PHYTOCHEMICAL AND ANTICONVULSANT STUDIES OF METHANOL LEAF EXTRACT OF *HYMENOCARDIA ACIDA*, TUL (EUPHORBIACEAE)

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BY

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DECLARATION

I declare that the work in this dissertation entitled 'PHYTOCHEMICAL AND ANTICONVULSANT STUDIES OF THE METHANOL LEAFEXTRACT OF *HYMENOCARDIA ACIDA*, Tul(EUPHORBIACEAE)'has been carried out by me in the Department of Pharmaceutical and Medicinal Chemistry. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree or diploma at this or any other Institution.

Haruna, Anas	_____	_____
Name of Student	Signature	Date

CERTIFICATION

This dissertation entitled 'PHYTOCHEMICAL AND ANTICONVULSANT STUDIES OF METHANOL LEAFEXTRACT OF *HYMENOCARDIA ACIDA,* Tul (EUPHORBIACEAE) byAnas HARUNA meets the regulations governing the award of the degree of Master of Science in Pharmaceutical and Medicinal Chemistry of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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ABSTRACT

The research was on the phytochemical and anticonvulsant studies of methanol leafextract of *Hymenocardia acida*, Tul, (Euphorbiaceae) used inNorthern Nigeria for the treatment of headache, rheumatic pain, sickle cell crisis, malaria, epilepsy and cancer. The preliminary phytochemical screening of crude methanol extract (CME) using standard methods revealed the presence of terpenoids, tannins, saponins, alkaloids and flavonoids.Phytochemical evaluations were carried out using silica gel column chromatography, preparative thin layer chromatography and gel filtration using sephadex LH-20. The CME was partitioned successively with n-hexane, chloroform, ethyl acetate and n-butanol to yield different fractions. Extensive phytochemical investigation of n-Hexane soluble fraction of the leaf using silica gel column chromatography, gel filtration and preparative thin layer chromatography led to the isolation of Lupeol. The structure of the isolated compound was elucidated with the help of ^{1}HMR and ^{13}C NMR analysis. The oral median lethal dose (LD_{50})in mice was found to be greater than 5000mg/kg, suggesting the crude extract is practically non-toxic. Anticonvulsant activity was studied using maximum electroshock test (MEST) in chicks and pentylenetetrazole (PTZ) induced seizure model in mice. The CME of *Hymenocardia acida* at doses of 150mg/kg, 300mg/kg and 600mg/kg did not exhibit significant activity against MES convulsion because none of the chicks was protected against the seizure but there was 90% protection with the standard drugPhenytoin at a dose of 20mg/kg while the extract produced a dose independent activity in the PTZ induced seizure in mice which was significant at (p<0.05) which was seen as percentage protection against seizure as 50, 33.33, 16.67% at doses of 150mg/kg, 300mg/kg and 600mg/kg respectively i.e the lower the dose of the extract the higher the protection. The standard control, Sodium Valproate 200mg/kg protected the mice 100% .

The finding of the study suggests that the CME of *Hymenocardia acida* possesses significant anticonvulsant activity which might be due to the phytochemical constituents. This provides some scientific rationale for the ethnomedicinal claim of the use of the plant in the management of epilepsy.

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LIST OF ABBREVIATIONS

1D NMR	One Dimensional Nuclear Magnetic Resonance
^{1}HNMR	Proton Nuclear Magnetic Resonance
2D NMR	Two Dimensional Nuclear Magnetic Resonance
A BU	Ahmadu Bello University
AEDs	Anti-Epileptic Drugs
AF	Aqueous Fraction
ANOVA	Analysis of Variance
BF	n-Butanol Fraction
CAT	Catalase
CH_3OD	Deuterated Methanol
CF	Chloroform Fraction
CME	Crude Methanol Extract
CNS	Central Nervous System
DEPT	Distortionless Enhancement by Polarization Transfer
EAF	Ethyl Acetate Fraction
ECF	Ethyl acetate Chloroform Fractions
GABA	Gama Amino Butyric Acid
HF	Hexane Fraction
HTLE	Hind Limb Tonic Extension

LD_{50}	Median Lethal Dose
MEHA	Methanol leaf Extract of *Hymenocardia acida*
MES	Maximal electro shock
MEST	Maximal electro shock test
NMR	Nuclear Magnetic Resonance
NNMDA	*Nigeria Natural Medicine Development Agency*
p.o	per oral
PH	Phenytoin
PTLC	Preparative Thin Layer Chromatography
PTZ	Pentylenetetrazole
R_f	Retardation Factor
SE	Status Epilepticus
SEM	Standard Error of Mean
SOD	Superoxide Dismutase
SV	Sodium Valproate
TLC	Thin Layer Chromatography
TMP	Traditional Medical Practitioner
UV	Ultraviolet
WHO	World Health Organization

CHAPTER ONE

1.0 INTRODUCTION

1.1 Natural Product

Natural Productscan be defined as organic compounds and other chemicals synthesized by plants through metabolic processes aided by sunlight, involving CO_2, H_2O vapour and chlorophyll. Generally, natural products are characterized by specific functions they perform in plants and animals. Classical natural product chemistry methodologies enabled the discovery of a vast array of bioactive secondary metabolites from various source materials including terrestrial plants, terrestrial micro-organisms, marine organisms, and terrestrial vertebrates and invertebrates (Tyler *et al.,* 1988). Natural products have been used since ancient times and in folklore for the treatment and prevention of many diseases and illnesses. They are the most successful source of potential drugleads (Haefner, 2003; Butler, 2004; Cragg andNewman,2005; Mishra andTiwari, 2011;Rey-ladino *et al.*, 2011).

Therefore, natural product continue to provide unique structural diversity in comparison to standard combinatorial chemistry, and presents opportunities for discovering mainly novel low molecular weight lead compounds. Since less than 10% of the world's biodiversity has been evaluated for potential biological activity, many more useful natural lead compounds await discovery with the challenge being how to access this natural chemical diversity (Cragg andNewman,2005).

The earliest records of natural products were depicted on clay tablets in cuneiform from Mesopotamia (2600 B.C.) which documented oils from *Cupressus sempervirens* (Cypress) and *Commiphora* species (myrrh) which are still used today to treat coughs, colds and inflammation (Cragg andNewman,2005). The Ebers Papyrus (2900 B.C.) is an Egyptian pharmaceutical record, which documents over 700 plant-based drugs ranging from gargles,

pills, infusions to ointments (Cragg andNewman,2005). The Chinese Materia Medica written sometimes around 1100 B.C. “Wu Shi Er Bing Fang” contains 52 prescriptions, Shennong Herbal (~100 B.C.) contain 365 drugs and the Tang Herbal (659 A.D) gave the records of 850 drugswhich are documented records of the uses of natural products (Cragg andNewman,2005). The Greek physician, Dioscorides, recorded the collection, storage and the uses of medicinal herbs, whilst the Greek philosopher and natural scientist, Theophrastus (~300 B.C.) dealt with medicinal herbs (Cragg andNewman,2005).

During the Dark and Middle Ages the monasteries in England, Ireland, France and Germany preserved this Western knowledge whilst the Arabs preserved the Greco-Roman knowledge and expanded the uses of their own resources, together with Chinese and Indian herbs unfamiliar to the Greco-Roman world (Cragg andNewman,2005).

It was the Arabs who were the first to privately own pharmacies in the 8th century with Avicenna, a Persian pharmacist, physician, philosopher and poet, contributing much to the sciences of pharmacy and medicine through works such as the *CanonMedicinae* (Cragg andNewman,2005).

1.2 Traditional Medicine

Traditional medicine refers to health practices, knowledge and beliefs incorporating plants, animals and mineral based medicines, spiritual therapies, manual techniques and exercises applied singularly or in combination to treat, diagnose and prevent illnesses or maintain well-being (WHO, 2005; NNMDA, 2008).The world Health organization estimated that 80 percent of people worldwide still rely on plant-based traditional medicines for some aspect of their primary health care (Farnsworth and Soejarto, 1985).

Medicinal plants are plants containing substances which can be used for medication or as precursor of drug synthesis (Sofowora, 1982). Medicinal plants can be referred to as: ‘all

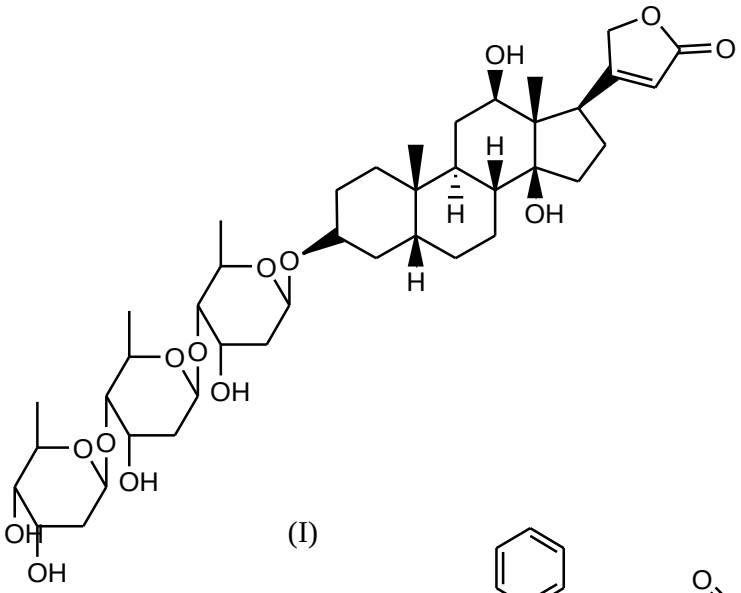
higher plants that have been alleged to have medicinal properties, i.e. effects that relate to health, or which have been proven to be useful as drugs by western standards, or which contain constituents that are used as drugs' (Farnsworth and Soejarto, 1991). The term 'medicinal' as applied to a plant indicates that it contains a substance or substances which modulate beneficially the physiology of sick mammals, and that it has been used by man for that purpose (Fellows, 1991). Medicinal plants have been a source of medicine to human health since ancient time, whereas about 60-75% of world populations require plant for carrying health (Farnsworth, 1994; Joy *et al*., 1998; Harvey, 2000). Plants and microbes are the main source of natural products (Hayashi *et al*., 1997; Armaka *et al.*, 1999; Lin *et al.*,1999a ;Lin *et al.*,1999b: Basso *et al*., 2005), and consistently become main source of the newest drugs (Harvey 2000). Many methods of investigation or drug development from natural sources are based on the bioassay-guided isolation of natural products on traditional uses of local plants (Ataur Rahman and Choudhary 1999). Ayurveda is the most ancient health caresystem and is practiced widely in India,Srilanka and other countries (Chopra and Doiphode, 2002). Atharvveda (around 1200 BC), Charak Samhita and Sushrut Samhita (100 - 500 BC) are the main classics that give detailed descriptions of over 700 herbs (Dash *et al.*, 2001). In the western world, documentation of use of natural substances for medicinal purposes can be found as far back as 78 A.D., when Dioscorides wrote "De Materia Medica", describing thousands of medicinal plants (Tyler *et al.*, 1988).

This treatise included descriptions of many medicinal plants that remain important in modern medicine, not because they continue to be used as crude drug preparations, but because they serve as the source of important pure chemicals that have become mainstays of modern therapy.

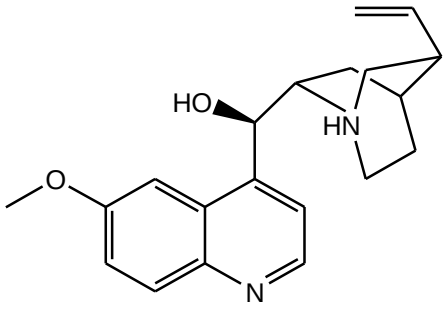
The knowledge associated with traditional medicine (complementary or alternative herbal products) has promoted further investigations of medicinal plants as potential medicines and

has led to the isolation of many natural products that have become well known pharmaceuticals. Many modern pharmaceuticals have been modeled on or derived from chemicals found in plants. An example is the heart medication digoxin (I) derived from foxglove (*Digtialis purpurea*), quinine (II), an antimalarial agent isolated from the bark of *Cinchona succirubra* tree. Paclitaxel (III) (Taxol®), a drug used for breast cancer was isolated from the bark of *Taxus brevifolia* (Pacific Yew) (Cragg, 1998). Taxol® is present in limited quantities from natural sources, its synthesis (though challenging and expensive) has been achieved (Cragg, 1998). It is widely known in ethnomedicine that various parts of a plant can possess different healing properties, for instance, the bark of *Rauwolfiamombasiana* is used for the treatment of malaria. The root of the same plant is used for the treatment of fever and anxiety states.The root, stem and leaves of another species of this plant, *R. vomitoria*, are used for fever (Iwu, 1993).

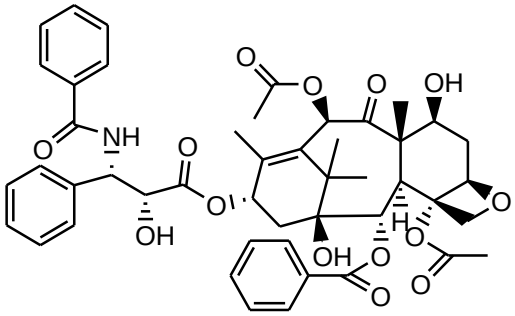
Morphine (IV) used for pain relief was derived from *Papaver somniferum*;a potent antimalarial drug named Artemisinin (V) was isolated from *Artemisia annua* as a remedy against the multidrug resistant strains of *Plasmodium*.



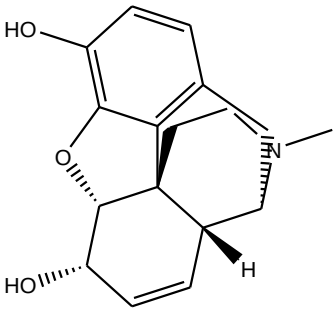
(I)



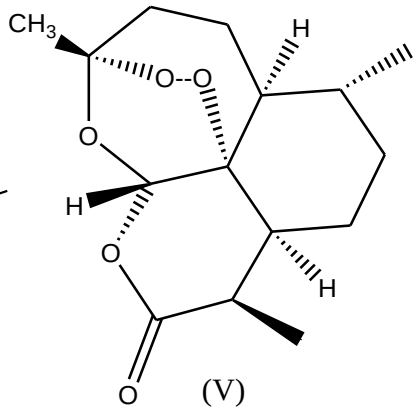
(II)



III



(IV)



(V)

Use of herbs as alternate medicine in developed countries has expanded sharply in the latter half of the twentieth century. Monographs on selected herbs are available from a number of sources, including the German Commission E (Blumenthal *et al.*, 1998), European Scientific Cooperative on Phytotherapy (ESCOP, 1999) and the World Health Organization (WHO, 1999).

The WHO monographs, for example, describes the herbs (including synonyms and vernacular names) and the herb part commonly used, its geographical distribution, tests used to identify and characterize the herb, the active principles (when known), dosage forms and dosing, medicinal uses, pharmacology, contra-indications and adverse reactions, which is found in every society irrespective of its level of development and sophistication (Odugbemi, 2006).

1.2.1 Medicinal plants and epilepsy

Plants have a long history in the management of epilepsy. A typical example of which is *Valariana officinalis* used as a herbal treatment of epilepsy in Europe and America (Murray, 1998). A number of African medicinal plants have been reported to possess bioactive constituents capable of exerting anticonvulsant action hence making them relevant in the management of seizure disorders. These plants include; *Glycyrrhyza glabra* (Ambawade *et al*., 2002), *Dalbergia saxatilis* (Yemitan and Adeyemi, 2006), *Cassia occidentalis, Heliotropium indicum* and *Xylopia aethiopica* (Mann *et al.*, 2003).

1.3 Epilepsy

Epilepsy, also known as the 'falling sickness', not only has a much older history than any of the other individual nervous or mental disorders, but it has also occupied people's minds to a much larger extent than the majority of ailments to which the genus *Homo sapiens* is susceptible to (Kinnier-Wilson and Reynolds, 1990).

Epilepsy is thought to be a common and diverse set of chronic neurological disorders characterized by seizures, as such there are various definitions of epilepsy but most definitions require that the seizure be recurrent and unprovoked (Chang and Lowenstein, 2003), while others require only a single seizure combined with brain alterations which increases the chances of future seizures (Fisher *et al.*, 2005). The term epilepsy has also been

defined as a disorder of brain function characterized by periodic and unpredictable occurrences of seizures.Brain dysfunctions, whether primary or secondary to malfunction of other systems, are a major concern of human society, and a field in which pharmacological intervention plays a key role (Rang *et al.*, 2003).

Epilepsy is estimated to affect about 50 million people worldwide and is the second most common neurological disorder after stroke (Harvey and Champe, 2006). Close to 80 % cases of epilepsy are found in developing countries (WHO, 2012).

1.3.1 Etiology

The causes of epilepsy are summarized in three general etiological groups:

The first one is the threshold, which determines the susceptibility of individual brains to generate seizures in response to epileptogenic perturbations. This will determine what is called "primary" or "idiopathic"epilepsy, when it is not the result of some other brain abnormality. They are usually benign and often remit spontaneously or after uninterrupted pharmacological treatment with Antiepileptic drugs(AEDs). The duration between onset and remission can vary from 2 to 12 years (ILAE, 1994; Engel and Pedley, 1997).

The second group is related to a specific epileptogenic abnormality, which could be an acquired lesion of the brain, congenital malformations of the brain or genetic disorders other than epilepsy. This"secondary" or "symptomatic" epilepsy is very common in developing countries, where it is responsible for the difference in terms of prevalence and prognosis. Risk factors are dominated by poor perinatal care, head trauma, and intracranial infections, including parasitic infestations (such as neurocysticercosis, neuromalaria), and these are far more common than in industrialized countries. Their control requires, in addition to AEDs, specific care of the aetiology which can either be medical and/or neurosurgical (ILAE, 1994; Engel and Pedley, 1997).

The third group represented by epileptic disorders that are probably symptomatic, but the causes have not been identified with existing diagnostic means, and are therefore called CRYPTOGENIC (which means hidden cause) with a high suspicion of a genetic (but no identifiable) factor (ILAE, 1994; Engel and Pedley, 1997).

1.3.2 Pathophysiology

Despite extensive researches that led recent breakthroughs in the understanding of the mechanisms involved in the pathophysiology of epilepsy, the specific causes of several types of epilepsy are still unknown (Engelborgs *et al*., 2000).

According to Ditcher and Brodie (1996), the hypersynchronous discharges during a seizure may begin in a very discrete region of cortex and then spread to neighbouring regions. Seizures initiation is characterized by two concurrent events: high frequency bursts of action potentials and hypersynchronization of a neuronal population.

1.3.2 Mechanisms of Ictogenesis and Epileptogenesis

Ictogenesis is a transient and direct event that induces seizures due to excessive discharges from groups of neurons. Such discharges are initiated by the sequential opening of the voltage-dependent Na^+ channels due to membrane depolarization resulting from K^+ and/or Ca^{2+} channel-mediated events or via neurotransmitters and/or activation of ionic glutamate receptors. On the other hand, epileptogenesis involves long-lasting and prolonged histological/biochemical alterations of neuron network and reorganization of neuronal matrices, with the process ranging from months to years (Sasa, 2006).

According to Sasa (2006), drugs currently used in the management of epilepsy (antiepileptic drugs) are classified as drugs against ictogenesis (anti-seizure) which is different from the concept of epileptogenesis because they are unable to stop the progression of epilepsy.

1.3.4 Classification of Epilepsy

Epilepsies have been classified as described below (Tripathi, 2008).

1.3.4.1Generalized seizures

Generalized tonic-clonic seizures (GTCS), major epilepsy, grand mal: This is the commonest and lasts for 1-2 minutes. The usual sequence is aura-cry-unconsciousness-tonic spasm of all body muscles-clonic jerking followed by prolonged sleep and depression of all CNS functions.

Absence seizures (minor epilepsy, petit mal): prevalent in children, lasts about 60 seconds. Momentary loss of consciousness, patient apparently freezes and stares in one direction, no muscular or little bilateral jerking.

Atonic seizures (Akanitic epilepsy): Unconsciousness with relaxation of all muscles due to excessive inhibitory discharges. Patient may fall.

Myoclonic seizures: Shock-like momentary contraction of muscles of a limb or the whole body.

Infantile spasms (Hypsarrhythmia): seen in infants, probably not a form of epilepsy. Intermittent muscle spasm and progressive mental deterioration.

1.3.4.2Partial seizures

Simple partial seizures (SPS, cortical focal epilepsy)

Complex partial seizures (CPS, temporal lobe epilepsy, psychomotor).

Simple partial or complex partial seizures secondarily generalized

1.3.5 Status Epilepticus

Status epilepticus (SE) is a life threatening emergency characterized by a prolonged continuous state of convulsions. It is defined as a continuous seizure activity or multiple

seizures without regaining consciousness for more than 30 min (Delgado-Escueta *et al*., 1983). If untreated can lead to brain damage and death. It can either be generalized convulsive SE or non-convulsive SE (Delorenzo *et al*., 1992). The pathophysiology of SE is not clearly understood but excess excitatory (glutamate) neurotransmission and loss of normal inhibitory (GABA) neurotransmission are thought to be the most likely mechanisms. The first-line therapies of choice are intravenous benzodiazepines (e.g. diazepam and lorazepam), which potentiate the inhibitory responses mediated by GABA-A receptors (Brophy *et al*., 2012).

1.3.6 Anticonvulsant Studies

1.3.6.1 Animal Models for Anticonvulsant Studies

The use of animal seizure models is essential in the discovery and development of new drugs for the treatment of epileptic seizures. These models can be either *in vivo* or *in vitro*. Discovery of a new therapeutic agent begins with the hypothesis that there is a relationship between the experimental seizure model and the initiation and propagation of the seizure and that the experimental seizure approximates the pathophysiology underlying the human condition. Phenytoin, carbamazepine and valproate were identified by relatively simple screening procedures that do not provide insight into a drug's mechanisms of action. Two concepts were assumed: either seizure spread or seizure threshold was affected. When animal models were developed that used electrical stimulation or chemoconvulsants, they were systematically validated using the then-known clinically effective compounds.

The pharmacologic activity of the potential anticonvulsants was then profiled to predict their utility in the various types of human epilepsy. The therapeutic activity as well as the toxicity of these new agents was then demonstrated in various animal models and species (Harvey, 2000).

The anticonvulsive pharmacology of novel test substances can be characterized using variations of two basic test methods of animals: blockade of electroshock-induced convulsive seizures and the blockade of chemically-induced convulsive seizures. Variations of the two basic methods have led to the classification of the experimental models into acute and chronic seizure models (Mody and Schwartzkroin, 1997).

1.3.6.2 Acute seizure models

Acute seizure approaches to the understanding of seizure-like activity which has been extremely important in the history of epileptic investigation, because they represent the first best method to explore basic mechanisms. In addition, some of these models have become the standards against which antiepileptic drugs are evaluated for efficacy (Mody and Schwartzkroin, 1997).For this study, one electrical (MEST) and one chemical method of acute seizure induction was employed i.e the PTZ-induced seizures.

The maximal electroshock test (MEST), developed by Toman and collaborators in 1946 and modified by Swinyard and Kupferberg (1985) and Browning (1992), is probably the best validated pre-clinical test that predicts drugs effective against generalized seizures of the tonic-clonic (grand mal) type (Loscher and Schmidt 1988; White, 2003; Mares and Kubova, 2006). It allows evaluation of the ability of an agent to prevent seizure spread through neural tissue of the CNS (Swinyard and Kupferberg, 1985). The MES test is simple and can be conducted easily with a minimal investment in equipment and technical expertise and is well standardized (Mares and Kubova, 2006).

Several standard and newly developed drugs are effective in MES test, hence making it possible to quantify their anticonvulsant potency after both single and combined application (White, 2003; Borowich, 2007).Agents that act on sodium channels e.g. carbamazepine, phenytoin, oxcarbazepine and lamotrigine are known to suppress hind limb tonic extension induced by maximal electroshock (Rho and Sankar, 1999).

Pentylenetetrazole (PTZ) is a chemoconvulsant agent that induces seizures in experimental animals by the non-competitive inhibition of Gamma Amino Butyric Acid (GABA) receptors and is a widely accepted experimental model for absence seizure (Loscher *et al*., 1991).

PTZ, a tetrazole derivative is the prototype agent in the class of systemic convulsants that when administered parenterally has consistent convulsant actions in mice, rats, cats and primates (DeDeyn *et al.,* 1992). PTZ was introduced as a screening test for anticonvulsant in part because the antiabsence seizure drug ethosuximide, which is effective against PTZ induced seizures, fails to alter MES thresholds. In contrast, some drugs effective against MES induced seizures such as phenytoin and carbamazepine are ineffective against PTZ induced seizures (DeDeyn *et al.,* 1992).

1.4 Antiepileptic Drugs (AEDs)

The term antiepileptic is used synonymously with anticonvulsantto describe drugs that are used to treat epilepsy (which does not necessarily cause convulsions) as well as non-epileptic convulsive disorders (Rang *et al.,* 2003). Management of epilepsy usually requires the use of antiepileptic drugs, however, in cases of refractory epilepsy, non-pharmacological methods can be employed and they include surgery, ketogenic diet and implantation of medical devices e.g. Vagus nerve stimulation (VNS).

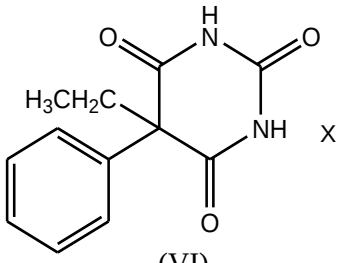
1.4.1 Mechanisms of Action of AEDs

AEDs protect against seizures through interactions with a variety of cellular targets. The actions on these targets are often categorized into three major classes: (Rang*et al.,*2007).

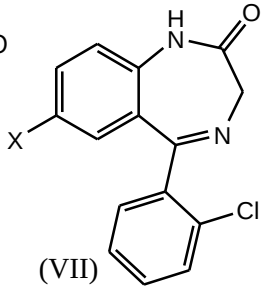
a) *Enhancement of GABA action* e.g. Phenobarbital (VI), Diazepam (VII).

b) *Inhibition of sodium channel function* e.g. Valproate (VIII), Phenytoin (IX).

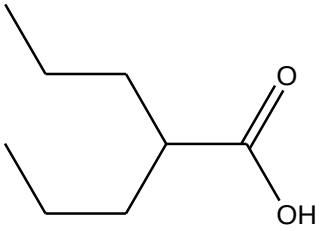
c) *Inhibition of calcium channel function* e.g. Ethosuximide (X), Gabapentine (XI).



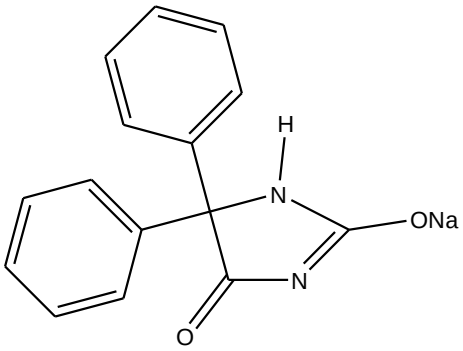
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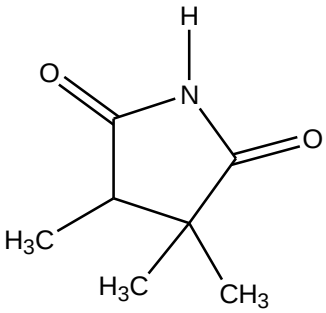
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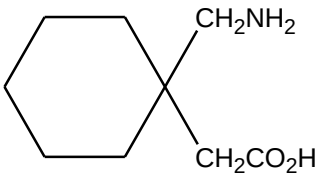
(VIII)



(IX)



(X)



(XI)

1.5 Statement of Research Problem

Epilepsy is one of the oldest conditions known to mankind and still the most common neurological condition affecting individuals of all ages. At any given time, it is estimated that 50 million individuals worldwide are diagnose of epilepsy (WHO, 2001).

Epilepsy is a major neurological disorder that accounts for 0.5 % of global burden of disease with close to 80 % of the cases worldwide found in developing countries (WHO, 2012). World Health Organisation estimates that the proportion of the general population with active epilepsy (i.e. continuing seizures or the need for treatment) at a given time is 4 to 10 per 1000 people. However, some studies in developing countries suggest that the proportion is 6 to 10 per 1000 (WHO, 2012). In developed countries, annual new cases are 40 to 70 per 100,000 people in the general population. In developing countries, this figure is often close to twice as high due to high risk of experiencing conditions that can lead to permanent brain damage (WHO, 2012).

The major causes of epilepsy include meningitis, tumours and traumas especially due to automobile traffic accidents. Statistics has shown that Nigeria and some East African countries have the highest automobile accident cases in the world with attendant increase in post-traumatic epilepsy (Ogunrin, 2006).

As with many other neurological disorders, epilepsy is usually managed not cured with AEDs. These pharmacological agents inhibit seizures and thus are also referred to as antiseizure drugs. Whether these drugs prevent the development of epilepsy (epileptogenesis) is uncertain (Cascino, 1994).

Approximately, 20 to 30 % of patients are refractory to therapies using currently available AEDs (Sasa, 2006) and 88 % of such patients suffer severe side effects like renal failure, due to long term management of the condition with such drugs (Baker *et al.,* 1997).

According to Meldrum (1997), plant extracts can be an important source of natural and safer drugs for the treatment of epilepsy. Medicinal plant extracts, fractions and pure compounds have been used traditionally for the treatment of epilepsy and have demonstrated anticonvulsant properties that need to be further investigated (Raza *et al.,* 2001; Kumar *et*

al.,2012). The study was therefore designed to investigate the activity of the plant extract and to contribute to drug development.

1.6 Justification for the Study

AEDs are the mainstay in management of epilepsy and may have great impact on the quality of life of epileptic patients. Despite the continued development and release of new antiepileptic drugs, many patients have seizures that do not respond to drug therapy or have related side effects that preclude continuous use (Perucca *et al*., 2007). Even in patients in whom pharmacotherapy is efficacious, current AEDs do not affect the progression of epilepsy (Loscher and Schmidt, 2006). These factors and more therefore, call for development and search for new AEDs especially from medicinal plant sources which may have fewer side effects and greater efficacy.

There are many medicinal plants employed locally in the management of epilepsy but with limited scientific evidences for their safety and effectiveness (WHO, 2008). This necessitates the need to scientifically evaluate the anticonvulsant profile of *Hymenocardia acida* in order to validate its folkloric use around Shika, Kaduna state of Nigeria.

1.7 Aim and Objectives of the Study

1.7.1 Aim: To isolate and characterize some ofthe bioactive compound(s) of the plant and to validate scientifically, the ethnomedicinal claim for the use of the plant in the management of epilepsy.

1.7.2 Specific Objectives

1. To identify the phytochemical constituents present in *Hymenocardia acida* leaf.
2. To isolate and characterize some of the bioactive compound(s).
3. To determine the median lethal dose (LD_{50}) of the crude methanol extract.
4. To determine the anticonvulsantactivity of the crude methanol extract.

1.8 Statement of Research Hypothesis

The methanol leaf extract of *Hymenocardia acida* contains bioactive constituent(s) with anticonvulsant activity.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 The Plant, *Hymenocardia acida,* Tul

2.1.1 Botanic Description

Hymenocardia acida is a small savannah tree or shrub about 9 m high. Branchlets become rusty brown as the bark peels. The bole is short, often flattened and usually crooked. The branches form a fairly heavy, rounded crown. Bark smooth or flaky, pinkish-brown when fresh but becoming pale brown or grey later. Leaves are thin, leathery, elliptic-oblong up to 8.75 cm long and 3.75 cm broad, apex obtuse to rounded, base obtuse; petiole slender, up to 1.8 cm long. Leaves usuallypubescent when young with a dense mat of fine hairs and with golden glands beneath (Schmelzer and Gurib-Fakim, 2008).

Flowers unisexual, male flowers reddish-yellow occurring in clusters of spikes up to 6.5 cm long; calyx cupular, red, anthers creamy white. Female flowers green, placed on axils of leafy lateral branches and bearing a prominent crimson stigma spreading about 1.25 cm. Fruit compressed, obcordate and reddish-brown, 2.5 cm long and 2.5-3.75cm broad. Developing in pairs along one edge, each with a thin pale brown nearly square wing. Seed flattened, glossy brown (Keay, 1989; Adjanohoun *et al.,* 1991; Schmelzer and Gurib-Fakim, 2008).

The generic name Hymenocardia is derived from the Greek words ‘hymen’ - membrane and ‘kardia ’- heart, in reference to the heart-shaped fruits which have a transparent covering membrane (hymen). The specific epithet acida describes the sour taste of its fruits. Some authors consider the genus under the family Hymenocardiaceae (Schmelzer and Gurib-Fakim, 2008).

2.1.2 Biology

H. acida is dioecious, male and female flowers ocurring on different trees. In Zambia and Nigeria, flowering starts from September-November and the seeds mature from June-September (Burkill, 1994).

2.1.3 Taxonomy

The genealogy of the plant is as follows;

Kingdom: Plantae

Order: Malpighiales

Family: Phyllanthaceae

Tribe: Hymenocardieae

Genus:*Hymenocardia*

Species:*Hymenocardiaacida*, Tul

2.1.4 Common Names

Hymenocardia acida (Euphorbiaceae) is very popular in African Trado-medicine. It is called "Heart-fruit" in English (Schmelzer and Gurib-Fakim, 2008), “ii-kwarto” in Tiv, "emela" in Etulo, "Uchuo" in Igede (Agishi,2004) "enanche" in Idoma (Abu and Uchendu,2011). It is commonly known as "jan yaro" in Hausa, "yawa satoje" in Fulani, "ikalaga" in Igbo, and "Orunpa” in Yoruba (Ibrahim *et al.*,2007).



Plate I: *Hymenocardia acida* in its natural habitat

2.1.5 Ethnomedicinal uses of *H. acida*

All parts of the plant are useful as remedies for many ailments (Abu *et al.*,2011). Decoction or infusion of leaf and other partsof this plant alone or mixed with other plant species are used for chest complaints, abdominal and menstrual painsand as poultices on abscesses and tumours (Sofidiya *et al.*,2007). Infusion of the leaf is used for small pox and together with the roots for deficiency diseases (Olotu *et al.*,2010). A decoction of the leafy twigs is used for bathing to treat tetanus, convulsion and exhaustion (Schmelzer and Gurib-Fakim, 2008).

The leaf infusion is used in the treatment of urinary tract infections and as topical application for skin diseases in Nigeria (Abu *et al.*,2011). Leaf is also used for the treatment of measles (Adjanohoun *et al.*,1991). Infusion of the leaf is takentwice daily for the treatment of inflammatory diseases (Sofidiya *et al.*,2007).

The leaf, bark and root of*H. acida* are used either in infusion or powdered form to treat hypotension, diabetes, sickle cell, epilepsy, schizophrenia (Ngo *et al.*,2011). It is one of the most common plants used in the management of sickle cell anaemia in Nigeria (Ibrahim *et al.*,2007 ; Ameh *et al.*,2012). Decoction of the leaf and bark is used to treat skin disease and as spice (Ige,2011).

Other reported ethnomedical uses of this plant include antifungal and antimycobacterial; anti-HIV and anti-inflammatory activity (Muanza *et al.*,1995), anti-sickling (Mpiana *et al.*,2009), antiplasmodial (Vothron *et al.*,2003), antimicrobial (Mann *et al.*,2008), anti-ulcer (Ukwe, 2004), in vitro trypanocidal activities (Hoet *et al.*,2004; Abu *et al.*,2009), antidiarrhoeal (Tona *et al.*,1999) and as a douche for female personal hygiene amongst the Idoma people of North Central Nigeria (Ada and Claffey,2003; Ibrahim *et al.*,2007; Abu *et al.*,2011).

It is clear that this plant is an important source of herbal medicine of human and perhaps of veterinary and agricultural importance too. Its ethnomedicinal significance may be as a result of a wide range of secondary metabolites (Mwine and Van Damme,2011) such as alkaloids, terpenoids, glycosides, flavonoids, saponins and tannins (Sofidiya *et al.*,2010; Abu *et al.*,2011).

2.1.6Pharmacological properties of*H. acida*

Antimalarial and anti-inflammatory activities have been reported. Hydroethanolic extract of *H. acida* stem bark in rats exhibited trypanocidal activity. Oral administration of the extract did not significantly affect the packed cell volume. However, the extract reduced the level of parasitaemia and prolonged the life span of infected rats. Also there is a report of the in vivo potential of hydroethanolic extract of *H. acida* in the treatment of African trypanosomiasis (Abu and Uchendu, 2011). In a similar study, methylene chloride, methanol and aqueous extract of the leaf and twigs of *H. acida* also exerted antitrypanosomal activity (Hoet *et al.,* 2004). However, antitrypanosomal activity study carried out by Yusuf *et al.,* (2012) revealed that both methylene chloride and petroleum ether extracts of *H. acida* leaves and root bark does not possess strong in vitro antitrypanosomal activity.

The aqueous and methanol leaf extracts of*H. acida*have been evaluated for their effect on superoxide dismutase (SOD), catalase (CAT) and Ca^{2+} concentrations in rats. The results of this study revealed that pharmacologicalaction credited to *H. acida* was due to effect on enzymatic antioxidants and Ca^{2+} metabolism (Ogbunugafor *et al.,* 2010).

This supports the use of this plant for the treatment of rheumatoid arthritis in traditional medicine practice in Nigeria (Ogbunugafor *et al.,* 2010).

In vivostudies revealed that *H. acida* stem bark is toxic to brine shrimps and caused chromosomal damage in rat lymphocytes; also that it was mutagenic and cytotoxic

(Sowemimo *et al.,* 2007). Aqueous stem bark extract of *H. acida* was also found to exhibit antiulcer activity in rats (Ukwe, 2004).

An antifertility test with ethanolic extract of *H. acida* stem bark suggests that the extract induced inhibitory effects on reproductive function in female albino mice (Abu and Uchendu, 2011). Its aqueous leaves extract demonstrated significant anti-inflammatory and anti-nociceptive activities explaining the use of the plant in folk medicine (Sofidiya *et al.,* 2010). Aqueous extract of this plant had an effect on motility of rat spermatozoa (Abu *et al.,* 2011). Oleic acid which has been isolated in the hexane extract of the stem bark of *H. acida* is believed to have antidiabetic effect (Abu and Uchendu, 2011). Methanol extract from the root bark exhibited moderate cytotoxic activity against 60 human cell lines (Schmelzer and Gurib-Fakim, 2008).

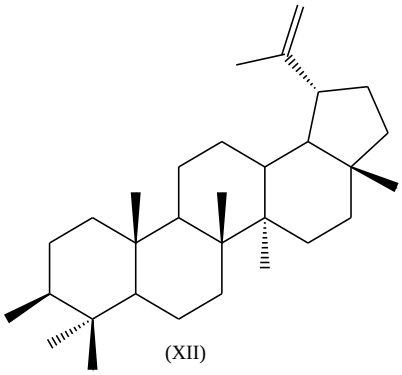
2.2 Phytochemistry of *H.acida*

Previous phytochemical studies of *H. acida* showed the presence of saponins, tannins (Schmelzer and Gurib-Fakim, 2008), flavonoids, flavonols, phenols, proanthocyanidins, steroids and triterpenoids (Ogbunugafor *et al.,*2010).

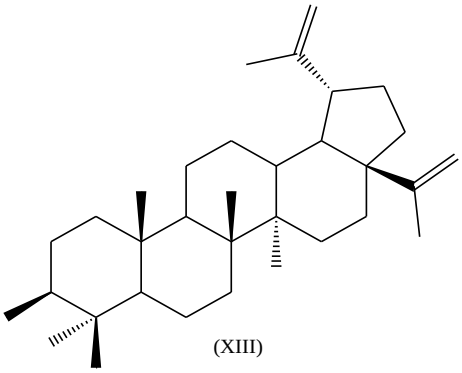
Hydroethanolic extract of *H. acida* stem bark revealed the presence of alkaloids, glycosides, flavonoids, saponins, tannins and terpenoids (Abu *et al.,*2011). Glycosides, saponins and tannins were also detected in the aqueous extract of *H. acida* stem bark (Ukwe, 2004).

To the best of our research, there is no report on isolation and characterization of any compound from the leaf of this plant, but Igoli and Gray (2008) have reported the isolation of five triterpenoids from *H. acida* stem bark; these triterpenoids are lupeol (XII),betulinic acid (XIII),β-sitosterol (XIV),stigmasterol (XV), friedelan-3-one (XVI)and the fatty acid, oleic acid(XVII).

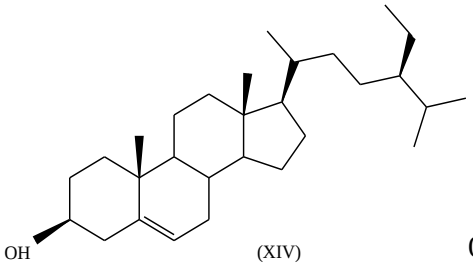
Preliminary studies of the chemistry of*H. acida*revealed the presence of saponins. Similarly, from thestem bark and root bark two cyclopeptide alkaloidshymenocardine (XVIII)and hymenocardinol (XVIII) were isolated respectively. This alkaloid was isolated together with five triterpenoids as mentioned earlier, but in this case no oleic acid (Mpiana *et al.*,2009).



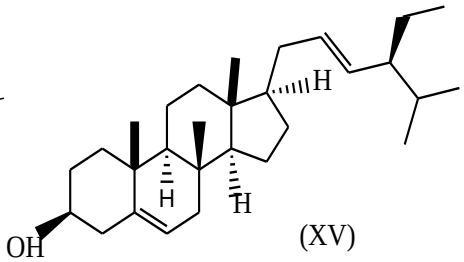
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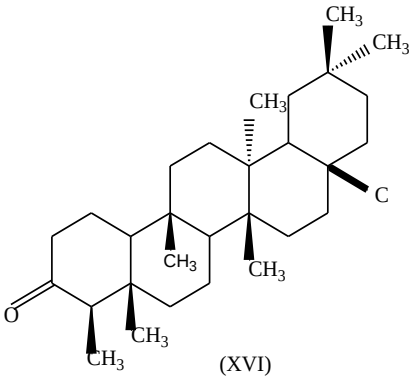
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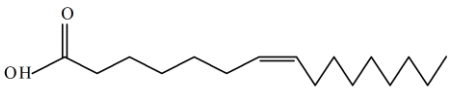
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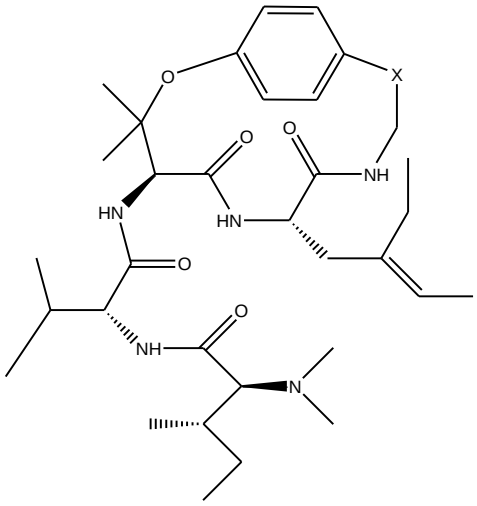
(XV)



(XVI)



(XVII)



(XVIII)

X= C═O ⟶ Hymemocardine

X= CHO ⟶ Hymemocardinol

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Solvents/Reagents and Chromatographic Materials

The solvents used include methanol, n- butanol, ethyl acetate, chloroform, and n-hexane. Reagents used were freshly prepared and those for phytochemical screening such as Molisch's reagent, Salkowski's reagent, Shinoda's reagent, Dragendorf's reagent, Mayer's reagent, Wagner's reagent and Borntrager's reagent. Pentylenetetrazole (Sigma Chemical Co. St. Louis, USA), Chromatographic materials included TLC plates (aluminum), Silica gel 60-120 mesh (Merck KGaA, Darmstadt, Germany), Glass Plates, Sephadex LH 20 (Sigma), Chromatographic tanks and open column (75 by 3.5cm). Standard drugs used includes, Phenytoin sodium (Parker-Davis and Co Ltd. Detroit), Sodium Valproate (Sanofi-aventis UK) and deionized water.

3.1.2 Equipment

Biomate 6 UV machine at the Department of Pharmaceutical and Medicinal Chemistry, Ahmadu Bello University, for UV spectroscopy. Ohaus digital weighing balance (Champ 11 CH15R, Ohaus Corporation, Pinebrook NJ, USA), Metler balance (Model P162 supplied by Gallenhamp), Syringes and needles, Mortar and pestle, Sample bottles, Beakers, Separating funnel, Petri dish and conical flasks. Bruker AVANCE III NMR spectrometer (400MHz) at the School of Pharmacy, University of Technology Malaysia (UTM).

3.1.3 Experimental Animals

Swiss albino mice of either sex weighing between 18-30 g obtained from Animal House of Department of Pharmacology and Therapeutics, Ahmadu Bello University, Zaria were used

for the study. In addition, day old ranger cockerels (28 – 41 g)were obtained from Chi Farms Ltd along Ibadan-Lagos express way, Ibadan, Oyo state. The animals were maintained in a well-ventilated room in cageswith stainless steel wire mesh covers in the laboratory animal house under ambient laboratory conditions of temperature and light. They were fed on standard animal feed and water *ad libitum*.

3.2 Methods

3.2.1 Collection, Identification and Preparation of Plant Material

The plant sample comprising the leaves, stem bark and root bark of the plant *Hymenocardia acida* were collected from Dajin Tohu, Shika, Zaria in Kaduna State of Nigeria in September 2015. The plant sample was authenticated by Mr. Musa Muhammad of the Herbarium Section of the Department of Biological Sciences, Ahmadu Bello University, Zaria, through comparison with herbarium reference voucher specimen (Number 1275). The leaves were removed, shade dried, pulverized maually using mortar and pestle.

3.2.2 Extraction and Partitioning

Six hundred and ninety grammes (690g) of the pulverized plant leaves was macerated with 70% methanol with occasional shaking for 72 hours and the solvent was removed using rotary vacuum evaporator to afford the crude extract (67 g), subsequently referred to as crude methanol extract (CME). The crude extract (62 g) was partitioned with n-hexane, chloroform, ethyl acetate and n-butanol leaving the residue aqueous fraction.

3.2.3 Preliminary Phytochemical Screening

Preliminary phytochemical test was carried out on the CME according to standard procedures as follows:

3.2.3.1 Test for Carbohydrates (Molisch's test)

To a small portion of the CME in a test tube, 3 drops of Molisch's reagent was added followed by concentrated sulphuric acid. The formation of a reddish colored ring at the interface indicates the presence of carbohydrates (Trease and Evans, 1996).

3.2.3.2 Test for Saponins (Frothing test)

To a small portion of the extract in test tube, 10 ml of distilled water was added and then shaken continuously for 30 seconds. The solution was allowed to stand for 5minutes, the formation of a persistent froth indicates the presence of saponins (Trease and Evans, 1996).

3.2.3.3 Test for Flavonoids

I. Shinoda test

The extract was dissolved in 2ml of methanol and pieces of metallic magnesium chips were added followed by few drops of concentrated hydrochloric acid, the formation of a pink, orange or red to purple coloration indicates the presence of flavonoid (Trease and Evans, 1996).

II. Sodium hydroxide test

Two drops of 10% Sodium hydroxide was added to the solution of the extract, yellow coloration indicates the presence of flavonoids (Trease and Evans, 1996).

III. Ferric chloride test

An amount of2 to 3 drops of Ferric chloride solution were added to the solution of the CME. Geen colour was observed (Trease and Evans, 1996).

3.2.3.4 Test for Tannins

I. Lead sub-acetate test

To a small portion of the CME, 4 drops of lead sub-acetate solution was added, the formation of a cream colored precipitate indicates presence of tannins (Trease and Evans, 1996).

3.2.3.5 Test for Terpenoids/Steriods

I. Salkowski's Test

A small portion of the CME was dissolved in 2ml of chloroform, 3 drops of concentrated sulphuric acid was added at the side of the test tube. A red brown coloration at the interface indicates the presence of terpenoids (Edeoga *et al*., 2005).

II. Liebermann-Burchard’s Test

To the portion of the CME equal voloume of acetic anhydride was added and mixed gently. 1ml concentrated sulphuric acid was added down the test tube. This was observed for instant colour changes and over a period of one hour. Blue to blue-green color in the upper layer and a redish,pink or purple color at the junction of the two layers indicates the presence of triterpene (Trease and Evans, 1996).

3.2.3.6Test for Alkaloids

I. Dragendoff’s Test

The CME was dissolved in 2 ml of 1% aqueous hydrochloric acid with continuous stirring in a water bath. The mixture was filtered and few drops of Dragendoff’s

reagent was added, rose red precipitate indicates the presence of alkaloids (Trease and Evans, 1996).

II. Mayer's Test

To 2 ml acidic solution of the CME in a test tube, few drops of Mayer's reagent were added, a cream precipitate indicates the presence of alkaloids (Trease and Evans, 1996).

3.2.3.7 Test for Anthraquinones (Bontrager's Test)

A small portion of the extract was dissolved in 5 ml chloroform,shaken and filtered. To the filtrate, an equal volume of 10% ammonia solution was added with continuous shaking, bright pink colour in the aqueous upper layer indicates the presence of anthraquinone (Trease and Evans, 1996).

3.2.3.8 Test for Cardiac Glycosides (Keller-Kiliani Test)

A small portion of the CME was dissolved in 1 ml glacial acetic acid containing traces of ferric chloride solution. The solution was then transferred into a dry test tube to which an equal volume of sulphuric acid was added, a brown ring obtained at the interface will indicate the presence of a deoxy sugar(Trease and Evans, 1996).

3.3 Chromatographic Procedures

3.3.1 Thin Layer Chromatographic Analysis (TLC)

In this procedure,precoatedTLC plates were used to carry out TLC by one way ascending technique. Capillary tubes were used to manually apply spots on the TLC plate and the spotted plate was developed in an air tight chromatographic tank at room temperature employing different solvent systems such as:

a. Hexane: Ethyl acetate (10:1 and 7:3)
b. Chloroform: Ethyl acetate (3:7, 1:5 and 5:3)
c. Ethyl acetate: Chloroform: Methanol: Water (15:4:4:1)

The spots were visualized under UV (254-366nm) and by the use of spray reagent (10% Sulphuric acid) followed by heating in an oven at 110^{o}C for about 5 minutes.

3.3.2 Column Chromatography

A 75 cm by 3.5 cm glass column and a stationary phase silica gel of 60-120 mesh size was used. Wet packing and dry sample loading was used with silica gel as adsorbent for the column chromatography.

3.3.3 Column Chromatography of Ethylacetate and Chloroform Fractions

Ethyl acetate fraction (3 g) and Chloroform fraction (2.5 g) were subjected to column chromatography using gradient elution technique; starting with chloroform 100 % (400 ml) followed by 95:5 chloroform: ethyl acetate to ethyl acetate 100% (400 ml), based on the TLC profile of the various collections and compound of interest the column was finally washed with methanol 100%. A total of 60 fractions of 100 ml each were collected. The collections werepooled together based on the similarities of their TLC profiles to obtain 10 pooled fractions coded ECF1-ECF10.

3.3.4 Gel Filtration Chromatography

The stationary phase used in this case was Sephadex LH-20 (Sigma) and was suspended in an eluting solvent (methanol) and left for 24 hours to swell prior to use. The suspended Sephadex was then poured into a 300 mm by 200 mm length by width burette column and allowed to set. The sample was dissolved in a small volume of the eluting solvent (methanol) and introduced into the column.

3.3.5 Preparative Thin Layer Chromatography (PTLC)

PTLC was carried out using Fluka silica gel precoated glass plates 20×20 cm with layer thickness of 0.25 mm. A thin line about 1.5 cm from the bottom of the plate was drawn with a pencil. The sample to be separated (D_3) was dissolved in minimum amount of solvent to give an approximate concentration of 20 mg/ml. It was then applied uniformly along the thin line using capillary tube. The plate was allowed to dry after which it was developed using n-hexane: ethylacetate (10:1) as developing solvent system. The developed plate was air dried in fume cupboard and visualized under UV machine after which the position of the band of interest was marked with pencil and scraped off the backing of the plate on to a foil. The scraped sorbent was size reduced using pestle and mortar, transferred in to a sintered glass funnel and washed repeatedly with methanol and the solution obtained was evaporated to give the compound (Gibbons and Gray, 1998).

3.3.6 Gel Filtration Chromatography of ECF10

ECF10having the distinct spots was subjected to gel filtration based. The elution was done using methanol 100 %, 21 collections of 2 ml were obtained and pooled into 5 fractions coded N_1-N_5based on their similarities on subjection to TLC. N_1 was further purified using Sephadex and 16 collections were made and pooled into 4 fractions based on similarities coded $N1_a$ –$N1_d$. N_5 and N_{1d} were furtherpooled together coded as N_8 and subjected to repeated gel filtration which yielded a yellowish amorphous compound coded AK. AK was subjected only to physicochemical tests, UV and IR analysis due to its low yield.

3.3.7 Column Chromatography of n-Hexane fraction

n- Hexane fraction (10.5g) was chromatographed over silica gel (60-120 mesh) packed column of dimension 75 by 3.5cm, the column was eluted continuously gradiently, beginning with Hexane, followed by Hexane: Chloroform mixture, Chloroform 100% and Chloroform:

Ethylacetate ratios. Seventy five (75) fractions of 50 ml each were collected. The fractions were pooled together based on the similarities of the TLC profiles to give five major fractions labelled as D_1-D_5 and the column was finally washed with methanol to give the sixth fraction (D_6). Preparative TLC of fraction D_3 led to the isolation of a white crystalline solid coded HA (12mg).

3.3.8 Melting Point (m.p) Determination

The melting point of the isolated compound was determined using Gallenkamp melting point apparatus.

3.3.9Spectral Analysis

The isolated compound HA was subjected to UV and 1D NMR analysis.

3.3.9.1 Proton and Carbon-13 Nuclear Magnetic Resonance

NMR spectra were obtained on a Bruker AVANCE (400 and 125 MHz for ^{1}H and ^{13}C) spectrometer, using the residual solvent peaks as internal standard. Chemical shift values (δ) were reported in parts per million (ppm) relative to appropriate internal solvent standard and coupling constants (J value) are given in Hz. The NMR solvents used for this measurements was deutrated methanol.

3.4 Pharmacological Studies of Crude Methanol Extract (CME)

3.4.1 Acute Toxicity Studies

The method of Lorke (1983) was adopted for the study. This method was carried out in two phases. Briefly, in the first phase, mice were divided into three groups each consisting three mice. They were treated with the geometric doses of the crude extract (10, 100 and 1000mg/kg body weight) via the oral route and observed for 24 hours for signs of toxicity and death. In the second phase, based on the outcome of the first phase, three groups with one

mice each were treated with doses at1600, 2900 and 5000mg/kg of the extract and observed for signs of toxicity and death. The median lethal dose was estimated as a geometric mean of the highest non-lethal dose (with no death) and the lowest lethal dose (where death occurred).

$$LD_{50} = \sqrt{minimum\ lethal\ dose \times maximum\ tolerated\ dose}$$

3.4.1.1 Maximal electroshock convulsion test in chicks

The methods of Swinyard and Kupferberg (1985) was employed. Day old chicks weighing between 29 and 41 g were randomly divided into five groups of ten each. The first group were treated with normal saline 10 ml/kg *p.o*. The second, third and fourth groups were treated with 150, 300 and 600 mg/kg doses of the extract *p.o* respectively, while the fifth group was treated with phenytoin 20 mg/kg *p.o* as positive control. Sixty minutes later, maximal electroshock was administered to induce seizure in the chicks using Ugo Basile electroconvulsive machine (Model 7801) with corneal electrodes placed on the upper eyelids of the chicks. The current, shock duration, frequency and pulse width was maintained at 80 mA, 0.8 s, 100 pulse per second and 0.6 ms respectively. The chicks were observed for hind limb tonic extension.

3.4.1.2 Pentylenetetrazole (PTZ) induced convulsion test in mice

The method of Swinyard *et al*., (1989) was employed. Thirty mice of either sex weighing between 16 and 21 g were randomly divided into five groups of six mice each. Mice in group I were treated with normal saline 10 ml/kg *p.o* .The second, third and fourth groups were treated with 150, 300 and 600mg/kg doses of the extract *p.o* respectively, while the fifth group was treated with 200 mg/kg Valproic acid. Sixty minutes later, mice in all groups were treated with 90 mg/kg body weight of freshly prepared PTZ orally. The mice were then observed for the presence or absence of clonic spasm of at least 5 seconds duration or death.

3.5 Statistical Analysis

Results of pharmacological studies were presented as mean ± standard error of mean (SEM) as well as percentages in form of tables where appropriate. Statistical analysis for differences between the means was carried out by one-way analysis of variance (ANOVA); when a statistically significant result was obtained with ANOVA, Dunnett's post hoc test for multiple comparison was carried out. The statistical analysis was performed using Statistical Package for Social Sciences (SPSS) software version 20. Values having $p< 0.05$ were considered significant.

CHAPTER FOUR

4.0 RESULTS

4.1 Yield

The extraction of 690 g of *Hymenocardia acida* leaf afforded a yield of 67g of the crude extract (9.7%). The percent yield from the partitioned crude methanol extract (67 g) are presented in Table 4.1

Table 4.1: Yield of Partitioned Fractions from 67 g Crude Methanol Extract

Solvent	Weight (g)	Yield (%)	Colour
Hexane	10.50	1.52	oily green
Chloroform	2.50	0.36	bright green
Ethyl acetate	3.00	0.43	Light brownish
Butanol	2.30	0.33	greenish brown
Aqueous	_	_	_
Total	18.3	2.64	

4.2 Phytochemical Studies

4.2.1 Preliminary Phytochemical Screening of CME of *Hymenocardia acida.*

Preliminary Phytochemical screening of the crude methanol extract revealed the presence of carbohydrates, anthraquinones, steroids/terpenes, flavonoids, tannins, saponins, alkaloids, and flavonoids (Table 4.2).

Table 4.2: Phytochemical Constituents of Crude Methanol Leaf Extract (CME) of *H. acida*

Constituents	**Test**	**Observation**
Anthraquinones	Bontrager	+
Alkaloid	Dragendoff	+
	Mayer	+
Carbohydrate	Molisch	+
Cardiac Glycosides	Keller-Kiliani	+
Saponin	Frothing	+
Flavonoid	Sodium Hydroxide	+
	Shinoda	+
Tannins	Ferric chloride	+
	Lead sub-acetate	+
Triterpenes/Steroids	Liebermann-Burchard	+
	Salkowski	+

Key: + = present

4.3 Thin Layer Chromatography

4.3.1 Thin-Layer Chromatography of HF, CF, EAF and BF

The TLC profile ofhexane, chloroform, ethyl acetate and n-Butanol fractions are shown in Plates II-IV using solvent system H: EA (5:1), the CF and EAF were pooled together because they had similar TLC profile. 10 % sulphuric acid was used as spraying reagent.

PLATE: II



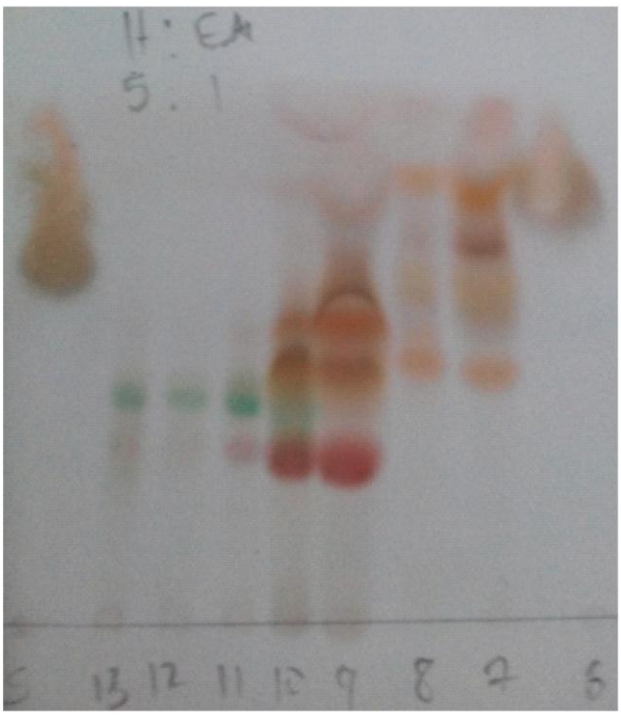
H:EA
5:1

PLATE: III



H:EA
5:1

PLATE:IV



H:EA
5:1

Plate II- IV: TLC profiles of Chloroform, Ethylacetate and Hexane fractions in Hexane: Ethylacetate (5:1) solvent system using 10 % Sulphuric acid as the spraying agent.

Table 4.3: Summary of TLC Profiles of Fractions

Fraction	**No. of Spots**	**R_f values of spots**
Hexane	5	0.15,0.25,0.30,0.43,0.51
Chloroform	7	0.18, 0.23, 0.33,0.41,0.49,0.58,0.82
Ethyl Acetate	3	0.12, 0.16, 0.70
n-Butanol	4	0.28, 0.32, 0.33, 0.37

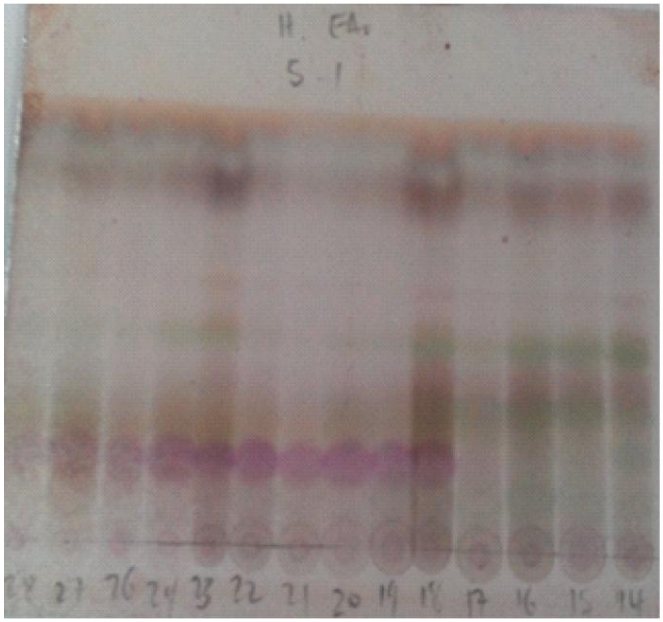
4.4 Column Chromatography

Table 4.4: Column Chromatography of Ethylacetate and Chloroform Fractions

No of beakers collected	Solvent ratio %	Number of spots
1-4	$CHCl_3$ 100	1 (low yield)
5-9	$CHCl_3$: Ethyl acetate 95:5	3
10-14	90:10	0
15-16	85:15	0
17-18	80:20	5
19-22	75:25	5
23-24	70:30	1 (low yield)
25-28	65:35	2
29-30	60:40	1 (low yield)
31-36	55:45	3
37-39	40:60	5
40-42	30:70	4
43-48	**20:80**	**5**
49-55	**10:90**	**6**
56-58	**5:95**	**4**
59-60	100	2

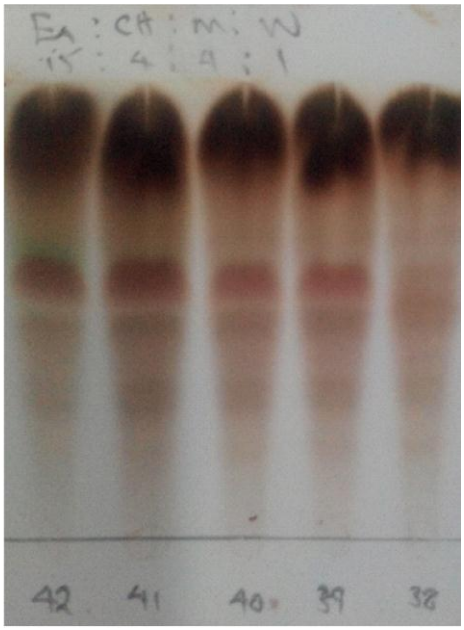
4.4.1: The TLC profiles of some of the collections of the pooled Hexane and Ethylacetatefractions from column are shown in Plates V and VI:

PLATE: V



EA:CH:M:W
15:4:4:1

PLATE: VI

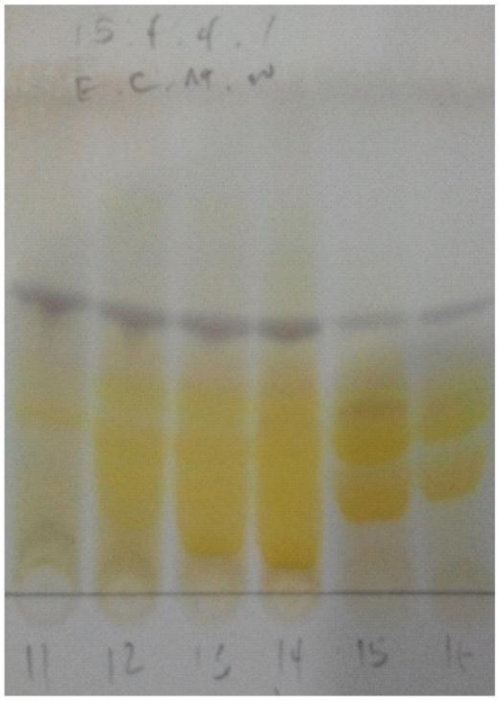


EA:CH:M:W
15:4:4:1

Plates V-VI: TLC profile of pooled fractions of Chloroform and Ethylacetate in Ethylacetate : Chloroform : Methanol : Water (15:4:4:1) solvent system using 10 % sulphuric acid as spraying agent.

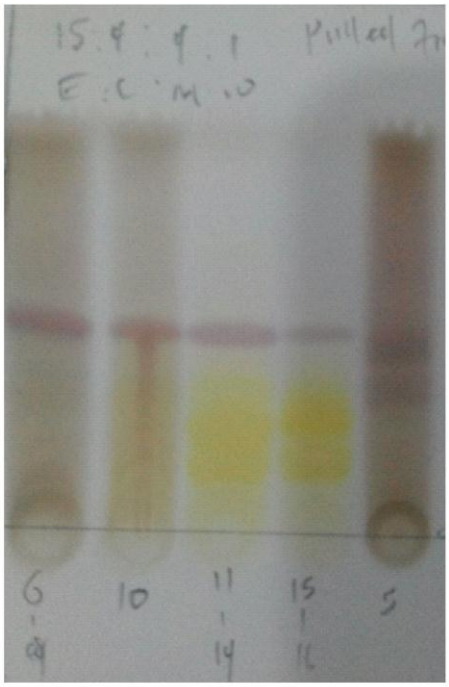
4.4.2: TLC profiles of some of the collections of the pooled fractions from the gel filtration are shown as Plates VII- VIII below:

PLATE: VII



EA:CH:M:W
15:4:4:1

PLATE: VIII



EA:CH:M:W
15:4:4:1

Plates VII - VIII: TLC profile of fractions 56-58 in EA:CH:M:W (15:4:4:1) solvent system using 10 % sulphuric acid as spraying agent.

PLATE:IX



CH:EA
5:3

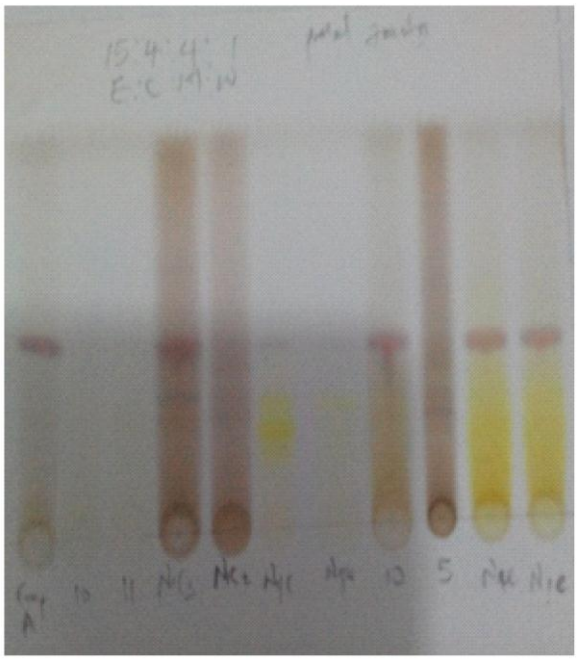
Plate IXa: TLC profile of pooled sub-fractions 6-9 from fractions 56-58 in Chloroform: Ethylacetate (5:3) solvent system using 10% sulphuric acid as spraying agent which afforded compound AK.

Table 4.5: Column Chromatography of n-Hexane Fraction

No of beakers collected	**Solvent ratio %**	**Number of spots**
(D_1) 1-12	Hexane100	5
(D_2) 13-25	**Hexane:$CHCl_3$ (20:1)**	**4**
(D_3) 26-33	**Hexane: $CHCl_3$ (10:1)**	**3**
(D_4) 34-45	Hexane: $CHCl_3$(9:1)	7
(D_5) 46-62	Hexane: $CHCl_3$(9:1)	5
(D_6) 64-75	CH_3OH (100)	5

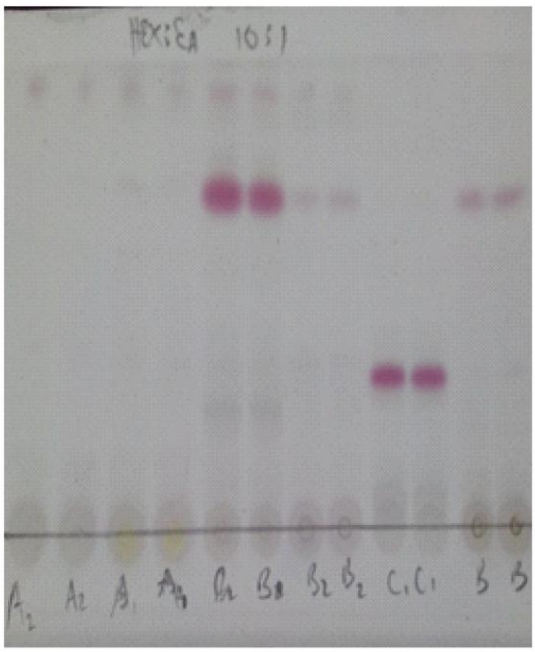
4.4.3: TLC profiles of Preparative TLC represented as plates IXb and X

PLATE: IX



EA:CH:M:W
15:4:4:1

PLATE: X

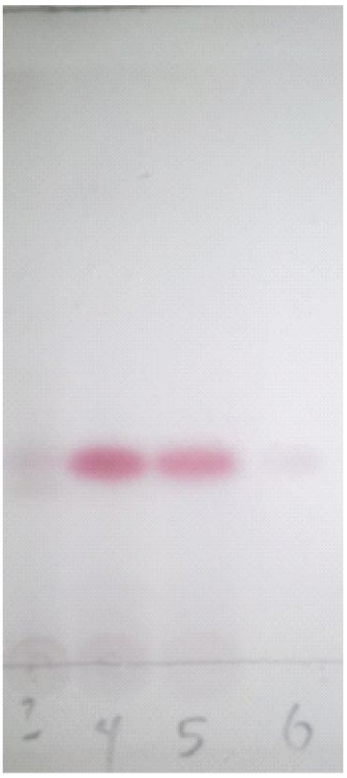


H:EA
10:1

Plates IXb - X: TLC profile of fractions 26-33 in solvent systems EA:CH:M:W (15:4:4:1) , HEX : EA (10:1) and HEX : CH (10:1) using 10 % sulphuric acid as spraying agent.

4.4.4: TLC plate of n- Hexane fractions 26-33 (D_3)

PLATE: XI



H:EA
10:1

Plate XI: TLC plate of n- Hexane fractions 26-33 (D_3) after scrapingin Hexane: Ethylacetate (10 : 1) solvent system using 10% sulphuric acid as spraying agent which afforded compound HA.

4.5 Spectral Analysis of AK

4.5.1UVanalysis of compound AK

The wavelength of maximum absorption (λmax) of AK was found to be 238 nm indicating the presence of a chromophore in the compound.

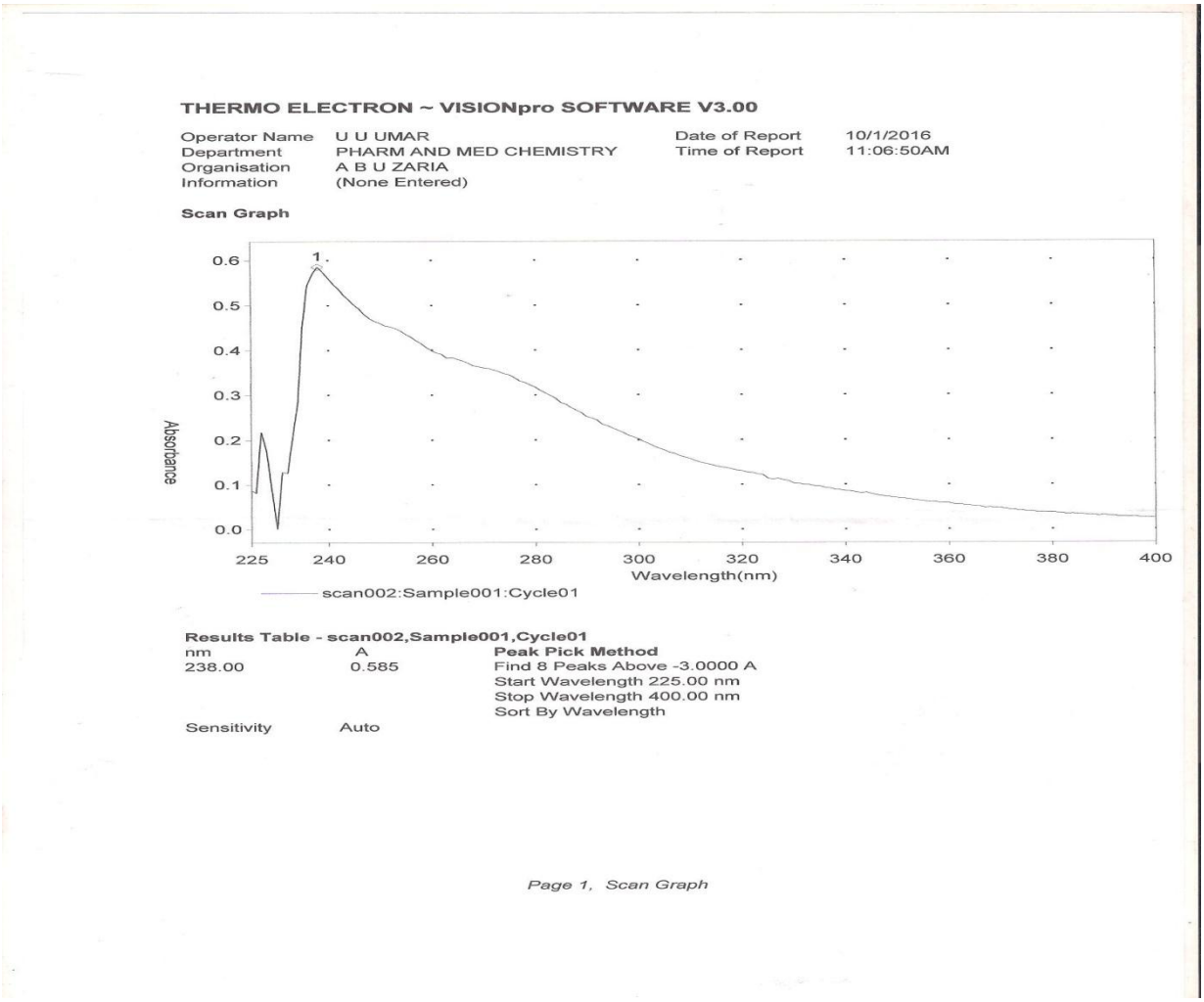


Figure 4.1: UV Spectrum of AK

4.5.2 IR Spectrum of AK

The IR spectrum of AK(Figure 4.2) showed a band at (3298 cm^{-1}) O-H vibration, a band at (1028 cm^{-1}) C-O bond vibration; the absorption observed at 764 $cm^{-1}$was due to an unsaturated out of plane C-H vibration; stretching and bending vibrations due to methyl groups were represented by the bands at 2922 $cm^{-1}$and 1606 cm^{-1}

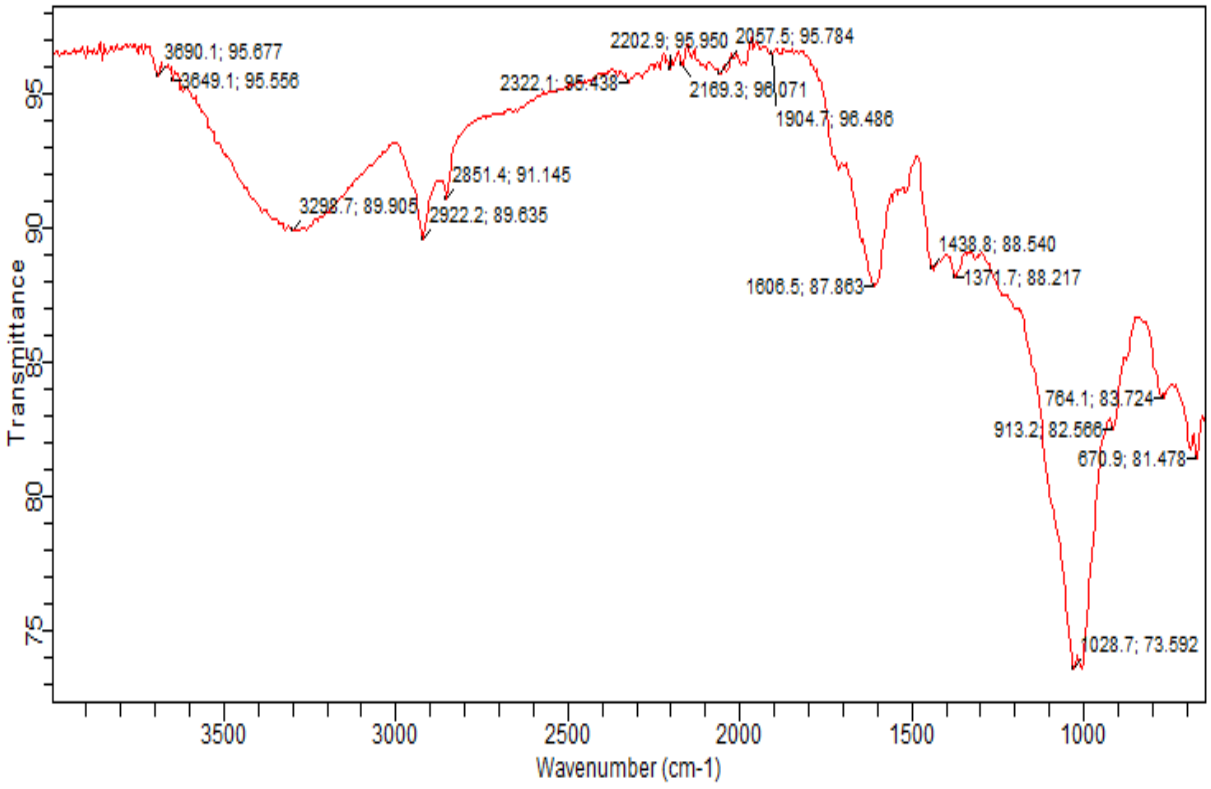


Figure 4.2: IR Spectrum of AK

4.6 Tests carried out on AK

4.6.1 Solubility Profile of AK

AK was completely soluble in methanol and acetone.

4.6.2 Melting point of AK

The isolated compound AK was found to melt between 157 - 159 ^{O}C.

4.6.3 Chemical Test on AK

AK produce orange colour when subjected to Liebermann Burchard's test.

4.7 Spectral Analysis of HA

4.7.1 UV analysis of HA

The wavelength of maximum absorption (λmax) of compound HA was found to be 236 nm indicating the presence of a chromophore in the compound.

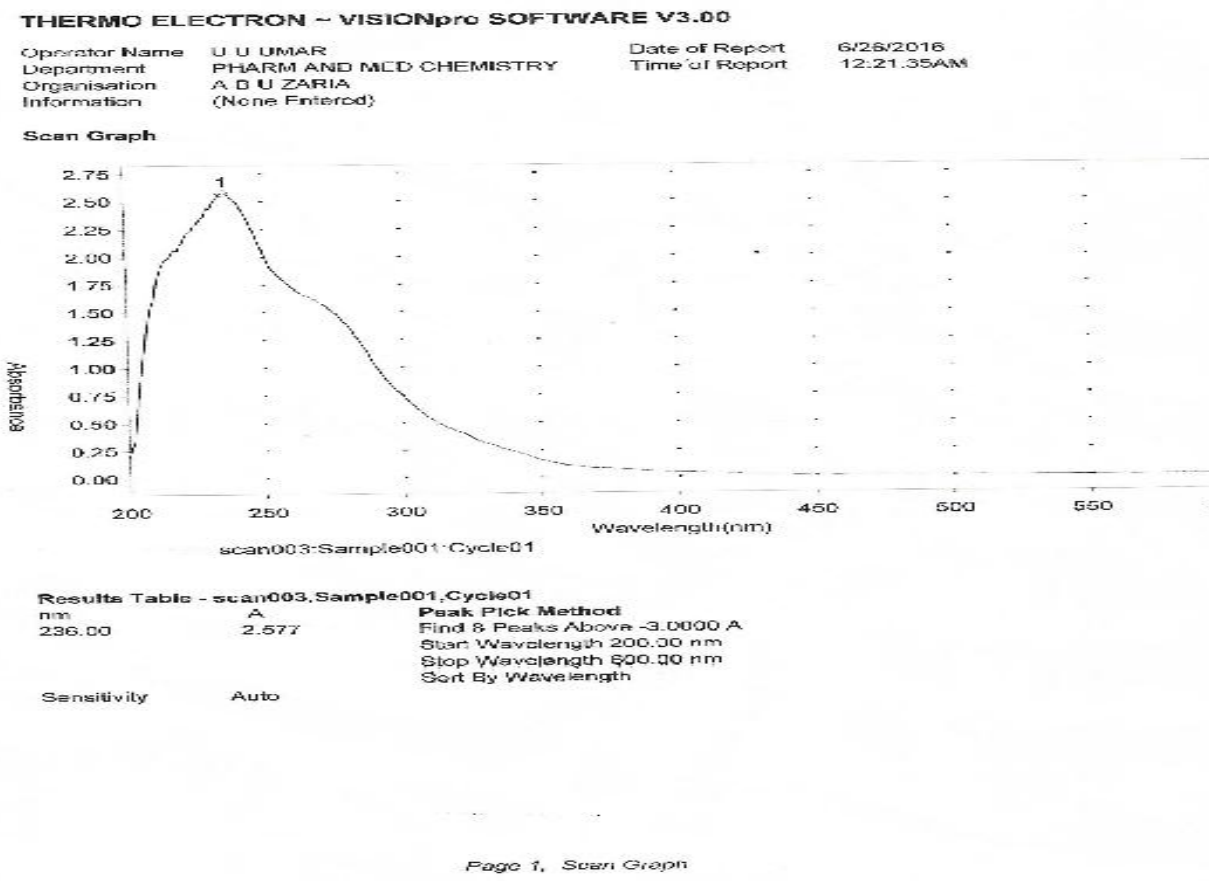


Figure 4.3: UV Spectrum of compound HA

4.7.2 Proton Nuclear Magnetic Resonance (^{1}H NMR) Analysis of HA

The [1]HNMR spectrum of H in CH_3OD (400MHz) revealed signal at δ 4.71(s, H-29), 4.59 (s, H-29), 3.21 (m, H-3), 2.41 (m, H-19), 1.70 (3H, s, H-30), 1.01(3H, s, 24), 0.99 (3H, s, 23), 0.85 (3H, s, 26), 0.81(3H, s,27), 0.72 (3H, s, 25) (Figure 4.4)

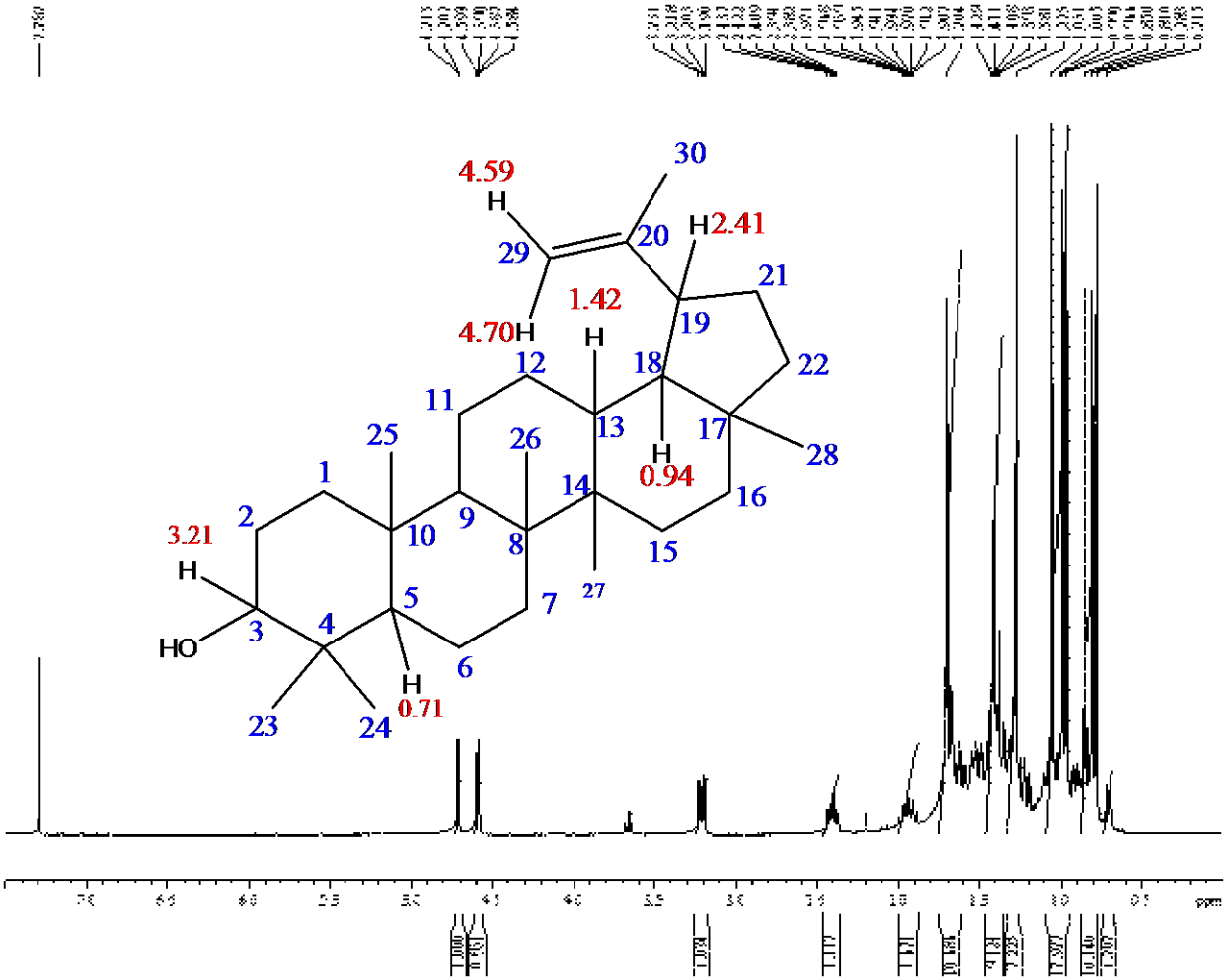


Figure4.4: [1]H NMR spectrum HA in CH_3OD

4.7.3[13]C and DEPT NMR Spectral Analysis of HA

The ^{13}C-NMR (δc ppm, 400MHz,CH_3OD) analysis revealed signals at 38.09 (C-1), 25.19 (C-2), 79.01 (C-3), 38.74 (C-4), 55.34 (C-5), 18.34 (C-6), 34.32 (C-7), 40.87 (C-8), 50.48 (C-9), 37.2 (C-10), 20.96 (C-11), 27.45 (C-12), 38.87 (C-13), 42.86 (C-14), 27.99 (C-15), 35.61 (C-16), 43.01 (C-17), 48.34 (C-18), 47.99(C-19), 150.96 (C-20), 29.88(C-21), 40.02 (C-22), 29.66 (C-23), 15.36 (C-24), 16.12 (C-25), 15.99 (C-26), 14.56 (C-27), 18.01 (C-28), 109.32 (C-29), 19.32 (C-30) in figure 4.5.

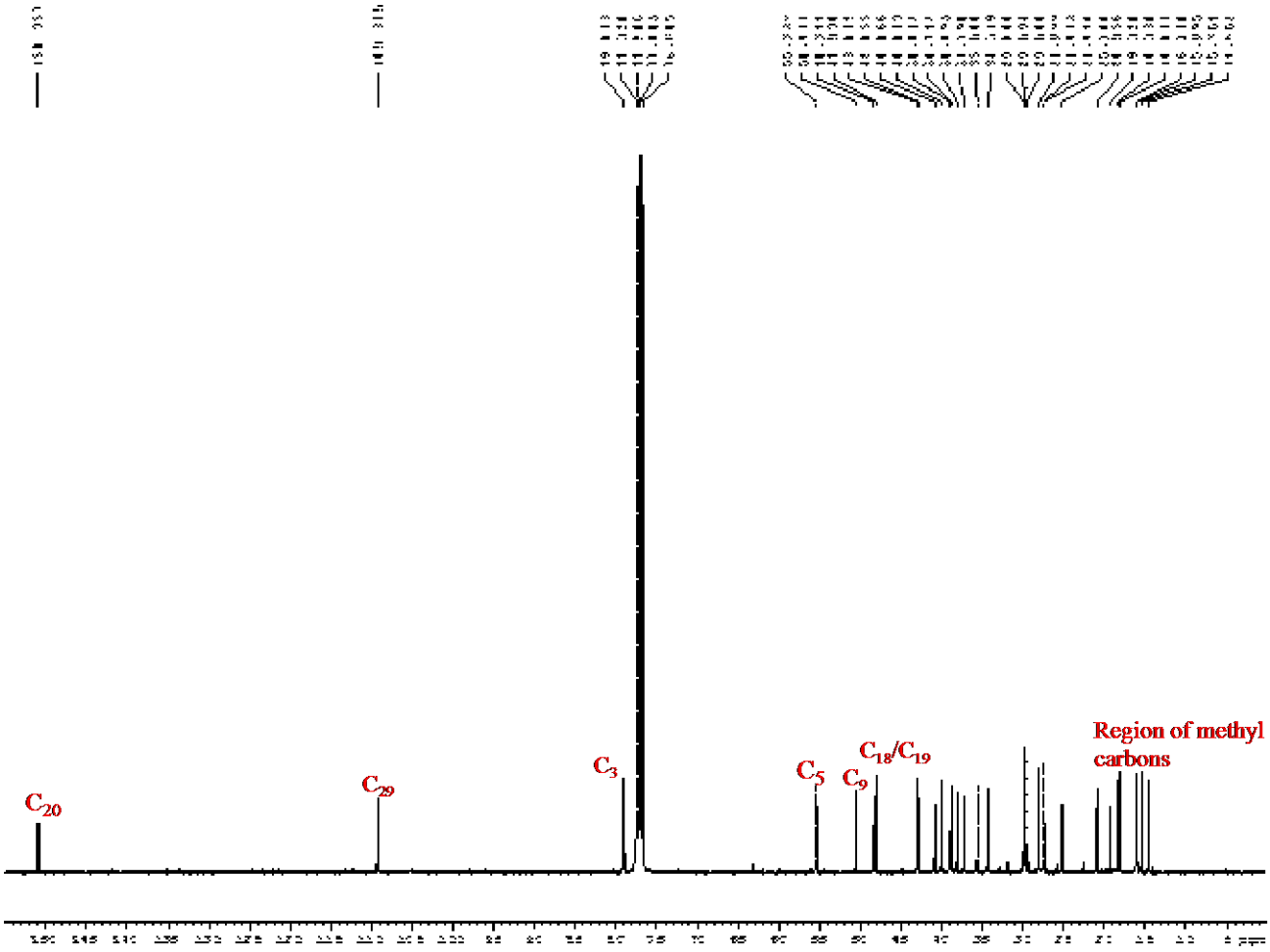


Figure 4.5: ^{13}CNMR Spectrum of HA in CH_3OD

The DEPT and ^{13}C NMR spectra revealed the presence of seven (7) methyl carbons viz: C-23, C-24, C-25, C-26, C-27, C-28 and C-30, eleven (11) methylene carbon viz: C-1, C-2, C-

6, C-7, C-11, C-12, C-15, C-16, C-21, C-22 and C-29, six (6) methine viz: C-3, C-5, C-9, C-13, C-18 and C-19 and six (6) quaternary carbons viz: C-4, C-8,C-10, C-14, C-17 and C-20 in figure 4.6.

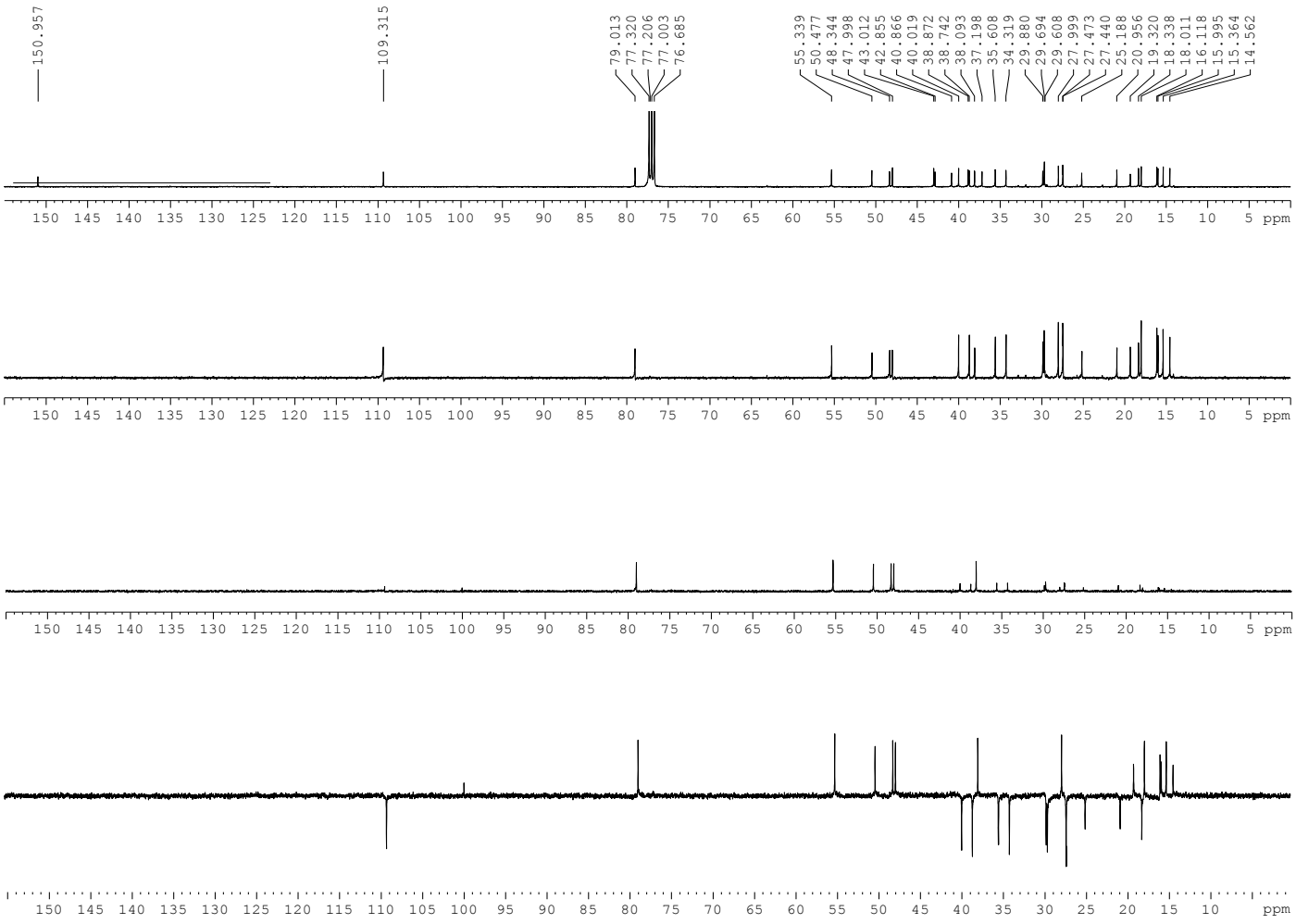


Figure 4.6: DEPT spectrum of compoud HA in CH_3OD

4.8 Tests carried out on HA

4.8.1 Solubility Profile of HA

HA was found to be sparingly soluble in chloroform but completely soluble in methanol and acetone.

4.8.2 Melting point of HA

The isolated compound HA was found to melts between 150-152^{O}C.

4.8.3 Chemical Test on HA

HA produces reddish colour when subjected to Liebermann Burchard's test and reddish brown colour at the interface when subjected to Salkowski's test.

4.9 Pharmacological Studies

4.9.1 Acute Toxicity Studies

The oral route median lethal dose of the crude extract was found to be above 5000 mg/kg in mice.

4.9.2 Anticonvulsant Studies

4.9.2.1Effect of Methanol Leaf Extract ofHymenocardia acida on Maximal Electroshock Induced Convulsion in Chicks

The methanol leaf extract of*Hymenocardia acida*did not protect the chicks against maximal electroshock induced seizures at all doses tested. There was no statistically significant difference*(p* <0.05) in the mean recovery periodwhen compared to the normal saline control group. The standard anticonvulsant drug (phenytoin) produced 90 % protection against HLTE induced by maximal electroshock (Table 4.6).

Table 4.6: Effect of CME of *Hymenocardia acida*on MES Induced Convulsion in Chicks

Treatment (mg/kg)	**Mean Recovery Period (Min.)**	**Quantal Protection**	**% Protection against seizure**

NS 10 ml/kg	8.30 ± 0.82	0/10	0.00
MEHA 150	8.56 ± 1.00	0/10	0.00
MEHA 300	10.60 ± 1.14	0/10	0.00
MEHA600	9.88 ± 1.74	0/10	0.00
PH 20	8.00 ± 0.00	9/10	90.00

Values are presented as Mean ± SEM, No significant difference compared to normal saline control group - One way ANOVA followed by Dunnett's post hoc test, n=10, NS = Normal saline, MEHA = Methanol Leaf Extract of*Hymenocardia acida*, PH = Phenytoin

4.9.2.2 Effect of Methanol Leaf Extract of Hymenocardia acida on PTZ-Induced Convulsion in Mice

In the pentylenetetrazole induced seizure model,*Hymenocardia acida* extract provided a non-dose dependent protection (50.00, 33.33 and 16.67 %) with statistically significant difference

($p<0.05$) at 150mg/kg and a decrease in the mean onset of seizures at doses of 300 and 600 mg/kg respectively (Table 4.7).

Table 4.7: Effect of CME of *Hymenocardia acida* on PTZ-Induced Convulsion in Mice

Treatment (mg/kg)	**Mean Onset of Seizures (min.)**	**Quantal protection**	**% Protection against seizure**	**% Mortality**
NS 10 ml/kg	6.50 ±0.43	0/6	0.00	100.00
MEHA 150	6.83 ± 1.70*	3/6	50.00	50.00
MEHA 300	6.17 ± 0.83	2/6	33.33	66.67
MEHA 600	5.17 ± 0.31	1/6	16.67	83.33
SV 200	-	6/6	100.00	0.00

Protection against seizure and mortality expressed as percentages; Mean onset of seizures presented as Mean ± SEM, * = $p<0.05$ compared to normal saline group - One way ANOVA followed by Dunnett's post hoc test of multiple comparison, n=6, NS - Normal Saline, MEHA = Methanol Leaf Extract of*Hymenocardia acida*, SV = Sodium Valproate.

CHAPTER FIVE

5.0 DISCUSSION

5.1 Phytochemical Studies and Spectra Analysis

A yield of 67 g of crude extract was obtained from the extraction of 690 g *Hymenocardia acida* leaf.The preliminary phytochemical screening of the CMErevealed the presence of saponins, tannins, glycosides, terpenoids, alkaloids and flavonoids. These phytochemical constituents have been reported to possess different kinds of pharmacological properties (Cowan, 1999) such as anti-oxidative (Okpuzor *et al*., 2009), anti-malarial (Ichino *et al.*, 2006) and anticonvulsant (Musa *et al.,* 2014).

Column chromatographic separation of ECF followed by gel filtration over sephadex LH-20 led to the isolation of a yellow amorphous compound coded AK. AK gave a single homogenous spot with two different TLC solvent systems indicating the purity of the compound. The IR spectrum of AK showed characteristic absorption frequencies at 3298 and 1028cm^{-1} typical of the O-H and C-O bond vibrations respectively; the absorption observed at 764$cm^{-1}$was due to an unsaturated out of plane C-H vibration; stretching and bending vibrations due to methyl groups were represented by the bands at 2922$cm^{-1}$and 1606cm^{-1}. The wavelength of maximum absorption (λmax) was 238 nm. AK was soluble in methanol and acetone, tested positive to Liebermann Burchard's test and melted at 157-159°C suggesting that AK might be a steroid or a triterpene (Pateh *et al.,* 2009 ; Baek *et al.,* 2010). AK was not subjected for spectral analysis due to its low yield.

Preparative Thin Layer chromatography carried out on the column fraction D_3 collected from column chromatography of n-Hexane fraction obtained from CME of *Hymenocardia acida* resulted in the isolation of a white crystalline solid coded compound HA which gave single homogenous spot with two different solvent systems.

The compound was soluble in methanol and acetone, absorbs at wavelength (λmax) of 236nm and tested positive to Liebermann Burchard's and Salkowski's tests and melted at 150-152^{O}C suggested that HA is a lupane triterpene (Baek *et al.,* 2010; Hamada *et al.,* 2012), the structure of HA was elucidated by spectroscopic analysis as well as comparison of its spectral data with previously reported values (Chaturvedula and Prakash, 2012;Abdullahi *et al.,*2013).

The ^{1}H NMR spectrum of HA revealed the presence of seven tertiary methyl protons at δ 0.72, 0.78, 0.81, 0.85, 0.99, 1.01 and 1.704(integrated for3H-each, s, CH_3). A sextet of one proton at δ 2.41 ascribable to 19β- H is characteristics of lupeol. The H-3 proton showed a multiplet at δ 3.21 while a pair of broad singlets at δ 4.58 and δ 4.71 (1H, each) was indicative of two olefenic protons (exocyclic double bond) at H-29 a and b. These assignments are in good agreement for the structure oflupeol (Imam *et al.,* 2007; Jain and Bari, 2010; Baek*et al.,* 2010*;* Abdullahi *et al.,* 2013).

The structural assignment of HA was further substantiated by the ^{13}C NMR experiments which showed 30 signals for the terpenoid of lupane skeleton viz ; seven (7) methyl groups at δc: 29.66 (C-23), 18.01 (C-28), 16.12 (C-25), 15.99(C-26), 15.36 (C-24), 14.56(C-27), and 19.32 (C-30); the signals due to an exomethylene group at δc: 109.32 (C-29) and 150.96 (C-20) respectively, of which the deshielded signal at δc 150.96 ppm was assigned for quartenary olefenic carbon C-20.The other deshieldedsignal at δc 79.01 was due to C-3 with a hydroxyl group attached to it, the eleven (11) methylene, six (6)methine and six (6) quaternary carbons were also assigned with aid of DEPT experiment. Comparing its UV, ^{1}H- and ^{13}C NMR spectral data with the literature values of reported compounds (Mahato and Kundu, 1994; Marina *et al.,* 1997; Rosenel *et al.,* 1998), the structure of HA was suggested to be a 20 (29) lupen-3β-ol (lupeol) (Fig:4.7)

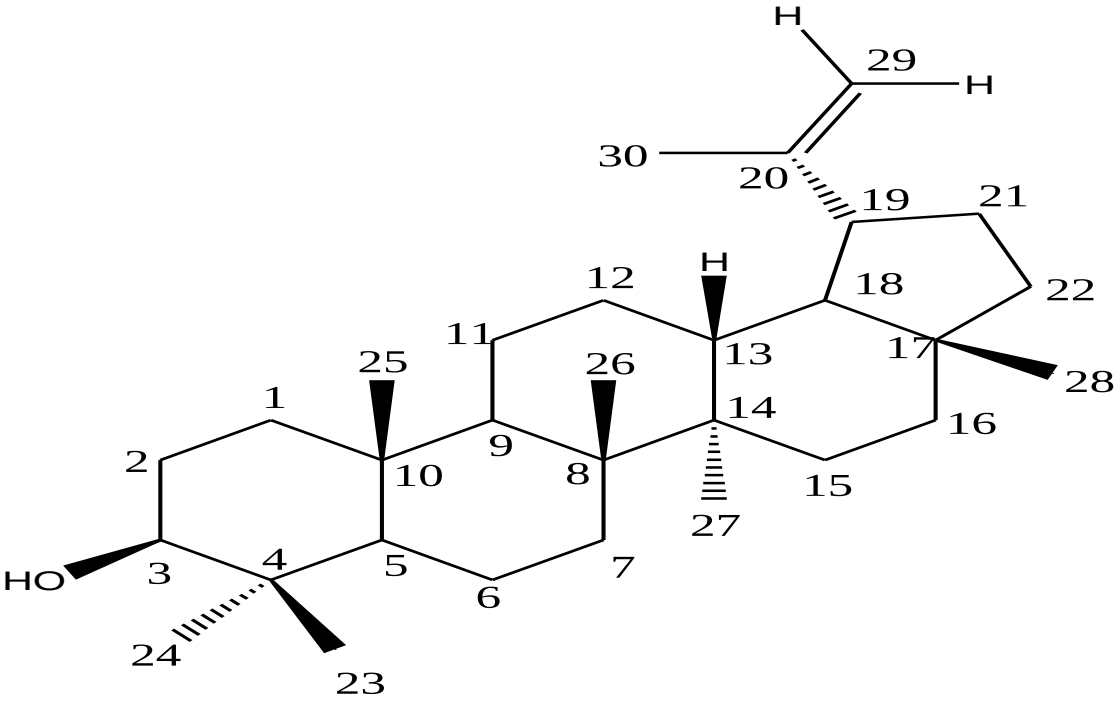


Fig 4.7: Proposed structure of compound HA 20 (29) lupen-3β-ol

Diverse range of biological activities triggered by lupeol have been reported including, the stimulation of programmed cell death in human leukemia cell line HL-60 (Aratanechemuge *et al.,* 2004). Lupeol is known to show anti-inflammatory and antiarthritic activities (Agarwal and Rangari, 2003). It inhibits the growth of highly aggressive human metastatic melanoma cells (Saleem *et al*., 2008), has antiangiogenic activity (You *et al*., 2003), antioxaluric and anticalciuric activities (Anand *et al*., 1995).

Lupeol derivatives have also been reported to have antimalarial activity (Fotie *et al*., 2006; Kumar *et al*., 2008), antimicrobial activity (Ragasa *et al*., 2005) and presented a gastroprotective effect on ethanol-induced gastric damage in mice in a dose response manner (Lira *et al.,* 2009). Triterpenes and steroids, among other phytochemicals have been reported to possess anticonvulsant activity (Musa *et al.,* 2014). Finally, lupeol and its related compounds have also been demonstrated to possess some activity in the nervous system (Martini *et al.,* 2007).

5.2 Pharmacological Studies

5.2.1 Acute Toxicity (LD_{50}) Studies

Determination of median lethal dose value of plants used by traditional medicine practitioners using acute toxicity study is of paramount importance because it provides information regarding the margin of safety of the plant. The oral median lethal dose (LD_{50}) value of the CME was found to be greater than 5000 mg/kg body weight in swiss albino mice. This LD_{50} value implies that the CME is relatively safe (Matsumura, 1985). Doses of less than or equal to 30 % of the LD_{50} which have been demonstrated to be relatively safe for ethnopharmacological research were used throughout the research procedure (Vongtau *et al*., 2004).

5.2.2 Anticonvulsant screening

The outcome of the study provides evidence that MEHA possess significant anticonvulsant activity. The effectiveness of the plant's extract in the experimental convulsion paradigm used probably suggests that the herb could be used to manage epilepsy especially *petit mal* seizures and human generalized absence seizures.

MEHA failed to alter MES thresholds at all doses tested in contrast to the positive control agent using phenytoin (Table 4.6).MEST is a standard AED test that evaluates the testing material's ability to protect against hind limb tonic extension (HLTE) phase of the MEST (DeLorenzo*et al.,* 1992). It is a model for generalised tonic clonic seizure, which is highly reproducible with consistent end- point (Stables and Kupferberg, 1997).

MEST is used to validate pre-clinical test that predicts drugs effective against generalized seizures of the tonic-clonic (grand mal) type (Mares and Kubova; 2006). Drugs that act on sodium channels e.g. carbamezapine, phenytoin, oxcarbazepine and lamotrigine are known to

suppress hind limb tonic extension induced by maximal electroshock (Rho and Sankar, 1999), thus, the study suggests that MEHA does not interfere with the sodium channels to elicit its anticonvulsant effect.

The study revealed that MEHA and Valproic acid inhibited pentylenetetrazole (PTZ) induced seizures. MEHA dose independently suppressed the onset and latency of seizure induced by PTZ. The PTZ test represents a valid model for human generalized and absence seizures (Loscher and Schmidt, 2006). Anticonvulsant activity in PTZ test identifies compounds that can raise the seizure threshold in the brain (White *et al.,* 1998; Raza *et al.*, 2001). PTZ has been shown to interfere with GABA neurotransmitter and the GABA receptor complex (DeDyn *et al.*, 1992).

Pentylenetetrazole has been used experimentally to study seizure phenomenon and to identify pharmaceuticals that may control seizure susceptibility. The exact mechanism of the epileptogenic action of PTZ at the cellular neuronal level is still unclear but it has been generally reported to produce seizures by inhibiting gamma-aminobutyric acid (GABA) neurotransmission (De Sarro *et al.*, 2003).

Enhancement of GABAergic neurotransmission has been shown to inhibit or attenuate seizures, while inhibition of GABAergic neurotransmission or activity is known to promote and facilitate seizure. Anticonvulsant agents such as diazepam, valproic acid and phenobarbitone inhibit PTZ induced seizure by enhancing the action of GABA-receptors, thus facilitating the GABA mediated opening of chloride channels (Gale, 1992; Olsen, 1981). Thus the inhibition of PTZ induced seizures by MEHA suggests that MEHA may produce this effect by enhancing GABAergic neurotransmission although it is also possible that it could have done so by depressing glutamate-mediated excitation.

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 Summary

The phytochemical screening of the methanol leaf extract of *Hymenocardia acida* revealed the presence of carbohydrates, glycosides, saponins, alkaloids, terpenoids, tannins and flavonoids. Column chromatography of n-Hexane fractions was followed by preparative thin layer chromatography and the isolation of lupane terpenoid (Lupeol).

The methanol leaf extract of *Hymenocardia acida*possessed significant ($p<0.05$) anticonvulsant effect(s) in PTZ induced convulsion model of epilepsy used and indicates a possible enhancement of GABA activity.

6.2 Conclusion

Preparative Thin Layer chromatography carried out on the column fraction D_3 collected from column chromatography of n-Hexane fraction obtained from the crude methanol leaf extract of *Hymenocardia acida* afforded lupeol which might be responsible for the observed anticonvulsant effect of the extract. The work has also validated the ethnomedicinal use of the plant in the management of epilepsy.

6.3 Recommendations

I. Bioassay guided isolation should be carried out to isolate the bioactive compounds responsible for the observed anticonvulsant activity.

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

III. Chronic toxicity studies should be done to clearly substantiate the reported claims of less toxicity.

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APPENDICES

Appendix I:Comparison of HA with Lupeol isolated by Chaturvedula and Prakash, 2012**.**

Position	**$\delta^{13}C$**	**$\delta^{1}H$, J (Hz)** **Compound HA**	**DEPT**	**$\delta^{13}C$**	**$\delta^{1}H$, J (Hz) Chaturvedula and Prakash, 2012**
1	38.09		CH_2	38.0	
2	25.19	a. 1.4, b. 1.28	CH_2	25.3	a.1.61, b. 1.54
3	79.01	3.21(multiplet, J=5.2Hz, 11.2Hz)	CH	78.4	3.21
4	38.74		C	38.6	
5	55.34	0.71	CH	55.1	0.69
6	18.34	1.39	CH_2	18.1	1.39
7	34.32		CH_2	34.1	
8	40.87		C	41.2	
9	50.48	1.25	CH	49.7	1.28
10	37.20		C	37.3	
11	20.96	1.43	CH_2	21.1	1.53
12	27.45	1.28	CH_2	27.5	1.29
13	38.87		CH	39.2	
14	42.86	1.42	C	42.6	1.42
15	27.99	1.01	CH_2	27.6	1.01
16	35.61		CH_2	35.6	
17	43.01		C	43.2	
18	48.34	0.94	CH	48.2	0.91
19	47.99	2.41(1H, ddd, J=6Hz, 11.2Hz)	CH	47.8	2.37
20	150.96		C	151.6	
21	29.88	1.94	CH_2	30.0	1.91
22	40.02		CH_2	40.2	
23	29.66	0.99	CH_3	28.2	0.98
24	15.36	1.00	CH_3	16.0	1.02
25	16.12	0.71	CH_3	16.8	0.64
26	15.99	0.85	CH_3	16.4	0.84
27	14.56	0.81	CH_3	15.1	0.97
28	18.01	0.71	CH_3	18.0	0.79
29	109.32	4.58(1H, brs), 4.71(1H, d, J=2.4Hz)	CH_2	108.6	4.56(1H, brs), 4.71(1H, d)
30	19.32	1.70	CH_3	19.5	1.69

Appendix II: Determination of oral median lethal dose (LD_{50})of the crude Methanol leaf Extract of *Hymenocardia acida*

Table X:Result of the First Phase of Acute toxicity studies of the Crude Methanol Leaf Extract of *Hymenocardia acida*.

Dose ($mgkg^{-1}$)	Number of mice used	Mortality
10	3	0/3
100	3	0/3
1000	3	0/3

Table Y:Result of the Second Phase of Acute toxicity studies of the Crude Methanol Leaf Extract of *Hymenocardia acida*.

Doses ($mgkg^{-1}$)	Number of mice used	Mortality
1600	1	0/1
2900	1	0/1
5000	1	0/1