the observations, the ethanolic extract of the stem bark of *Vitex doniana* may be a mild CNS depressant. It did not cause a significant reduction in spontaneous motor activity but induced mild staggering. The effects on motor activity were further investigated using the inclined plate screen.

As expected of CNS depressants, it caused loss of the pineal and corneal reflexes. It however did not cause loss of the righting reflex indicating that it may be a mild CNS depressant.

There were no significant effects on observations related to activation or depression on various parts of the autonomic nervous system. However it cannot be ruled out that the extract does not have effects on ANS function because the mouse model is not appropriate for such observations. More specific tests are required to evaluate the effects on the ANS.

The mice had a blunted response to manipulations such as touch. This may indicate anxiolytic activity. On pain induction the mice did not respond indicating that the extract may have anti-nociceptive activity. This was further investigated using a series of tests.

From the general observational screen, it was concluded that the extract may be a CNS depressant and this was evaluated by determining its effects on pentobarbitone sleeping time.

3.2.1.2 Anticonvulsant activity of extracts of *Vitex doniana*

The ability of various extracts and fractions of *V. doniana* to delay convulsions induced by strychnine and 4-aminopyridine was evaluated. All extracts were screened against 4-aminopyridine induced convulsions since it is thought to be the best model epilepsy. It is a centrally acting chemoconvulsant. The anti-convulsant effects of various extracts of plant are presented in Table 12. The latency of convulsions increased with dose up to maximum activity at dose 2000mg/kg. The anti-convulsant activities of lower doses were similar to that of pentobarbitone at a dose of 20mg/kg. However, at the highest dose of 2000mg/kg of extracts, positive control (20mg/kg) delayed the onset of convulsions by about 12.5 minutes. The activity of the crude extract was less than that of pentobarbitone since it delayed the onset of the

convulsions by 8.00 minutes. However this activity was significantly higher than that of the negative control ($P=0.07$). The activities of fractions of the crude extract were significantly better. The ethyl acetate fraction had the best activity and delayed the onset of convulsions by 36.5 minutes. This was superior to the activity of the pentobarbitone. The chloroform had no statistically significant activity. This was attributed to poor water solubility leading to poor bioavailability. There was no statistically significant difference in activities of the water extract and petroleum ether fraction ($P>0.5$).

The anticonvulsant activity of crude extract and water extract was evaluated against strychnine induced convulsions. The activities of the petroleum ether, chloroform and ethyl acetate fractions were not evaluated for strychnine induced convulsions because they were not available in sufficient amounts. Strychnine is a peripherally acting chemoconvulsant that acts by inhibiting the glycine receptor. The activities are presented in Table 9. Though pentobarbitone and the crude extract delayed the onset of convulsions, their activity was not statistically significant when compared to the effect of normal saline. This apparent lack of activity may be attributed to diminished activity at spinal level where strychnine exerts most of its effects. On the other hand, the water fraction was active and prolonged/ delayed the onset of convulsions by 33 minutes.

It was concluded that the crude ethanolic extract of *Vitex doniana* has anticonvulsant activity especially against 4-aminopyridine induced convulsions. In both the models of chemo-convulsion, fractions of the crude extract were generally more active than the crude extract except for chloroform fraction which had less activity. From the foregoing, it is concluded that the polar fraction of *Vitex doniana* extract has the greatest anticonvulsant activity. Bioassay guided fractionation should be carried out on polar fractions (ethyl acetate) to isolate and evaluate compounds with anticonvulsant activity present in *Vitex doniana* stem bark.

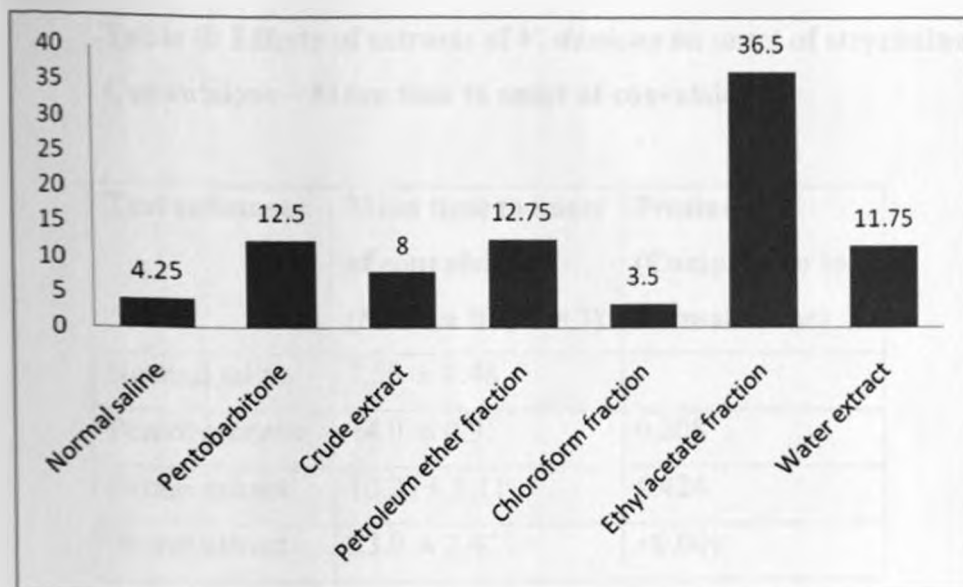


Figure 8: Effects of extracts of *V. doniana* on onset of 4- aminopyridine induced convulsions – mean time to onset of convulsions

Table 7: Effects of extracts of *V. doniana* on onset of 4- aminopyridine induced convulsions – Mean time to onset of convulsions.

Test substance	Mean time to onset of convulsions (Mean \pm SD, n = 3)	P-value (Comparison to normal saline)	P-value (Comparison to Pentobarbitone)
Normal saline	4.25 \pm 0.48	-	
Pentobarbitone	12.50 \pm 1.04	<0.001	
Crude extract	8.00 \pm 0.41	0.007	0.001
Petroleum ether fraction	12.75 \pm 0.48	<0.001	1.0
Chloroform fraction	3.50 \pm 0.65	0.942	<0.001
Ethyl acetate fraction	36.50 \pm 0.87	<0.001	<0.001
Water extract	11.75 \pm 0.85	<0.001	0.942

Table 8: Effects of extracts of *V. doniana* on onset of strychnine induced Convulsions – Mean time to onset of convulsions.

Test substance	Mean time to onset of convulsions (Mean \pm SD, n = 3)	P-value (Comparison to normal saline)
Normal saline	7.50 \pm 0.48	-
Pentobarbitone	14.0 \pm 0.91	0.208
Crude extract	10.25 \pm 1.11	0.424
Water extract	33.0 \pm 2.42	<0.001

3.2.1.3 Effect on Pentobarbitone sleeping time

The effect of co-administration of pentobarbitone with normal saline, the crude ethanolic extract and diazepam (5 mg/kg bwt.) on sleeping time was evaluated. Diazepam and the crude extract both prolonged sleeping time about 20 minutes (Figure 12). One-tail heteroscedastic t-test showed that sleeping time of diazepam and the crude extract was longer than that of pentobarbitone co-administered with normal saline ($P < 0.05$).

These findings confirmed that the ethanolic extract of *V. doniana* has mild CNS depressant activity comparable to that of diazepam. This property may be responsible for its claimed anticonvulsant effect. The mild CNS depressant effects make it ideal for chronic management of epilepsy since it may lack significant hypnotic effects.

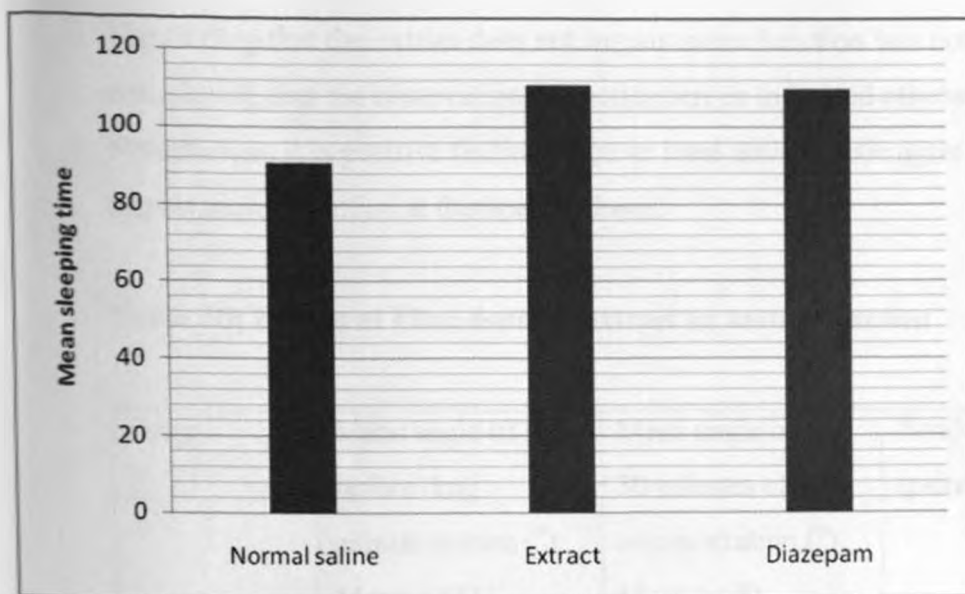


Figure 9: Effects of the ethanol extract of *Vitex doniana* on the sleeping time of Pentobarbitone treated mice

Table 9: Effects of the ethanol extract of *Vitex doniana* on the sleeping time of Pentobarbitone treated mice

Sleeping time (minutes) Mean \pm SD, n=4		
Normal saline (200 μ l)	Extract	Diazepam (5 mg/kg)
90.75 \pm 2.99	110.50 \pm 9.54	109.75 \pm 9.11

3.2.1.4 Effect of the ethanol extract of *Vitex doniana* on Motor function

The inclined plane test was used to determine if the extract can impair motor function. CNS depressants have the undesired property of impairing motor function. The motor activity measured as the angle at which a mouse begins to slip before intraperitoneal administration of the extract and 30 minutes after administration. The test results are presented in Table 15.

Though the extract caused a slight reduction in the mean angle of slip, this finding was not statistically significant. On the other hand, the positive control caused a significant reduction in the angle at which mice began to slip.

The finding that the extract does not impair motor function was not expected considering that the observational function screen indicated otherwise. Nonetheless, it is positive finding since an ideal antiepileptic agent should not impair motor function at therapeutic doses.

Table 10: Effects of *Vitex doniana* extract on motor function

Drug	Mean angle of slip before drug administration (°) Mean ± SD	Mean angle of slip 30 minutes of drug administration (°) Mean ± SD	P-value (paired t-test)
Extract	81.8 ± 0.8	78.0 ± 0.7	0.464
Tubocurarine	79.2 ±	67.2 ± 3.9	0.016

3.2.2 Anti-inflammatory and analgesic activities

3.2.2.1 Acetic acid writhing test

Figure 12 shows that *vitex doniana* extract had more inhibitory effect on acetic acid induced writhing compared to sodium salicylate. There was no significant difference between the two doses of Vitex extract. The effects showed a slight tapering off with time.

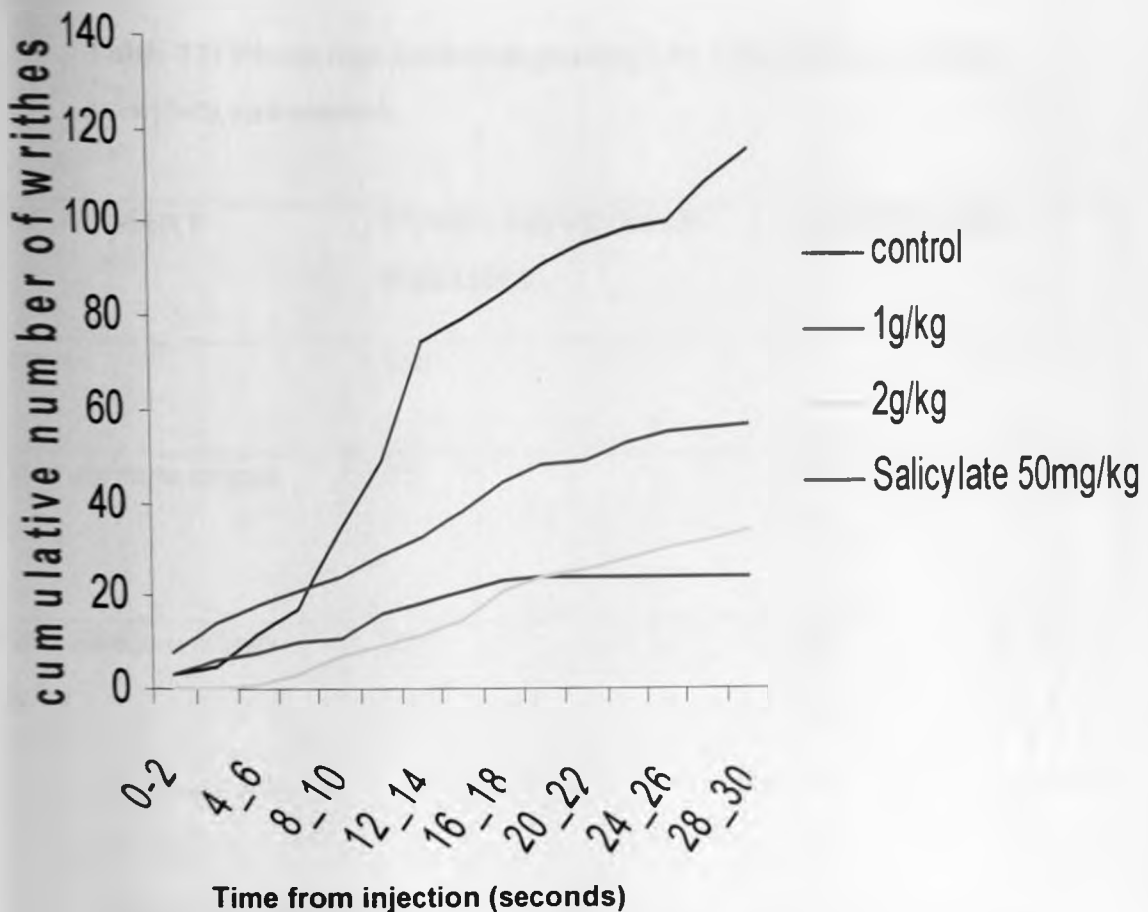


Fig.10: Effects of sodium salicylate and *Vitex doniana* on acetic -acid induced writhing.

Table 16 shows that both *Vitex* extracts (2 g / kg)and (1 g /kg) had greater effects than sodium salicylate (50 mg /kg) .The *Vitex* extract of concentration (2 g / kg) inhibited acetic acid induced writhing by 80% while sodium salicylate caused an inhibition by 50%.*Vitex doniana* extract (2 g /kg) had an almost equipotent effect with the lower concentration (1 g/ kg) which inhibited writhing by 75%.

Table 11: Percentage inhibition produced by *Vitex doniana* extract, salicylate and control.

TREATMENT	CUMULATIVE NO OF WRITHES	%INHIBITION
Control	110	
<i>Vitex doniana</i> extract 1g/kg	30	75
<i>Vitex doniana</i> extract 2g/kg	22	80

3.2.2.2 Hot plate test

The *Vitex doniana* extract had very little analgesic effect on the rats in the hot plate assay. The analgesic effect was completely absent after twenty five minutes of drug administration (Fig 14.)

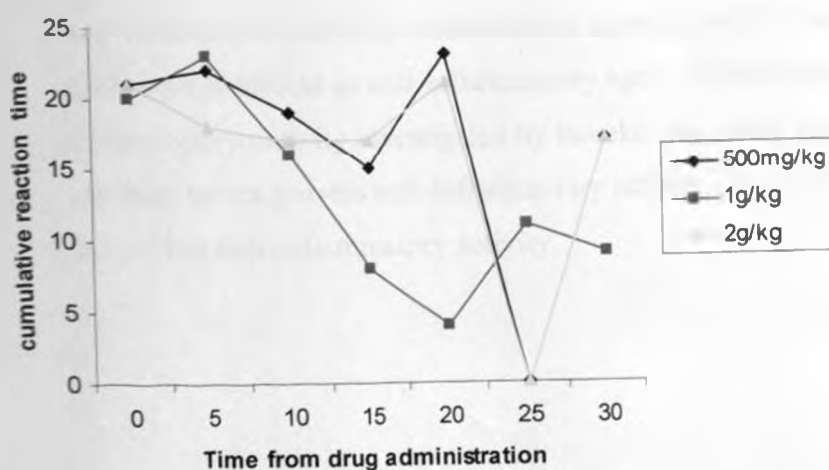


Figure 11: Effect of the extract against cumulative reaction time

3.2.2.3 Anti – Inflammatory activity

Table 12: Effect of extract on carrageenan induced edema

Drug	Dose/Kg	Edema volume after 3 hours
Normal saline	1ml	2.00 ± 0.41
Indomethacin	10mg	1.13 ± 0.52
Extract	2g	1.25 ± 0.25
P value (ANOVA)		0.305

Percentage inhibition of inflammation

$$\% \text{ inhibition of inflammation} = \frac{\text{Volume of control} - \text{Volume of test}}{\text{Volume of control}} \times 100$$

The percentage inhibition of oedema by extract was 37.5% while that of indomethacin was 43.5%. This suggests that anti-inflammatory activity of extract is slightly less than that of indomethacin. Statistically there was no significant difference among the extract and indomethacin in terms of anti-inflammatory effect of ethanol stem bark extract (P=0.305) (Table 17). The sample size should be increased to magnify effect of the study. However, the anti-inflammatory activity demonstrated seems to justify the folkloric use in traditional medicine as anti-inflammatory agent. Furthermore the anti-inflammatory activity investigated by Iwueke and others suggested that *Vitex doniana* leaves possess anti-inflammatory activity (46). This suggests that the extract has anti-inflammatory activity.

3.3 Effects on the isolated ileum and heart

3.3.1 Effect on the isolated rabbit ileum

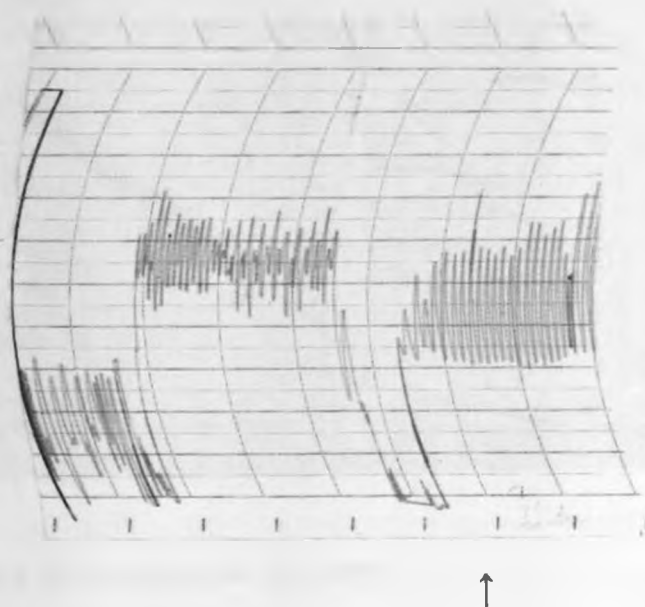


Figure 12: Stem bark extract activity in rabbit ileum.

The extract increased the amplitude of contractions (it had contractile activity in the rabbit ileum). This suggested that extracts of *Vitex doniana* has contractile effect on Rabbit ileum and other smooth muscles like uterine muscles. Studies on uterine muscles (42) using water extract of *Vitex doniana* stem bark showed that the extract induced graded uterine muscle contractions and also potentiated the contractile effects of prostaglandins, ergometrine and oxytocin. The study further revealed that effect of *V. doniana* stem bark extract may act via uterotonic receptors. Therefore the use of *V. doniana* to control postpartum bleeding after child birth may be justified.

3.3.2 Effect of the extract on the heart

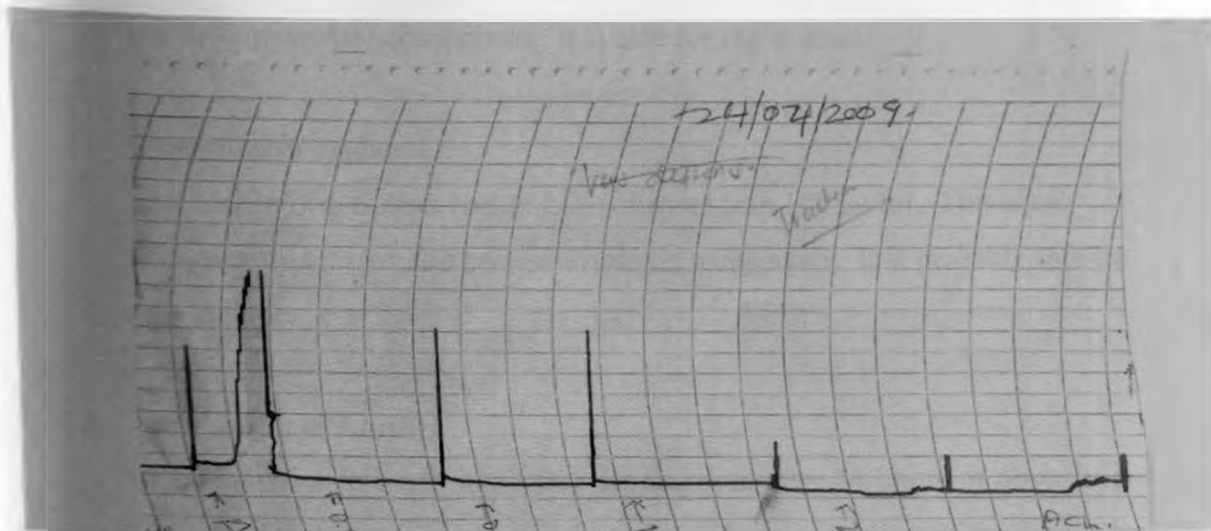


Figure 13: Effect of the extract on the heart.

The extract caused contraction of the heart muscles. This caused an increase in the heart rate. This may be due to β -adrenergic activity or high calcium levels in *Vitex doniana* extract. Further investigation may be necessary in this action.

3. 4. Toxicity of the ethanol extract of the stem bark of *Vitex doniana*.

3.4.1 Brine shrimp lethality assay

In the brine shrimp lethality test, the ethylacetate extract of the stem bark killed nauplii with an LD_{50} of 81.48 $\mu\text{g/ml}$. This is an indicator that the extract may contain cytotoxic compounds. This test may be used to monitor toxins and tumor agents etc. The test is also used to study insecticidal activity. Therefore this extract may also possess insecticidal activity

Brine shrimp assay in ethanol, petroleum ether and chloroform extracts:

Ethanol extracts

The extracts of stem bark killed nauplii with LD_{50} of more than 100 $\mu\text{g/ml}$. They probably do not contain cytotoxic compounds.

Pet ether extract

The pet ether extract had an LD₅₀ of more than 100 µg/ml. Probably it does not have cytotoxic compounds. It is safe for use in mice.

Chloroform extract

The chloroform extract had an LD₅₀ of more than 100 µg/ml. This is an indicator that it may not contain cytotoxic compounds. It is probably safe for use in mice.

3.4.2 Acute toxicity in mice

No death was reported in all five groups even after 24 hours of observation. The animals became calm after 5 minutes of administration of the extract but recovered with lapse of time. The animals were kept for three more days to enable the detection of delayed toxicity. The extract did not cause death in doses as high as 4 g/kg of bwt. This indicated that it is safe for use in rodents.

CHAPTER 4

4.0 CONCLUSION AND RECOMMENDATIONS.

The ethanolic extract of *Vitex doniana* was obtained by cold maceration of stem bark using 85 % ethanol. Different fractions were obtained by sequential maceration. The crude extract and different fractions were subjected to anticonvulsant screening using chemoconvulsion models in mice (using 4-aminopyridine and strychnine). Both crude extract and fractions showed activity with ethyl acetate having the highest activity in the 4-aminopyridine chemoconvulsion model. This study appeared to justify the use of *Vitex doniana* in the folkloric treatment of epilepsy.

Two compounds VD1 and VD2 were isolated from the stem bark of this plant. No pharmacological screening was carried out on these compounds. However, literature search shows that flavonoids isolated from *Passiflora incarnata* in Iran have anticonvulsant activity. None of the compounds was conclusively identified. Further work is required to conclusively identify the structures of these compounds.

The tests on potentiation of pentobarbitone sleeping time indicated that the extract has a mild CNS depressant activity. This suggests that *Vitex doniana* is a good antiepileptic since it lacks hypnotic activity.

From these findings it is recommended that more work be done to isolate and screen more compounds from this plant responsible for antiepileptic activity. The new compounds may act as lead compounds for the development of new AEDs for treatment of refractory seizures (in line with folkloric claim- that *Vitex doniana* cures epilepsy). On the other hand, it is known that the Pharmacological action of many herbal remedies is additive and synergistic of the components thereof. It was observed that the crude extract and various fractions had anticonvulsant activity. Further work need to be done in line with folkloric use to standardize and develop this folk medicine for the treatment of epilepsy. The extract was also tested for anti-nociceptive and spasmolytic activity. It showed peripheral analgesic activity in the acetic acid writhing test. The active compound need be isolated and mechanism of action be determined so that it can be developed into an alternative to

ulcerogenic NSAID and CNS acting narcotic agents. The extract showed contractile activity in the isolated Rabbit ileum. This appeared to justify the folkloric use of *Vitex doniana* in the management of postpartum bleeding. This action need be investigate so that active compounds responsible for activity can be isolated and be developed for clinical use.

This study also stimulates research on this plant for bioactive compounds responsible for anticancer, antimalarial and other ethno-medical uses.

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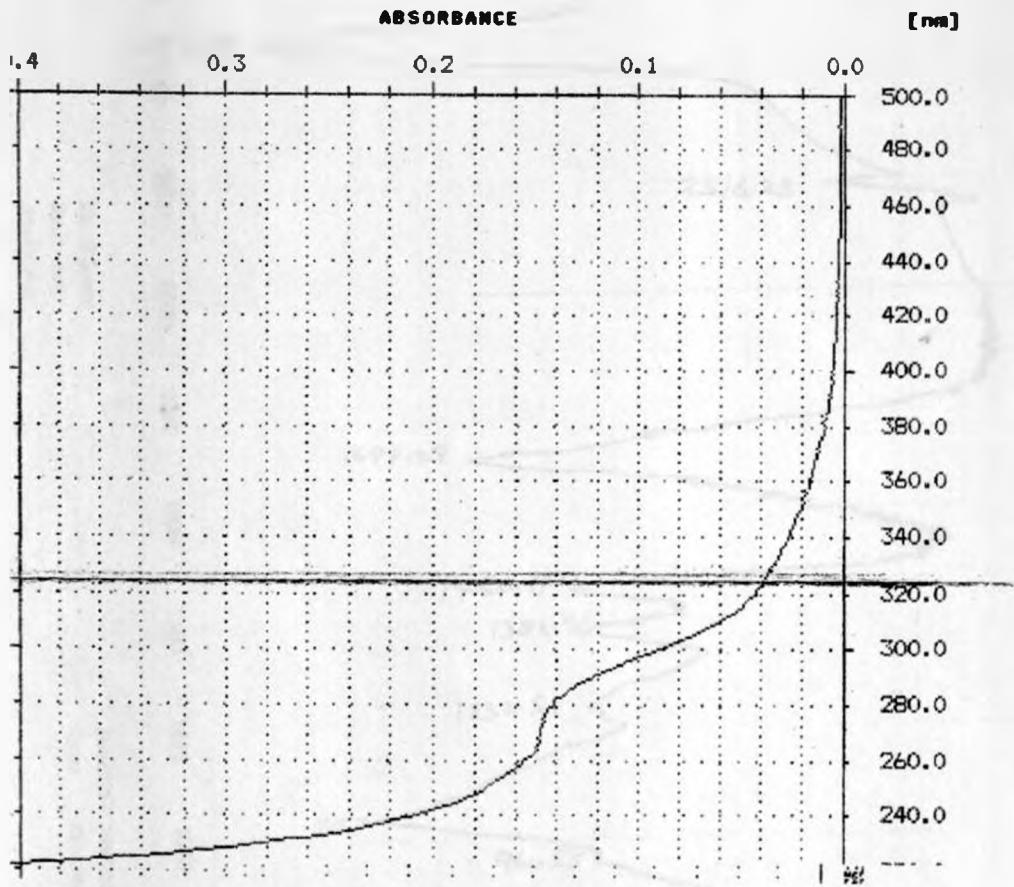
Appendix 1

UV/VIS spectrum of VD -1

PERKIN-ELMER LAMBDA 12/2.0 nm (1.3) UV/VIS SPECTROMETER
 DATE: 10/02/18 THURSDAY TIME: 17:29:24

METHOD NO. 2: SCAN

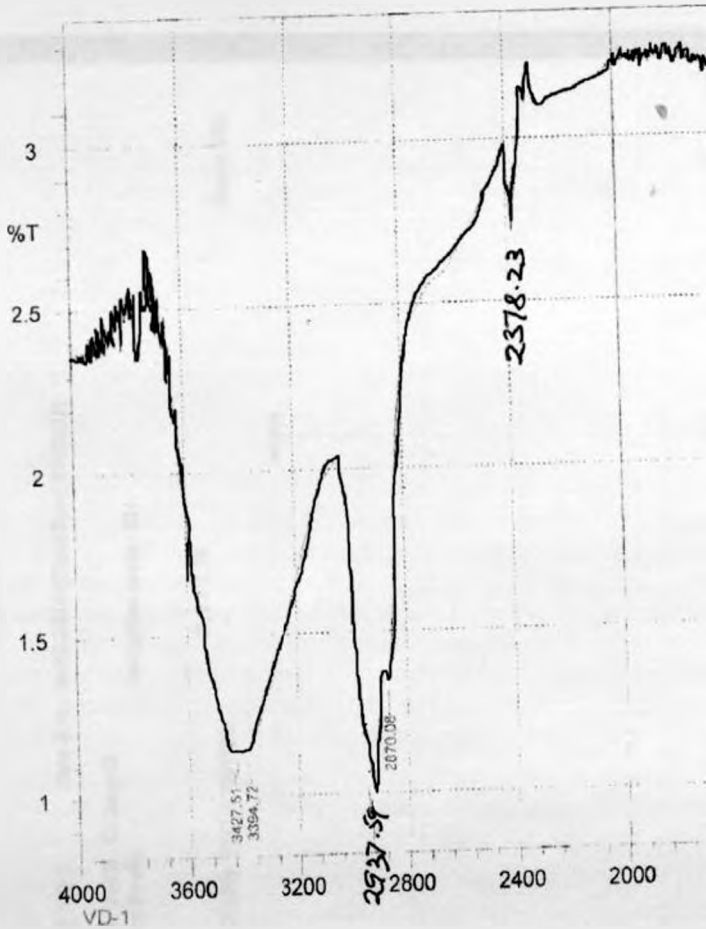
SAMPLE ID: *V.D.-1.* OPERATOR ID:



THRESHOLD : 0.100

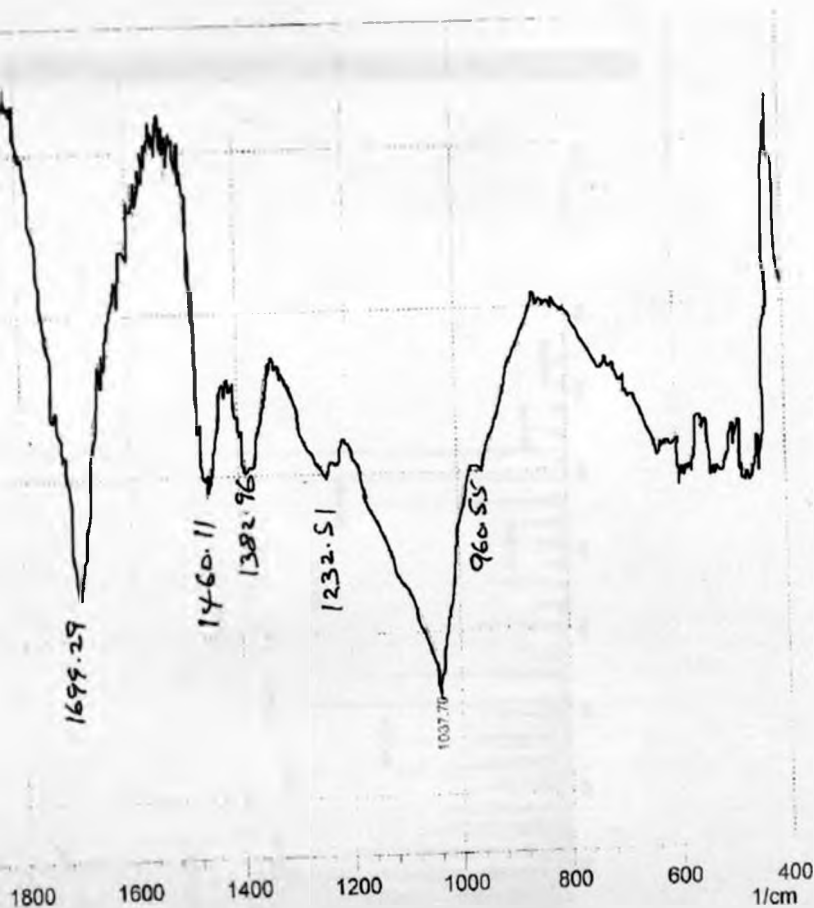
SAMPLE	CYCLE	WAVELENGTH	DATA
001	17:29	288.8 nm (MAX)	1.087 ABS
		288.4 nm (MIN)	0.827 ABS
		287.2 nm (MAX)	6.404 ABS
		286.8 nm (MIN)	6.000 ABS
		286.0 nm (MAX)	6.540 ABS
		284.4 nm (MIN)	-2.323 ABS
		283.2 nm (MAX)	5.148 ABS
		282.4 nm (MIN)	-1.252 ABS
		281.3 nm (MAX)	2.927 ABS

Appendix 2 IR spectrum of VD-1



Comment;
VD-1

No. of Scans;
Resolution;
Apodization;



Date/Time; 2/16/2010 2:21:55 PM
User; The User

Appendix 3 MS of VD-1

Univ Cape Town
4/22/2010

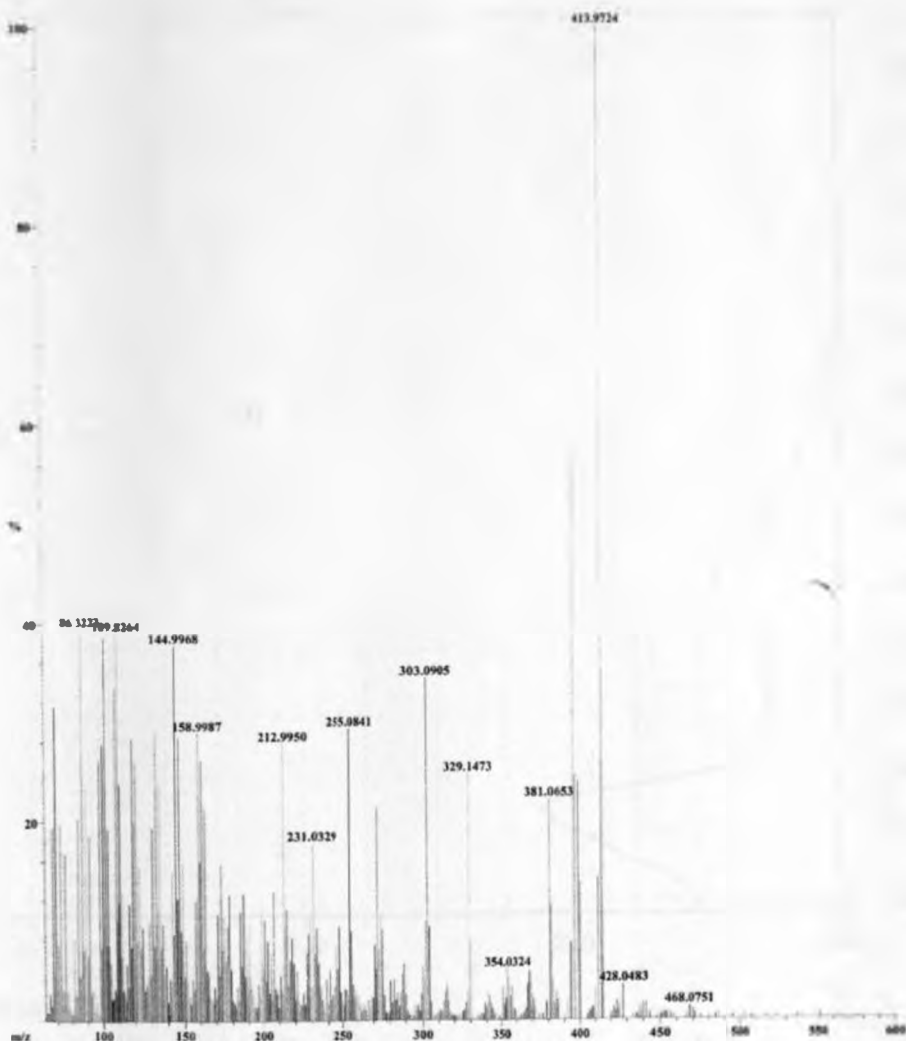
Page 1

File: 10D22-VD 1 Date Run: 04-22-2010 (Time Run: 09:53:33)
Sample:
Instrument: JEOL GCmatell
Inlet: Direct Probe Ionization mode: EI+

Scan: 151
Base: m/z 414; 17%FS TIC: 67267328

R.T.: 2.98

#Ions: 390



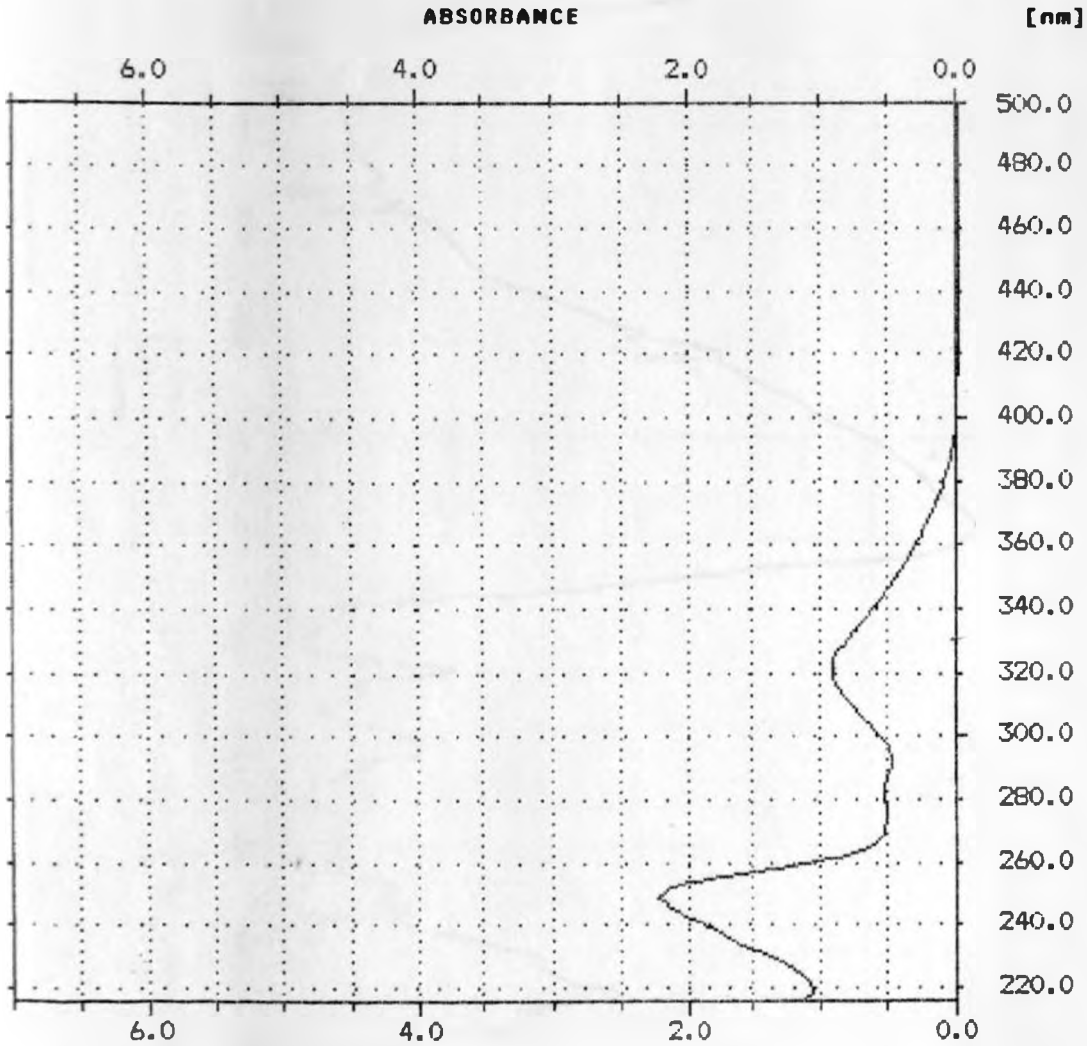
Appendix 4 UV/VIS spectrum of VD-2

PERKIN-ELMER LAMBDA 12/2.0 nm (1.3) UV/VIS SPECTROMETER
 DATE: 10/02/18 THURSDAY TIME: 18:07:32

METHOD NO. 2: SCAN

SAMPLE ID: VD-2... OPERATOR ID:

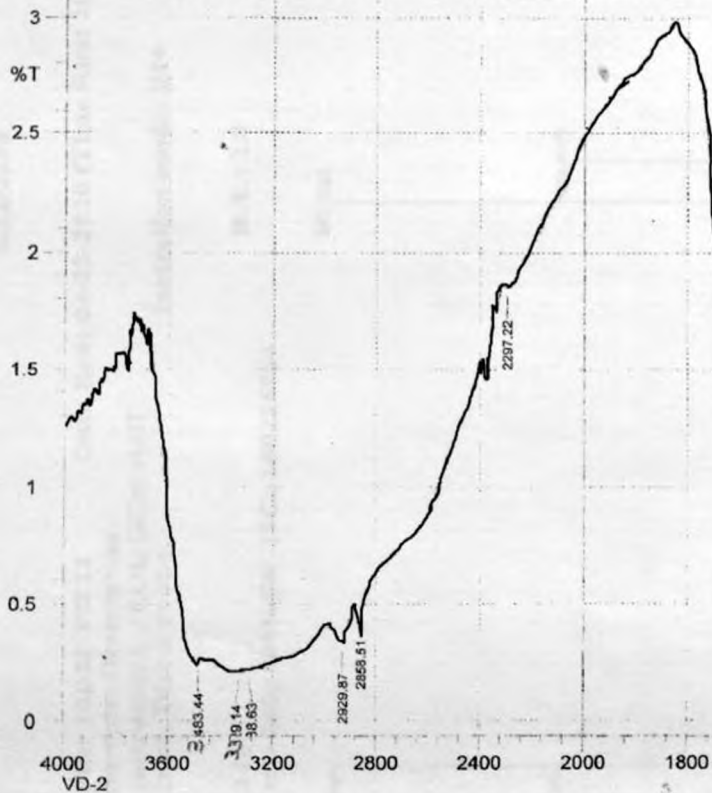
500-220



THRESHOLD : 0.100

SAMPLE	CYCLE	WAVELENGTH	DATA
001	18:08	321.6 nm (MAX)	0.936 ABS
		292.0 nm (MIN)	0.476 ABS
		248.4 nm (MAX)	2.226 ABS
		218.8 nm (MIN)	1.073 ABS

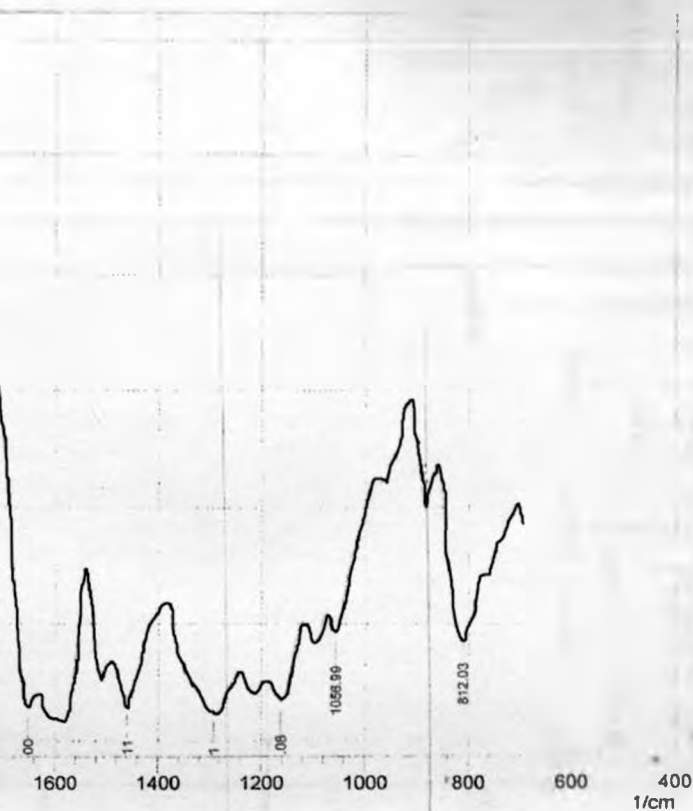
Appendix 5 IR spectrum of VD-2



Comment;
VD-2

No. of Scans;
Resolution;
Apodization;

SHIMADZU



Date/Time: 2/16/2010 2:30:33 PM
User: The User

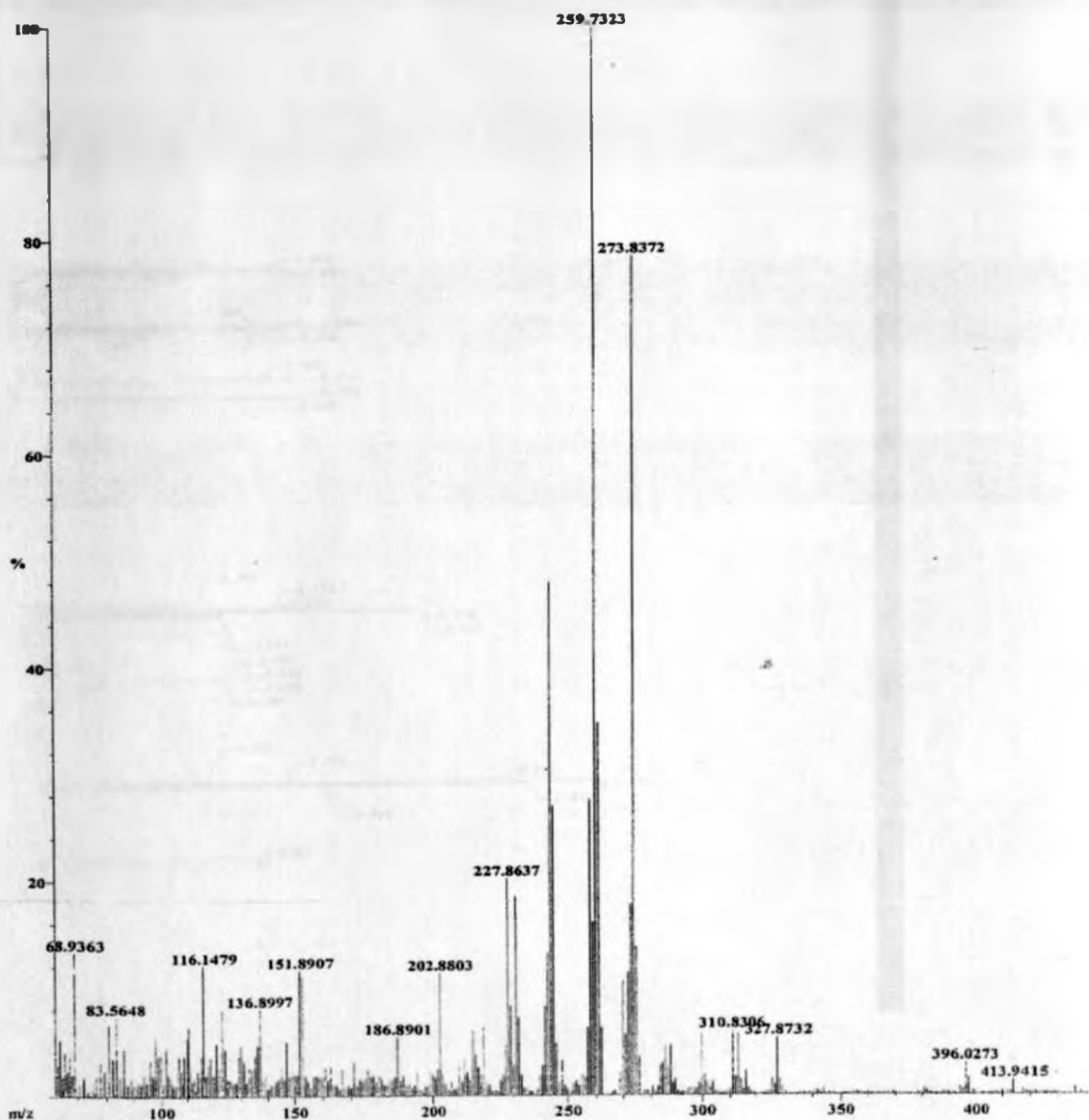
Appendix 6 MS of VD-2

Univ Cape Town
4/22/2010

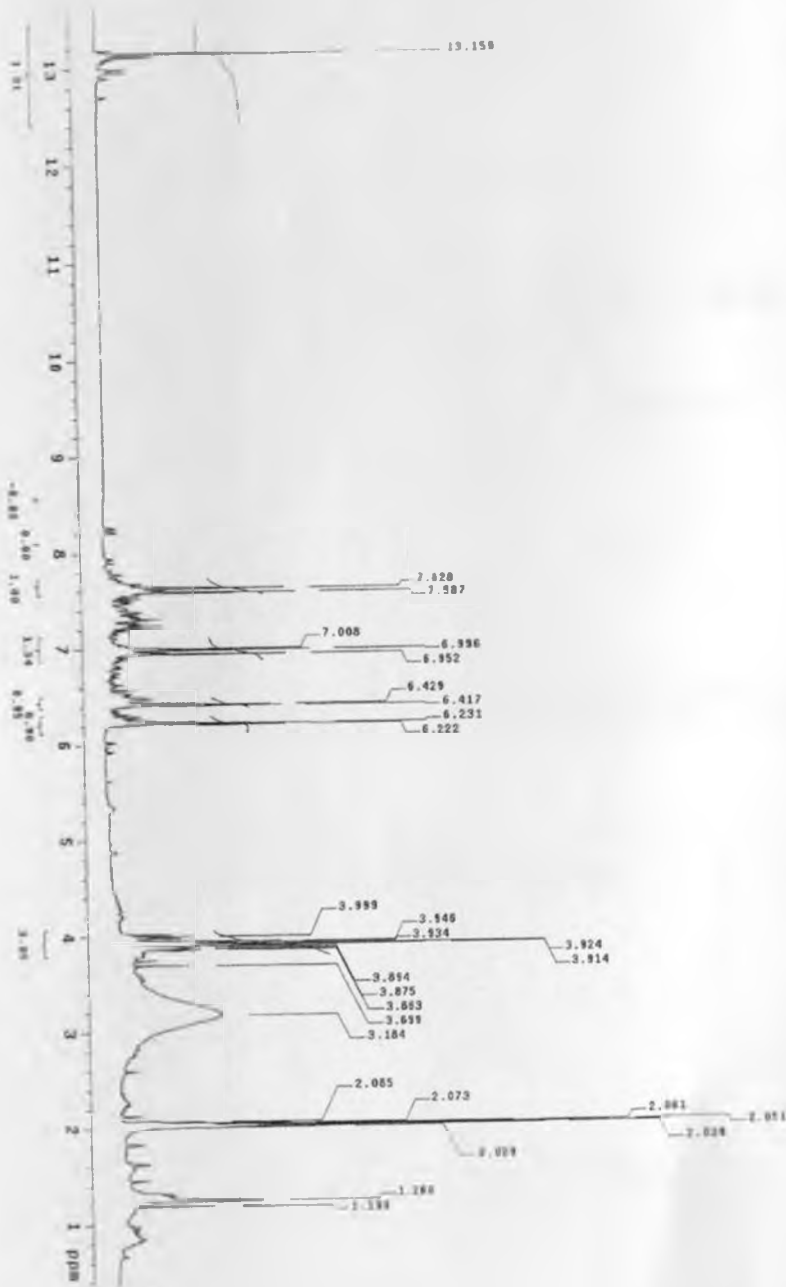
File: 10D22-VD 11 Date Run: 04-22-2010 (Time Run: 10:00:02)
Sample: Description
Instrument: JEOL GCmateII
Inlet: Direct Probe Ionization mode: EI+

Scan: 142
Base: m/z 260; 100%FS TIC: 180216064

R.T.: 2.8

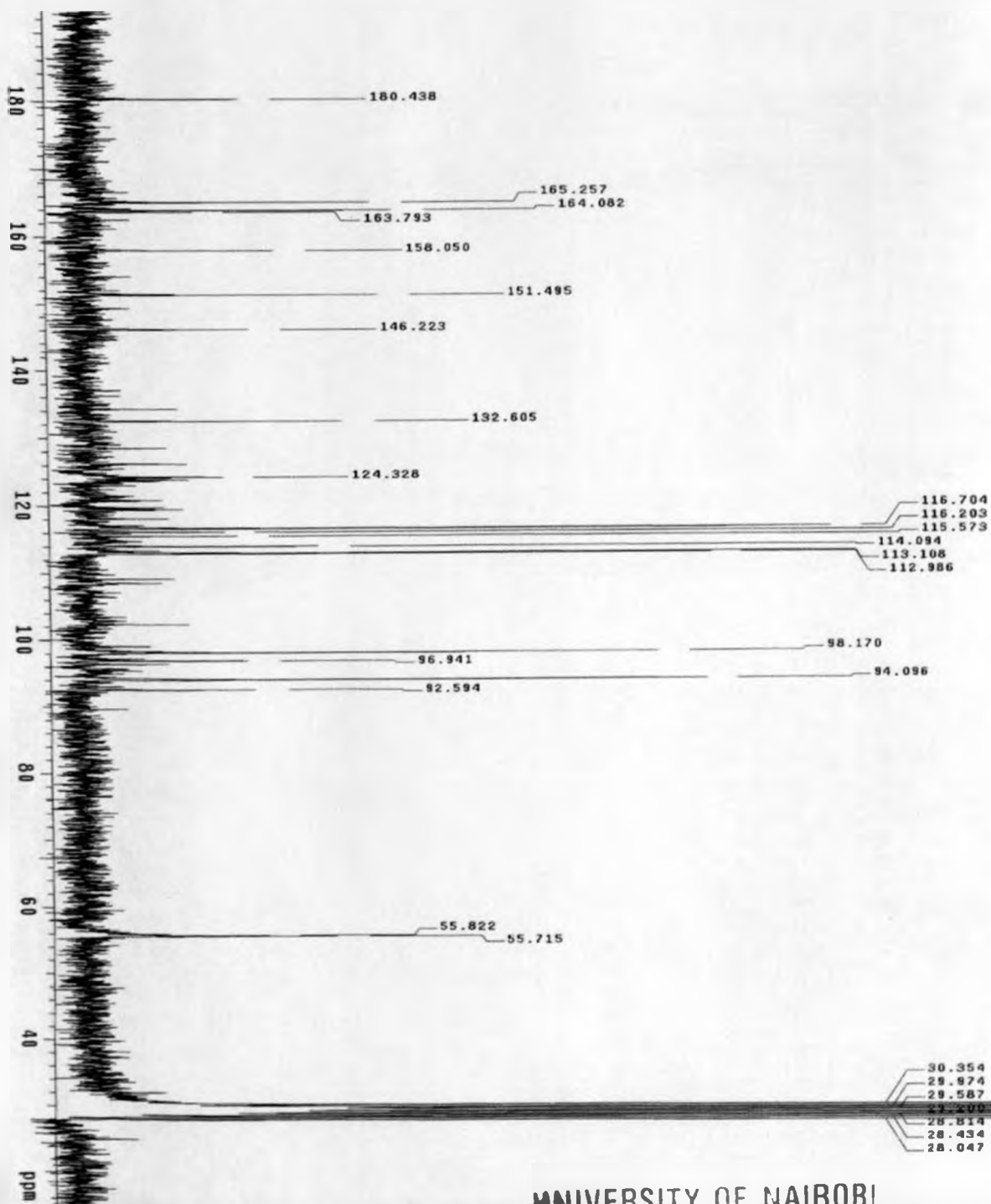


APENDIX 7 proton NMR of VD - 2



111000
Pulsed Spectrometry
VD-11
100 MHz
11-1-78
Pulsed Spectrometry 12001

Appendix 8 Carbon 13 NMR of VD-2



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