PHYTOCHEMICAL AND SOME PHARMACOLOGICAL STUDIES OF THE AERIAL PART OF *ALYSICARPUS OVALIFOLIUS* Schumach & Thonn FAMILY: FABACEAE

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DEPARTMENT OF PHARMACEUTICAL AND MEDICINAL CHEMISTRY, FACULTY OF PHARMACEUTICAL SCIENCES, AHMADU BELLO UNIVERSITY, ZARIA.

NIGERIA

MARCH, 2013

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DECLARATION

I declare that the work in the thesis titled “PHYTOCHEMICAL AND SOME PHARMACOLOGICAL STUDIES OF THE AERIAL PART OF *ALYSICARPUS OVALIFOLIUS* Schumach.& Thonn FAMILY: FABACEAE” has been carried out by me in the Department of Pharmaceutical and Medicinal Chemistry, under the supervision of Dr.U.U. Pateh and Prof. M. Ilyas. The information derived from the literature has been duly acknowledged and a list of references provided. No part of this thesis was previously presented for another Degree or Diploma at this or any other Institution.

Oluwakemi Abidemi Banwo …….. …….

Signature Date

‘

CERTIFICATION

This thesis entitled “PHYTOCHEMICAL AND SOME PHARMACOLOGICAL STUDIES OF THE AERIAL PART OF *ALYSICARPUS OVALIFOLIUS* Schumach. & Thonn” by OLUWAKEMI ABIDEMI BANWO meets the regulations governing the award of the degree of Masters 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

*Alysicarpus ovalifolius (*Schum.&Thonn) Family: Fabaceae is a protein rich fodder plant that is widely distributed across Africa, Asia and Australia and medicinally used to treat cough and wounds. Recently, it has been reported to be used in the Northern part of Nigeria as tea by commercial motor cycle riders popularly known as “okada riders”, labourers and young people .The dried powder of the plant was initially defatted with petroleum ether, the marc was further subjected to extraction with methanol and partitioning with petroleum ether, ethyl acetate and n-butanol. The preliminary phytochemical screening shows the presence of steroids, triterpenes and cardiac glycosides in the petroleum ether fraction while cardiac glycosides, saponins, steroids, triterpenes and flavonoids are present in the methanolic extract. The n-butanol fraction was subjected to column chromatography, gel filtration and preparative thin layer chromatography to yield two compounds coded D7 and E17. D7 was characterized as a glycosidic flavone (3,5-dihydroxy-4^{1}-methoxy-7-O-β-glycosyl flavone,($C_{19}H_{22}O_{10}$) and E17 was characterized as 3 β –cholest-5-ene-3-diol using chemical tests and spectral analysis (^{1}H-NMR) and extensive literature review. The pharmacological studies carried out on the extract to show it has central nervous stimulant activity are, the drug (diazepam) induced- sleep in mice and the hole-board experiment. The acute toxicity test of the extract was also carried out. The diazepam induced-sleep study showed decreased onset of sleep and and increase in the duration of sleep while the hole board suggest that the n-butanol fraction of methanolic extract contain constituents which may possess central nervous system stimulant activity as seen in the exploratory behavior of the mice. It can be said that the plant *Alysicarpus ovalifolius* (Schum&Thonn) possess some central nervous stimulant activity and therefore the plant could be exploited for its stimulant property.

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LIST OF ABBREVIATIONS

O^c	degree Celsius
$CDcl_3$	chloroform
cm	centimeter
d	doublet
dd	doublet of doublets
g	gram
g/kg	gram per kilogram
HZ	hertz
J	coupling constant in Hertz
m	multiplet
mg/ml	milligram per milliliter
ml	millilitre
NMR	Nuclear Magnetic Resonance Spectroscopy
ppm	parts per million

‘

q	quartet
s	singlet
t	triplet
TLC	thin layer chromatography
δ	chemical shift in ppm
PE	Petroleum ether
ME	Methanol extract
PEF	Petroleum ether fraction
EAF	Ethyl acetate fraction
nBF	n-Butanol fraction
AF	Aqueous fraction
H_2S0_4	Sulphuric acid
^{1}HNMR	Proton nuclear magnetic resonance
1. P	Intraperitoneal

CHAPTER ONE

1.0 INTRODUCTION

Plants have formed the basis of sophisticated medicine practices that have been used for thousands of years by people of different traditional countries and cultures.

Recently, much focus has been placed on plant research all over the world and evidence has shown immense potential of medicinal plants use in various traditional systems (Bhat, 1997). Medicinal plants are plants which in one or more of its organs contain substances that can be used for therapeutic purposes or which are precursors for chemo- pharmaceutical semi-synthesis. When a plant is designated as medicinal it is implied that the said plant is useful as a drug or therapeutic agent or an active ingredient of a medicinal preparation (Addis *et al.,* 2001).

Medicinal plants play a major role in rural areas of the third world. In Nigeria, medical therapy using herbal remedies have succeeded in the treatment of several diseases including breast cancer, sickle cell, diabetes, skin disorders, some viral diseases and some form of mental disorders (Trease and Evans, 2002).

According to the World Health Organization (WHO) 80 percent of the world's population is dependent on medicinal plants for their primary health care source (Evans, 2002). Besides serving medicinal and cultural functions, they have played an important economic role in developing countries.

Therefore, the study on natural products is always an interesting target for scientists over the decades especially on plants. Historically, plants (fruits, vegetables, medicinal herbs, etc.)

have provided a good source of a wide variety of compounds, such as phenolic compounds, nitrogen containing compounds, vitamins, terpenoids and some other secondary metabolites, which are rich in valuable bioactivities, e.g., antioxidant, anti-inflammatory, antitumor, anti-mutagenic, anti-carcinogenic, antibacterial, or antiviral activities. In many oriental countries (China, Thailand, Japan, etc.), the traditional herbal medicines have been widely used for thousands of years. Herbal plants have become the main object of chemists, biochemists, and pharmacists. Their research plays an important role for discovering and developing new drugs, which hopefully are more effective and have no side actions like most modern drugs (Ly, 2002).

Traditional medicine has remained the most affordable and easily accessible source of treatment in the primary healthcare system of resource poor communities. In Ethiopia, medicinal plants have been used as traditional medicines to treat different human ailments by local people from time immemorial. These medicinal plants are estimated to be over 700 species. (Kebebew and Addis, 1996) and most of them are confined to the Southwestern regions of the country (Abbink, 1995).

There is high expectation of enormous traditional knowledge and use of medicinal species due to the existence of diverse cultures, languages and beliefs among people.However, since cultural systems are dynamic (Cumingham,2001), the skills are fragile and easily forgettable as most of the indigenous knowledge transfer in the country is based on oral transmission (Abebe and Ayehu, 1993).

Most medicinal plant species used to treat human ailments grow wild. This implies that majority of plants of medicinal importance were not cultivated by traditional healers. Shrubs

and trees were the most represented growth form for remedy preparation, this is due to the fact that these growth forms are available in all seasons as they are relatively drought resistant and not affected by seasonal variations.

Marketed plants are very important items of trade in many parts of the world. The communities use these for a variety of purposes such as food, cosmetics, flavors, spices, and medicines. It seems that plants used for medicinal purposes form the most common category (Juntachote *et al*., 2006**).**

In industrialized countries plant based traditional medicines or phytotherapeutics are often termed "complementary or alternative medicine (CAM) and their use have increased steadily over the last 10 years. In the USA alone the total estimated "herbal" sales for 2002, was $ 4.4 billion a significant increase from $2.5 billion in 1995. However, such 'botanical dietary supplements are regulated as food rather than drugs by the United State Food and Drug Administration. The World Health Organization recently recognized many herbal remedies and it encourages Developing Countries to incorporate the use of folkloric plants for obvious reasons of availability and affordability apart from being effective alternative to allopathic practice. In Nigeria medical therapy using herbal remedies succeeded in the treatment of several diseases like sickle cell anemia, viral disease, diabetes and some form of mental disorders (Evans, 2002).

In conclusion plants have provided human with many of the essential needs including life saving pharmaceutical agents. Recently the World Health Organization estimated that 80 percent of people worldwide rely on herbal medicines for some aspects of health care. Many

developing countries have intensified their efforts in documenting ethno medicinal data and scientific research on medicinal plants.

It has been estimated that more than 400 traditional plants or plant-derived products have been used for the management of type 2 diabetes. Galegine, a substance produced by the herb *Galega officinalis,* provides an excellent example of such a discovery. Experimental and clinical evaluations of galegine, provided the pharmacological and chemical basis for the discovery of metformin which is the foundation therapy for type 2 diabetes (Bailey and Day, 1989).

Plant derived agents are also being used for the treatment of cancer. Several anticancer agents including taxol, vinblastine, vincristine, the camptothecin derivatives, topotecan and irinotecan, and etoposide derived from epipodophyllotoxin are in clinical use all over the world.

Plants can provide biologically active molecules and lead structures for the development of modified derivatives with enhanced activity and reduced toxicity. The small fraction of flowering plants that have so far been investigated have yielded about 120 therapeutic agents of known structure from about 90 species of plants.(Butlet, 2004). Some of the useful plant drugs include digitoxigenin, gitoxigenin, digoxigenin, tubocurarine, morphine, codeine, aspirin, atropine, pilocarpine, capscicine, allicin, curcumin, artemesinin and ephedrine among others. In some cases, the crude extract of medicinal plants may be used as medicaments. About 121 (45 tropical and 76 subtropical) major plant drugs have been identified for which no synthetic one is currently available.

‘

Information on ethno medical uses of plants has provided an important lead in the discovery of a great number of chemically useful plants drug worldwide**.** According to the available literature, some of the reported medicinal plants species were found to have some phytochemical and biological activities (Macia et *al.,* 2005).

1.2 STATEMENT OF THE RESEARCH PROBLEM

The plant *Alysicarpus ovalifolius* and some other of its species have been reported to be consumed in the northern part of Nigeria as tea. This is especially true of young people and commercial motor cycle riders popularly known as “okada riders” who use it as tea and also for its stimulant effect, based on this report, the plant is being investigated for its stimulant activity.

1.3 SIGNIFICANCE OF THE STUDY

Alysicarpus ovalifolius has been documented to be a nitrogen fixing plant and also used to treat wounds and cough in some places but not much work has been conducted on its central nervous activity relating to its stimulant activity.

1.4 AIM AND OBJECTIVES

1.4 Aim: To justify the use of *Alysicarpus ovalifolius* as a stimulant in traditional medicines.

1.5 Objectives

1. to establish the safety and CNS activities of *Alysicarpus ovalifolius*
2. to isolate compound(s) from the n-butanol fraction of the methanolic extract.
3. to elucidate the structure of the isolated compound(s) using spectroscopic means.

‘

1.6 HYPOTHESIS

The *Alysicarpus ovalifolius* plant contains phytochemical constituents that have central nervous system stimulant effects.

‘

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 GENERAL OVERVIEW

Kingdom: Plantae- Plants

Family: Fabaceae-Pea family

Genus: Alysicarpus

Species: *Alysicarpus glumaceus* (vahl) DC

Alysicarpus ovalifolius (Schum.&Thonn)

Alysicarpus rugosus (Wild) DC

Alysicarpus vaginalis (L) DC and over twenty more species.

The family Fabaceae is of great ethno botanical importance in indigenous and urban communities throughout the world. Fabaceae is a family of cosmopolitan distribution, with approximately 730 genera and 19,400 species lying in third place after Asteraceae and Orchidaceae with respect to species richness at a global level.

This high species richness is reflected in great morphological and chemical diversity from which multiple uses are derived **(**Waterman, 1994**)**

In socioeconomic terms, their importance for health and human alimentation has been documented, although they also provide wood resources and dyes, resins, insecticides fibers, fodders and so forth (Isely, 1982**)**

The nutritional value of Fabaceae is to a great extent due to their ability to fix nitrogen for protein synthesis. This advantage has led to protein concentration in leaves and seeds which vary between 20% and 40% dry weight, depending on species.(Simpson and Ogorzaly, 1986).With regards to medicinal use, it has been pointed out that they are found amongst the five

‘

botanical families richest in therapeutic properties of pharmacopeia of indigenous and rural populations in Holarctic (Moerman *et al*., 1999)

Their medicinal value lies partly in their effectiveness in the treatment of a wide variety of human ailments. (Munoz *et al.,* 2001)

2.2 Description of *Alysicarpus ovalifolius*

Alysicarpus ovalifolius (Schum.& Thonn.) also known as Alyce clover, is an erect or spreading annual herbaceous (sometimes woody at the base) legume, very variable in size (2–60 cm tall) and in shape**.** The stems are decumbent, slightly pubescent when young and almost glabrous with age. Leaves are alternate, unifoliolate; leaflets are oblong or narrowly lanceolate, 1-10 cm long × 0.5-3 cm broad. Inflorescences are pseudoracemes, sometimes paniculate. Flowers are orange-buff to pink or reddish-violet. Fruits are indehiscent oblong pods, 18-25 mm long, Seeds are oblong-ellipsoid, 2.5 mm × 1.5 mm × 1.2 mm. *Alysicarpus ovalifolius* is very similar to *Alysicarpus rugosus* or *Alysicarpus glumaceus* during their vegetative stages, thus difficult to identify. It is widely distributed in tropical Africa, Madagascar, Afghanistan, India, and Vietnam and in the tropics. It is spread across West and East regions of the tropics. It is known locally by the;

Hausa as Gadagin (Burkill, 1997)

Swahili as Mpakapaka

Pare as Silubua

Tanganyika as Selubua

‘



(Oval-leafed Alysicarpus)

2.3 Ethno Medicinal Use

The whole plant of *Alysicarpus ovalifolius* provides protein rich fodder and a palatable feed for livestock grazing on rangeland in Niger. It is a valuable component of vegetation collected and traded as fodder in Niger and in Nigeria it is reported as a wound medicine and for treating cough in some Asian countries.

The leaf of *Alysicarpus glumaceus* DC is used by the people in East Africa as a remedy for thrush and sores. In Arabia and India the herb is used as an external application to swollen feet.

‘

The leaf and root of *Alysicarpus* r*ugosa* DC are used medicinally in Tanganyik for fever and cough and thrush in South Africa. In Northern Rhodesic feeding tests with the leaf and stem in the bovine and the sheep have given negative toxicological results.

Alysicarpus vaginalis (Linn.) DC. [Family Leguminosae-Papilionoideae]

Common names English: alyce clover; one-leaved clover.
West African: Mali Dogon Sɔ̀kámaána (CG), Nigeria Ful-Fulfulde (Nigeria)

Senkello (MM) Hausa Gàdágíí (ZOG; Etkin) Kanuri lussak (C&H), Sierra Leone

Mende Tolubele (FCD), Upper Volta Ful-Fulfulde (Upper Volta) Bundiya (K&T)

Buyaayi (K&T) Gadaji (K&T)

Uses General

- Agri-horticulture: composting, manuring; fodder; land conservation
- Phytochemistry: miscellaneously poisonous or repellent

Root

- Medicines: kidneys, diuretics; leprosy; pulmonary troubles
- Products: chew-sticks, etc.

Seed

- Phytochemistry: alkaloids; mucilage

English: Clover, Alyce; Clover, One-leaved; Alyce clover; One-leaved clover

Description A perennial herb from a tough root-stock, erect or straggling to 50 cm, in waste places of the savanna zone of Sierra Leone, Mali, Upper Volta and Nigeria, and dispersed over tropical Africa and into tropical Asia and the western Pacific.(Burkill 1997).

The leaf and twig of *Alysicarpus vaginalis* DC are under suspicion of being the cause of criminal poisoning in Swaziland. It also contains alkaloids and mucilage. (Burkill 1997)

While, *Alysicarpus zeyheri Harv. And Sond* is a South African remedy for impotence.

‘

Alysicarpus monolifer Linn has been used in indigenous system of medicine for the treatment of leprosy, cough, stomach pain, also as a purgative and expectorant.

2.4 Phytochemistry of Genus Alysicarpus

Alysicarpus monolifer Linn (Fabaceae) grows throughout India, Pakistan and Ethiopia in sandy and sub-sandy soils and in lawns especially along the coast (Varadarajan., 1985). The study indicates the potential hepato-protective activity of *Alysicarpus monolifer* whole plant through its bioactive constituents as there was no report on the hepato-protective activity of this plant.

Bioflavonoids known for their anti-oxidant and anti-inflammatory activities are implicated in the maintenance of health. They also inhibit LDL (Low Density Lipoproteins) oxidation and impact cardio protective effects (Kondo *et al*., 1996). These compounds were also known to reduce inflammation, tumurgenesis and cell damage caused by oxidation. (Dempke *et al.,* 2001).

Phytochemical analysis of *Alysicarpus monolifer* Linn, revealed C-glycosyl flavones such as vitexin and isovitexin, the bioassay with methanolic extract of whole plant recorded significant hepato-protective activity thus the scientific rationale of its traditional cure against jaundice and other ailments can be substantiated.

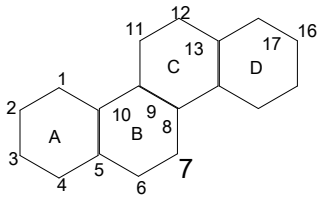
Recently, a phytochemical study was carried out on the plant genus *Alysicarpus glumaceus* (Vahl) DC which resulted in the isolation of two flavonoids .

Phytochemical studies on the whole plant of *Alysicarpus monolifer* Linn collected in Karachi (Pakistan) has resulted in the isolation and structure elucidation of a new triterpenoid of oleanic series known as alysinol (3f,22g –dihydroxyolean-12-ene) by spectroscopic techniques.

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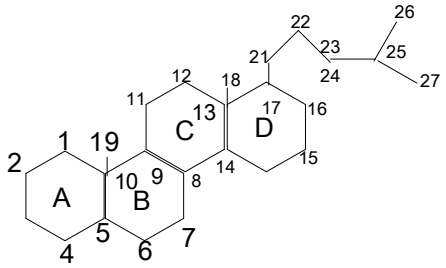
2.4.1 STEROIDS

Steroids are a class of organic compounds with a chemical structure that contains the core of gonane or a skeleton derived from it. Usually, methyl groups are present at the carbons C-10 and C-13 – an alkyl side-chain at carbon C-17 may also be present.



Steroids are structurally related compounds and widely distributed in plants and animals there are so many groups of compound belonging to the phytochemical class steroid. Example includes sterols, vitamin D, bile acids, sex hormones, adrenal cortex hormones. The steroidal nucleus is a saturated tetracyclic hydrocarbon. The nucleus, partially or completely hydrogenated, is generally substituted by methyl groups at C-10 and C-13. According to the chemical structure, the wide array of steroid molecules may be divided into several groups (Rossier, 2006).

2.4.1.1 Stereochemistry of steroids

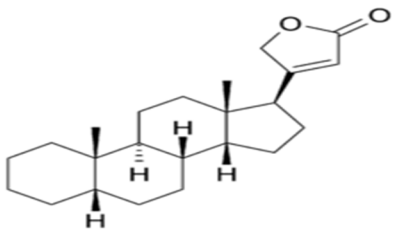


(Fully saturated sterol compound)

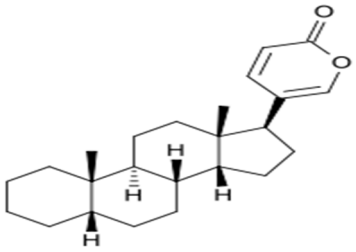
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In all natural steroids, the fusion of B/C is a *trans* because the steroid molecule is long, thin and flat, the fusion of A/B or C/D may be *ci s*or *trans* but B/C is always *trans* and thus flat. The fusion of C/D is *trans* in some steroids such as bile acid, whereas it is *cis* in cardiac glylocosides and toad poison exemplified by cardenolides and bufadienolides

cardenolides



Bufadenolides



2.4.1.2 Sterols

Sterols are formed in plants and animals in their fats and oils. The molecules of steroids are characterized by alcoholic OH group. They are solid crystalline compounds. They are isolated from un-saponable portion of oils and fat. Plant sterols and plant stanols are phytoesterols (small but essential components of certain plant membranes). They are found naturally (in very small amounts) in some vegetable oils, nuts, grains, legumes, fruits, and vegetables. Research has shown that plant sterols and plant stanols have the ability to help lower cholesterol, which is the animal cholesterol. Cholesterol is a very important sterol with molecular formula $C_{27}H_{46}O$

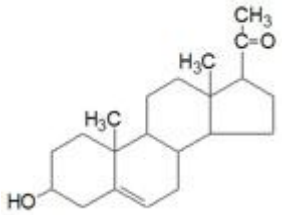
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MP149^{O}C obtained from higher animals i.e. mammals. The main sources of cholesterol are fish – liver oil, the brain and spinal cord of cattle.

Sex hormones

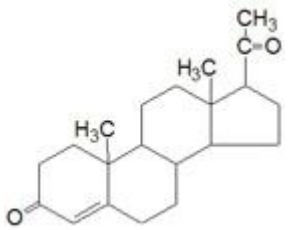
These hormones are very important and are subdivided into estrogen, progestagen and androgen. Androstenedione produced in the adrenal glands and the groups (ovary and testicles), is the common precursor of all sex hormones

Estrogens



It is also known as the female hormones or follicular hormones .The sex hormones is general are responsible for sexual processes and also responsible for the secondary characteristics which differentiates male from female. Estrogen are secreted by the ovary, it has estrogenic activity but is also present in plants pollens and seeds of date palms and seeds of pomegranate (Rulkpaul and Rein, 1990**)**

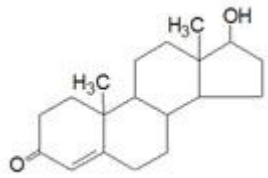
Progestagens



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Example is progesterone which is a steroid hormones involved in the female menstrual cycle, pregnancy and embryogenesis of humans and other species and the major naturally occurring human progesterone (Duport *et al,* 1998).

Androgen

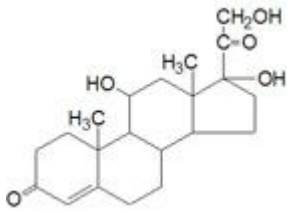


The major androgen is testosterone which is responsible for the development of male organs. This hormone is present in human male secretion such as sweat, saliva and semen and has been implicated as putative human pheromones. (Stanmorth and Jones, 2008).

Corticosteroids

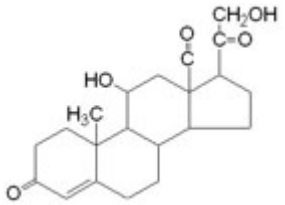
The major corticosteroids in vertebrates are cortisol and aldosterone, these steroids contains three or more oxygen atoms and have an oxygenated function at C-20.

Cortisol



‘

Aldosterone



Colour test for steroids

These are simple tests usually to infer the presence of sterol, this include; salkowski and Lieberman- Burchard test

a) Salkowski test: when concentrated H_2S0_4 is added to a solution of sterol in chloroform, a red colour at the interphase indicate the presence of steroid

b) Lieberman – Burchard Reaction: when the sample is in chloroform and a solution of concentrated H_2S0_4 in $(CH_3CO)_2$ is added, valid test which change from red to blue and finally bluish green over a period of some minutes is an indication of steroid presence

2.4.2 Saponins

Plants materials containing saponins have long been used in many parts of the world for their detergent properties these plants contain a high percentage of the glycosides known as saponin (latin sapo, soap) which are characterized by their property of producing a frothing aqueous solution. They also have haemolytic properties and when injected into the bloodstreams are highly toxic.

Saponins have a high molecular weight and their isolation in a state of purity presents some difficulties. Saponins can be divided into two classes: (a) steroidal and (b) pentacyclic-triterpenoids.

‘

a. Steroidal saponins

The Steroidal saponins are less widely distributed in nature than the pentacyclic triterpenoid type. Steroidal saponins are of great importance and interest because of their close relationship with compounds such as the sex hormones, cortisol, diuretics, steroids, vitamin D and the cardiac glycoside. Some of the steroidal saponins are used as starting materials for the synthesis of these compounds, diosgenin is the principal sapogenin employed by industries but most yams contains a mixture of sapogenin in the glycosidic form.

b.Pentacyclic Triterpenoid saponins

Unlike the steroidal saponins, pentacyclic triterpenoid saponins are rare in monocotyledons but abundant in many dicotyledons. These saponins consist of two units and a sugar. The pentacyclic triterpenoid saponin are attached to a chain of sugar or uronic acid unit or both often in the 3 position.

2.4.3 FLAVONOIDS

Flavonoids (from the Latin word flavus meaning yellow i.e. their colour in nature) are a class of plant secondary metabolites. Flavonoids were referred to as Vitamin P (probably because of the effect they had on other permeability of vascular capillaries) from the mid 1930’s to early 50’s but the term has since fallen out of use.

Flavonoids are compounds which all possess the same C15 skeleton (C6-C3-C6) flavone nucleus (Harborne, 1988), two benzene rings (A and B) linked through oxygen containing pyran or pyrone ring (C). This structure is common to 3-deoxyflavonoids (flavones, flavanones, and

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isoflavones) and 3-hydroxyflavonoids (anthocyanin, flavan-3-ol, flavan-3,4-diols, flavanonols, and flavonols) (Khnau, 1976**).**

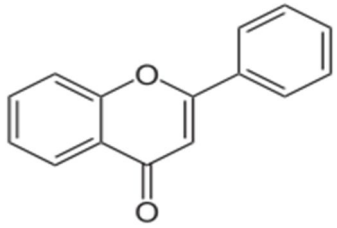
According to the IUPAC nomenclature, they can be classified into

a) Flavones: derived from 2-phenylchromen -4-one (2-phenyl-1, 4-benzopyrone) structure example: quercetin, rutin).

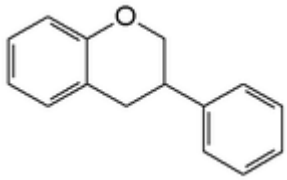
b) Isoflavonoid: derived from 3-phylchromen -4-one (3phenyl-1,4-benzopyrone structure)

c) Neoflavonoid: derived from 4-phylcoumarine (4-phyl-1,2-benzopyrone) structure .These three flavonoid classes above are all ketone-containing compounds and as such are flavonoids and flavonols.

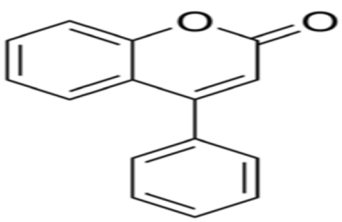
flavone structure



isoflavan structure



neoflavan structure



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Flavonoids are widely distributed in plants fulfilling many functions. They are secreted by the roots of their host plant in the infection stage of their symbiotic relationship with legumes. Flavonoids (especially flavonoids such as the catechins) are the most common group of polyphenolic compounds in the human diet is found in plants. Flavonoids are most commonly known for their antioxidant activity in vitro. The antioxidant abilities of flavonoids in vitro may be stronger than those of vitamin C and E depending on concentration tested.

Most of the flavonoids occur as glycosides in actively metabolizing plant tissues. There are hundreds of different glycosides, with glucose, galactose, rhamnose, xylose and arabinose as the most frequently found sugar moieties (Strack, 1997). The two major types of linkages are O- and C - glycosides (Harborne, 1994). Glycosyl transferase catalyzes the glycosylation of flavonoids. Flavonoid conjugation is not restricted to glycosylation, many flavonoids contain acylated sugars. The acyl groups are either hydroxyl cinnamates or aliphatic acids such as malonate.

2.5 Plants / CNS Activity

Plants have been used by human beings since immemorial times to cure diseases and to promote relief from ailments. There were times when they were the most important sources of medicines for people However, beginning in the late 1940s, this old form of therapeutics began to lose its importance, being more and more replaced by synthetic remedies The lessons from millennia were forgotten and were considered ‘‘unscientific.’’. On the other hand, such ancient use of plants was a lead for scientists in their search for new substances endowed with therapeutic properties It is estimated that nearly 25%of the modern drugs directly or indirectly originated from plants (De Smet, 1997) Several are the examples concerning the CNS: Caffeine, ephedrine, cannabinoids, opioids and reserpine are a few of them However, for the majority of CNS active

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plants, the active principles are not yet known.3. The present review shows that nature provided hundreds of CNS active plants covering the whole spectrum of activity such as psychoanaleptic, psycholeptic and psychodysleptic effects.

2.6 Diazepam

Diazepam is a fast-acting benzodiazepine agent with intermediate duration of action. It is quite likely the most commonly prescribed sleep medication in the US. As far as its pharmacological action is concerned, Diazepam is a potent sedative-hypnotic agent, with marked anti-anxiety and muscle relaxant effects.The pharmacological action of diazepam enhances the effect of the neurotransmitter GABA by binding to the benzodiazepine site on the $GABA_A$ receptor (via the constituent chlorine atom) leading to central nervous system depression. (Mandrioli *et al.,* 2008)

Diazepam binds to a specific subunit on the $GABA_A$ receptor at a site distinct from the binding site of the endogenous GABA molecule, known as an allosteric site. The $GABA_A$ receptor is an inhibitory channel which, when activated, decreases neuronal activity. Benzodiazepines do not supplement for the neurotransmitter GABA, rather benzodiazepines such as diazepam bind to a different location on the $GABA_A$ receptor, resulting in enhanced GABA effects. Benzodiazepines cause an increased opening of the chloride ion channel when GABA binds to its site on the $GABA_A$ receptor, leading to more chloride ions entering the neuron, which in turn leads to enhanced central nervous system depressant effects. Diazepam binds non-selectively to alpha1, alpha2, and alpha3 and alpha5 subunit containing $GABA_A$ receptors. (Atack, 2005)

‘

CHAPTER THREE

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Chemicals and Reagents Used

All organic solvents used were of analytical grade; all equipment and their capacity were quoted accordingly and operated using standard procedure. The silica gel for column chromatography was of mesh size (60-120).

3.1.2 Collection and Identification of the Plant Material

The plant *Alysicarpus ovalifolius* was collected around Zaria, Nigeria in October, 2009. The plant was identified and authenticated at the Herbarium of Biological Science Department, Ahmadu Bello University, Zaria by comparison with a herbarium specimen (voucher number 433)

The plant material was air-dried under shade for weeks, the aerial part was grounded into powder form and a total of 500g of the powder was obtained..

3.1.3 Instruments Used

Ultraviolet light (254 and 366): Ultraviolet product insurance

Nuclear magnetic resonance (NMR) spectra on Bruker (Billerica, MA, USA) DRX 400 spectrometer operating at 400 MHZ.

3.2 METHODS

3.2.1 Extraction Procedure

The air-dried powder was exhaustively defatted with petroleum ether (60-80^{O}C) using soxhlet apparatus for 24 hours. The extract was concentrated using Rotary evaporator. The concentrated petroleum ether extract was evaporated to dryness to give 2.0g of very dark green residue. The defatted marc was dried to give 30.31g of dark brown residue dissolved in methanol.

3.2.2 TLC profile for the methanolic extract

Various TLC solvent systems were tried so as to have information on the methanolic extract. Ascending technique was employed and the silica gel precoated glass plate was the stationary phase. The spots were applied manually with capillary tubes and the plates were developed at room temperature in an air tight chromatographic chamber containing the solvent system. Ethyl acetate: methanol: water (5:1:1), ethyl acetate: methanol (5:2), ethylacetate: methanol: water (5:4:1), n-butanol: ethyl acetate: water (1:2:3), ethyl acetate: chloroform: methanol: water (15:8:4:1) and several others were tried but ethyl acetate: methanol: water (5:4:1)gave the best separation.

The developed chromatogram were dried in the air and visualized under ultraviolet light (254 and 366nm), sprayed with 10% sulphuric acid and heated for about 5 minutes in the oven and also the iodine chamber was similarly employed for visualization.

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3.2.3 Preliminary Phytochemical Screening

Both the petroleum ether extract and the methanol extract were subjected to preliminary phytochemical screening using standard procedures.

a. Test for Anthraquinone Derivatives

i. Test for free Anthraquinones (Borntrager’s Test)

About 0.5g of the methanol extract was shaken with 10mls of benzene and filtered.5mls of 10% ammonia solution was and shaken, presence of a pink-red or violet colour indicates the presence of free anthraquinones.(Trease and Evans, 2002)

b. Test for Cardiac Glycosides.

i. Legal Test

Solution I 0.5% of a recently prepared sodium nitroprusside in water and solution II 0.2M NaOH. About 0.2g of methanol extract was dissolved in 3 drops of pyridine, 1 drop of solution I was added followed by four drops of solution II one at a time. A deep red colour confirms presence of cardiac glycosides.(Trease and Evans,2002)

ii) Keller – Killiani Test

Test: 0.5g of the methanolic extract was dissolved in 2ml of glacial acetic acid containing I drop of ferric chloride solution. A reddish brown colour ring obtained at the interphase indicates the presence of steroids.(Trease and Evans,2002)

iii) Salkowskii Test About 0.2g of methanolic extract was dissolved in 2mls of chloroform and 1ml of concentrated H_2S0_4 was added down the test tube to form a lower layer. A reddish brown

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or yellow colour at the interphase indicates the presence of steroidal ring. (Trease and Evans, 2002)

c. Test for Saponins

i) Frothing Test

About 0.5g of the methanolic extract was shaken with water in a test tube, formation of froth that persisted for more than 15minutes indicates the presence of saponins. (Sofowora, 1993).

d. Test for Steroids and Triterpenes

i) Lieberman – Burchard Test

About 0.2g of methanolic extract was dissolved in 1ml chloroform and 1ml of acetic anhydrous, all were cooled on ice. The solution was transferred into test tube and a drop of concentration H_2SO_4 was added down the side of the test tube. Violet, pink or red colour indicates the presence of triterpenes while blue or blue-green is taken as positive test for steroids and triterpenes (Silva *et al*., 1992).

e) Test for Flavonoids

i) Shinoda Test

About 0.3g of methanolic extract was dissolved in 2 mls of 50% methanol, warmed and filtered, three pieces of magnesium chips were added followed by a few drops of concentrated hydrochloric acid. Orange or red colour indicates the presence of flavonoids. (Trease and Evans, 2002)

‘

ii) Sodium Hydroxide Test

A small quantity of the methanolic extract was boiled with water and filtered. 2mls of the filtered extract was dissolved in 10% aqueous sodium hydroxide solution to give a yellow colour, a change in colour from yellow to colourless on addition of dilute HCl indicates the presence of flavonoid.(Trease and Evans, 2002)

f) Test for Tannins

i. Ferric Chloride Test

A small quantity of the methanolic extract was boiled with water and filtered, two drops of ferric chloride were added to the filtrate, formation of a blue-black precipitate indicates hydrolysable tannins and green precipitate indicates the presence of condensed tannins. (Trease and Evans, 2002)

g. Test for Alkaloids

i) Wagner’s Test

Few drops of Wagner’s reagent were added to 2mls of the methanolic extract. Formation of whitish precipitate indicates the presence of alkaloids. (Richard, 1998).

ii) Dragendoff’s Test

Few drops of Dragendoff reagent were added to 2mls of the methanolic extract. Formation of rose-red precipitate indicates the presence of alkaloids. (Richard, 1998**).**

‘

3.2.4 Partitioning of Methanolic Extract

The dried methanolic extract was suspended in distilled water, stirred very well; about 200ml of petroleum ether was added and then shaken. The mixture was allowed to stand for some minutes so as to obtain clear separate layers of aqueous and petroleum ether. This procedure was repeated until the petroleum ether used became colourless. The petroleum ether was collected in an evaporating dish to dry and it was labeled as PEF (Petroleum ether fraction).

This procedure was repeated using ethyl acetate and n-butanol in order of increasing polarity and they were labeled appropriately as follows:

Petroleum ether fraction PEF

Ethyl acetate fraction EAF

n-Butanol fraction BF

Aqueous fraction AF

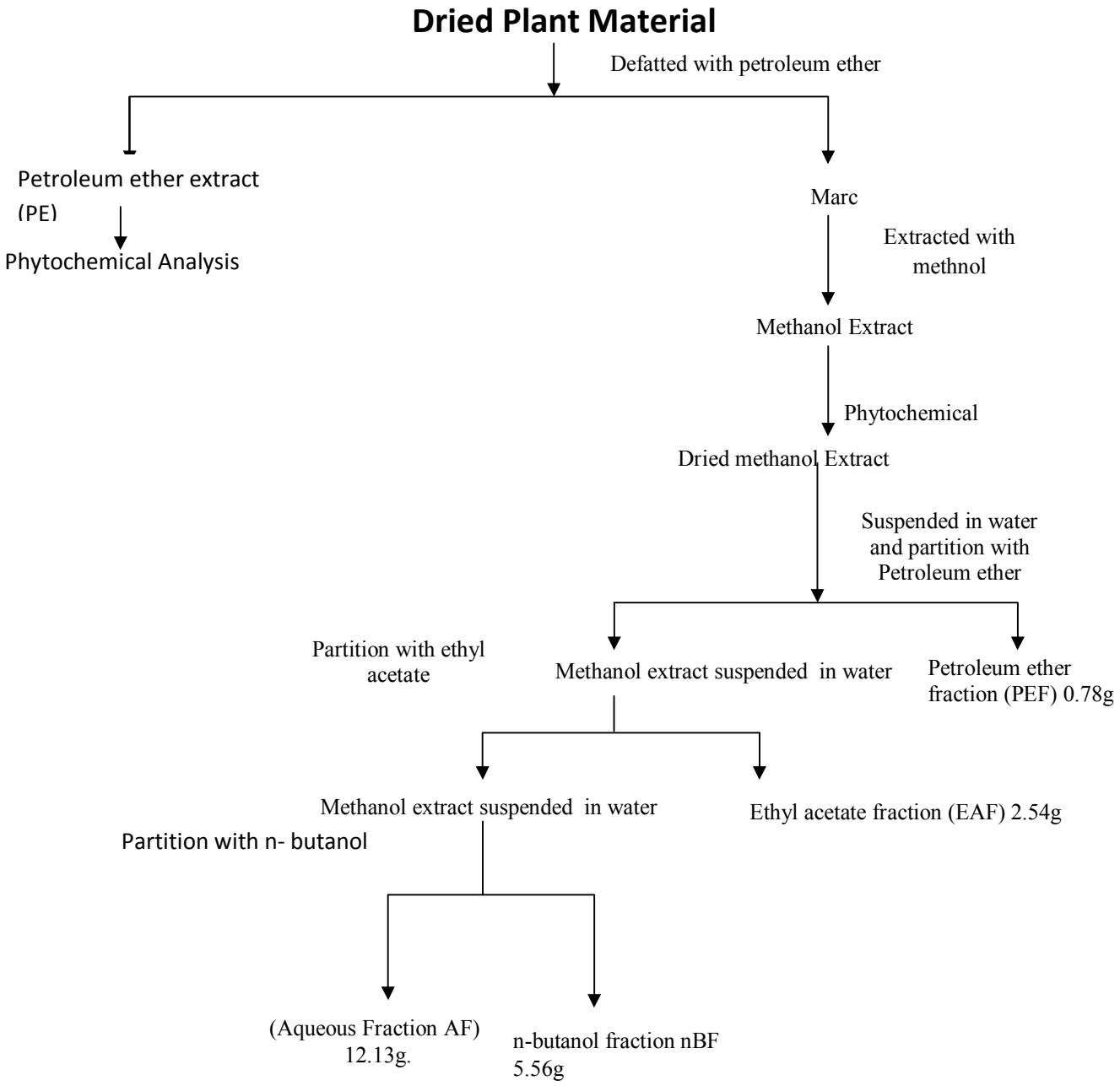


Fig. 3.1: Scheme of extraction and partition of the methanolic extract.

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3.2.5. Column Chromatography

Silica gel (60-120 mesh size) was used as the adsorbent and packed by slurry method into a sintered glass column (4.0 × 75cm).

Small quantity of n-butanol fraction (n-BF) was dissolved in a little portion of n-butanol and was absorbed on silica gel. This was allowed to dry properly then grounded into a very fine powder. This fine powder was introduced into the well packed and stabilized silica column. Small amount of chloroform was added and a little amount of cotton wool was used to cover it. More of the chloroform was used to top the column and the column was run. Starting with 100% chloroform, a gradient elution technique was employed with methanol added to gradually increase the polarity. Eluents were collected in 50mls beakers and the progress of the separation was monitored by thin layer chromatography, consecutive fractions that showed similar TLC profiles were combined and allowed to dry at room temperature.

‘

Table 3.1 Column Chromatography separation of components of the n-butanol fraction of t methanolic extract

S/N	Elements	Eluting Solvents (%) chloroform: Methanol	No of spots on TLC	Code
1	1-17	100:0	-	B1
2	15-25	100:0	3	B 2
3	26-48	100:0	4	B3
4	49-63	95:0	4	B4
5	64 -71	90-10	4	B5
6	72-80	80-20	5	B6
7	81-86	70-30	5	B7
8	87-111	60-40	6	B8
9	112-138	50-50	6	B9
10	139 – 147	40-60	7	B10
11	148 – 164	30-70	6	B11
12	165 – 181	30-70	6	B12
13	182 – 196	20-80	7	B13
14	197 – 217	10-90	6	B14
15	218 - 227	0-100	7	B15

3.2.6 Thin Layer Chromatography

Ascending technique was employed and the stationary phase was the glass plate precoated with silica gel. Spots were applied manually using capillary tubes and the plates developed at room

‘

temperature in an air tight chromatographic tank containing solvent system (n-butanol: acetic acid: water,4:1:5 (upper layer). The developed chromatograms were dried in the air and visualized under the ultraviolet light (254 and 366nm), sprayed in the fume cupboard with 10% sulphuric acid and heated in the oven for about five minutes.

3.2.7 Gel Purification

B11 from pooled fractions was severally run in sephadex column eluted with 100% methanol. The eluents were monitored with thin layer chromatography and solvent system n-butanol: acetic acid: water (4:1:5 upper layer) was used. A sub fraction was collected with two spots which gave yellow colour on spaying with sulphuric acid. This fraction was coded C2 whereas fractions of B8 produced white crystals, this was run consecutively on sephadex to yield another sub fraction with one spot monitored with thin layer chromatography and solvent system ethylacetate: methanol: water, (3:2:1), this fraction was coded E17.

3.2.8 Preparative Thin-Layer Chromatography

The sub-fraction C2 was loaded on silica gel precoated glass plate about 1.5 cm from the edge of the plate and allowed to dry. This loaded plate was put in a chromatographic tank containing the solvent system n-butanol : acetic acid : water (4:1:5 upper layer).The plate was air-dried and viewed under UV light.

Each distinctive spot was scrapped separately and eluted with methanol,eacheluate was air-dried and monitored with thin layer chromatography, two distinct spots were collected and coded D7 and D10.

‘

3.2.9 Spectral Analysis of D7

The UV spectra were recorded in methanol and the ^{1}H-NMR spectra was run in hexa deuterium dimethyl sulphoxide DMSO-d6.

3.2.10 Pharmacological Studies

The studies involved whole animal which were carried out on mice to ascertain the plant for central nervous system activity (stimulant and exploratory behavior)

3.2.10.1Acute toxicity study

The Intraperitoneal LD50 study was performed in mice using the Method of Lorke (1983). The study was divided into two phases; using thirteen mice (the first phase had nine mice and the second phase had four mice).

In the first phase, mice of either sex were divided into three groups of three mice each. About 0.3g of n-butanol fraction was dissolved in 3ml of water to make stock solution. Group I received 1000mg/kg of stock solution while group II received 100mg/kg and group III received 10mg/kg of the stock solution respectively. The mice were observed for signs and symptoms of toxicity and mortality for twenty four hours after treatment with the n-butanol fraction.

In the second phase; four mice were divided into four groups of one mouse each. The first received plant extract at a dose of 140mg/kg while the second received 225mg/kg the third and fourth received doses at 370mg/kg and 600mg/kg respectively based on the result of the first phase. The median lethal (LD_{50}) was calculated as the square root of the product of the lowest lethal dose and the highest non-lethal dose i.e. the geometric mean of consecutive doses for which 0 and 100% survival rate was recorded

‘

The Interperitoneal LD_{50}= $\sqrt{a \quad + \ b}$

a = lowest -lethal dose

b = highest non- lethal dose

3.2.10.2. Diazepam Induced – Sleep Test

Twenty four mice were divided into 4 groups of six mice each. Group I received 2mls/kg of Diazepam and normal saline (control). Groups II, III and IV received 2mls/kg of diazepam treated with extract at doses of 50mls/kg, 25mls/kg and 12.5mls per kg respectively after one hour of administering the extract. The onset and duration of sleep were duly observed and recorded.

3.2.10.3. Hole Board Experiment

Thirty mice of 5 groups with each group having six mice each. Group I was treated with normal saline as control group while groups II, III and IV received extract at doses of 50mls/kg, 25mls/kg and 12.5mls/kg. Group V received Diazepam 2mls/kg (small dose of diazepam used here as a sedative). The number of head dip per time (5mins) was recorded.

‘

CHAPTER FOUR

4.1.0 RESULTS OF PHYTOCHEMISTRY

4.1.1 Percentage yield of extraction

The percentage yield of petroleum ether extract was calculated to be 0.4% and 6.1% for the methanolic extract.

The petroleum ether extract produced a very dark green mass (2g) and the methanol extract gave a brown mass (30.31g).

4.1.2 Phytochemical screening

The phytochemical screening of petroleum ether extract revealed the presence of cardiac glycosides, steroids and triterpenes. Methanol extract revealed the presence of saponins, flavonoids, cardiac glycosides, steroids and triterpenes .

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4.1.2 Result of the Preliminary Phytochemical Screening

Table 4: 1 Phytochemical constituents of methanolic extract and petroleum ether extract

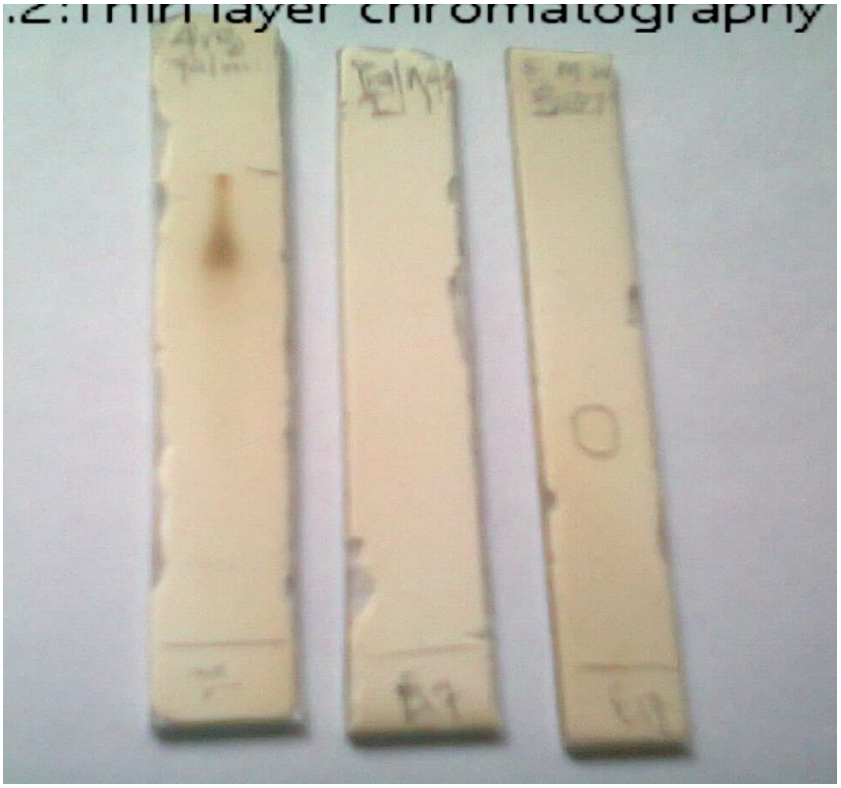
S/N	Compound	Test	Observation	Inference	
				Methanol	**petroleum ether**
1	Anthraquinones	a. Free anthraquinone b. Combined anthraquinone	No colour change No colour change	- -	- -
2	Cardiac Glycosides	a. Legal test b. Keller – Killiani c. Salkowski	Deep red colour Reddish brown Reddish brown	+ + +	+ +
3	Saponins		Frothing	+	-
4	Steroid/ Triterpenes	a. Lieberman-Burchards	Pink – blue	+	+
5	Flavonoids	a. Shinoda test b. Sodium hydroxide	Orange colour Yellow	+ +	- -
6	Tannins	a. Ferric Chloride	No precipitate	-	-
7	Alkaloids	a. Mayer’s reagent b. Dragendoff test c. Wagner’s test	No precipitate No colour change No colour change	- - -	- - -

+ = Presence of constituents

= absence of constituents

‘



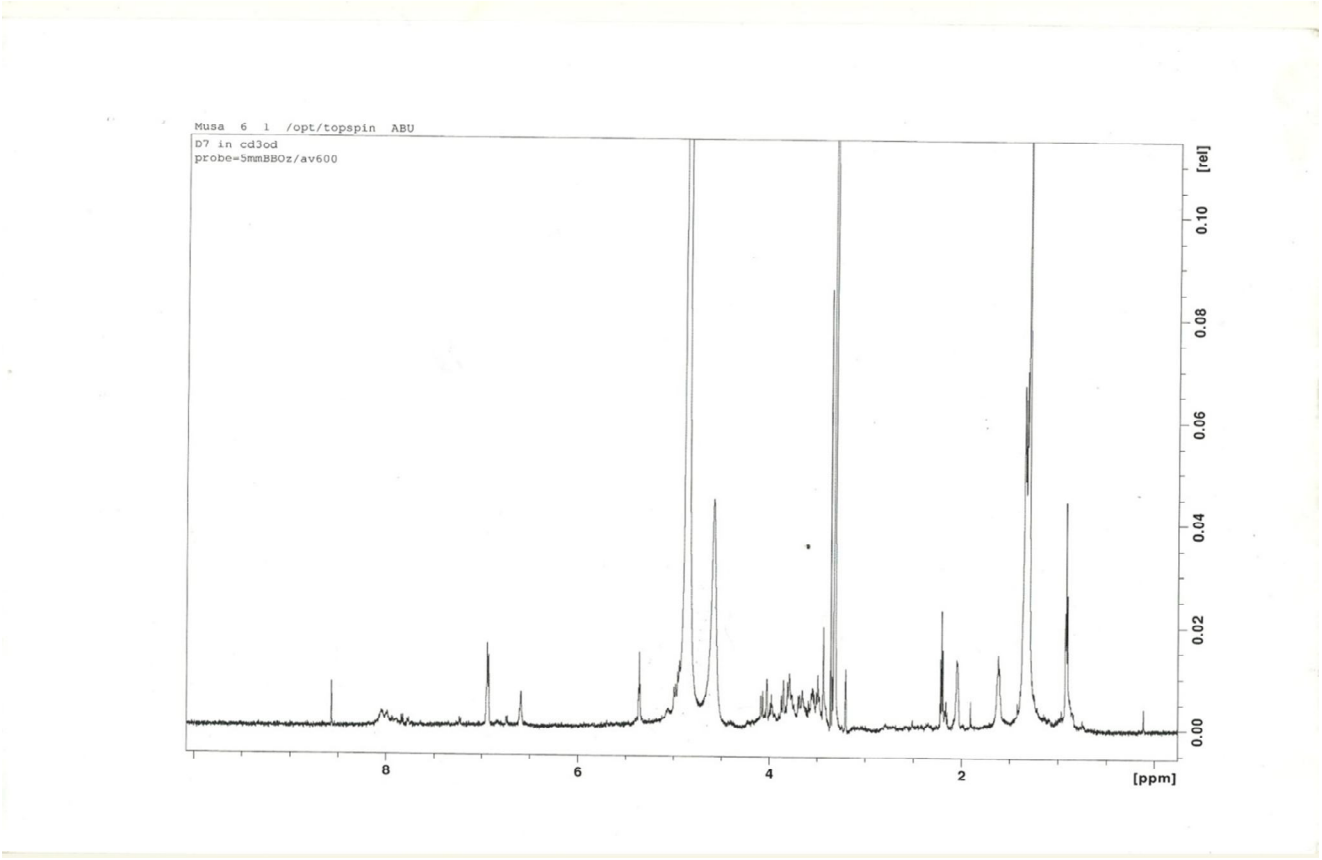


TLC solvent system; n-butanol: acetic acid: water (4:1:5 upper layer)

Fig.4.1 TLC chromatogram of the isolated compounds (D7 and E17)

‘

4.2 Proton nuclear magnetic resonance (^{1}H-NMR, CD3od) spectra for D7



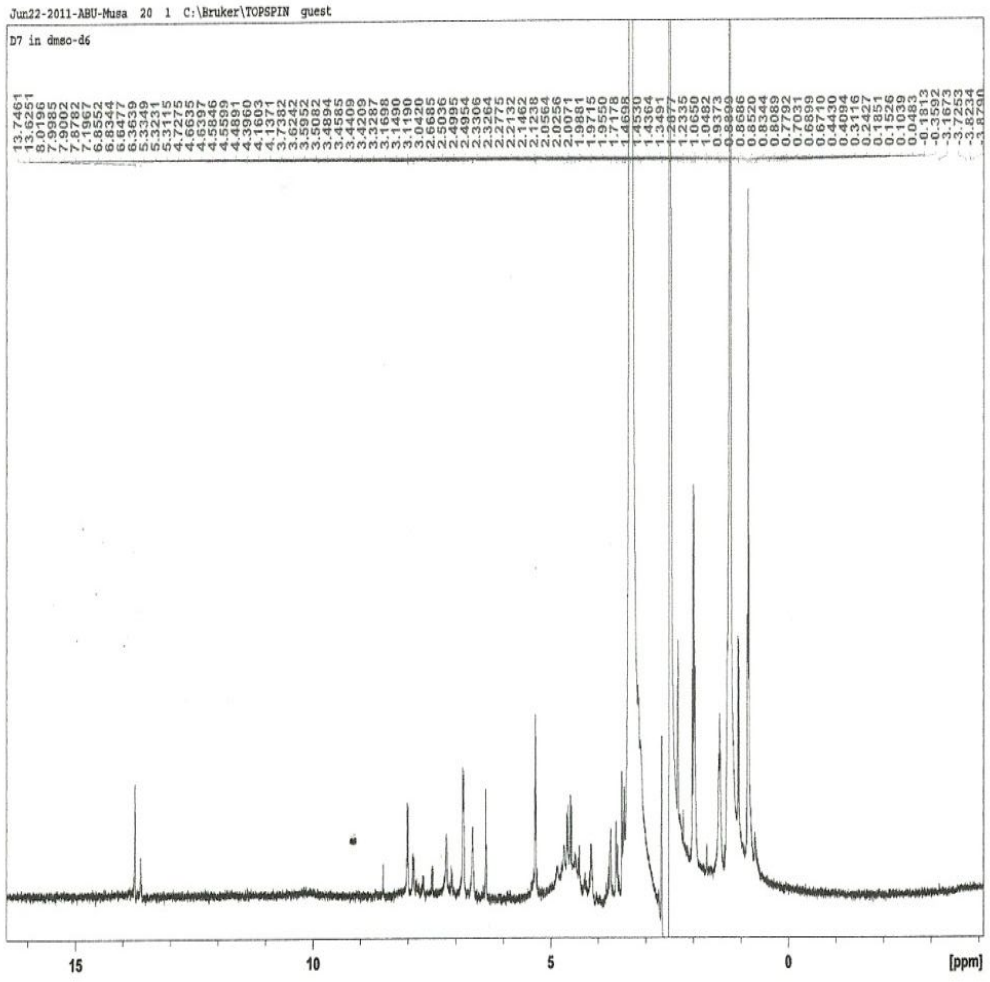


Fig.4.2. Expanded proton nuclear magnetic resonance of D7

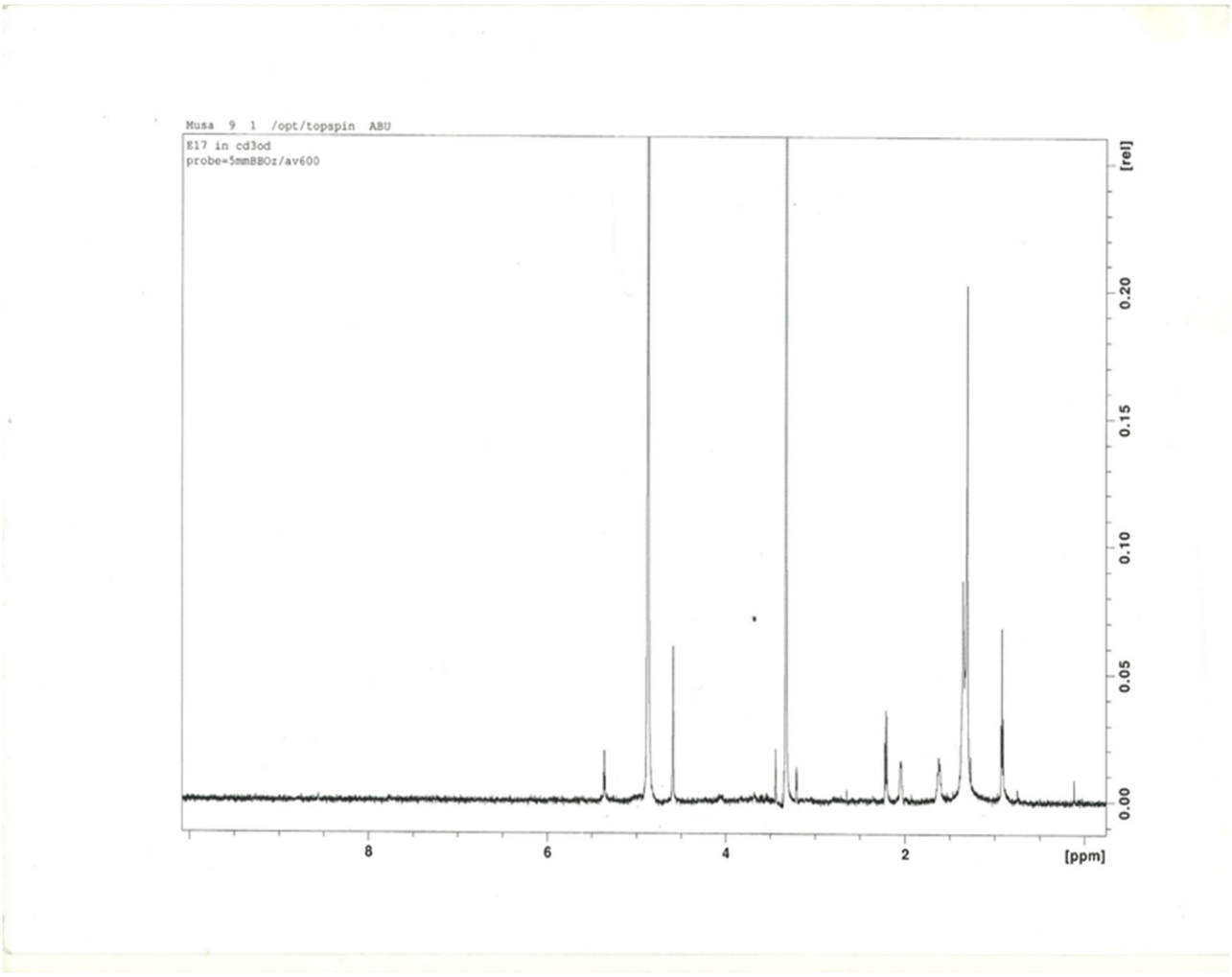


Fig4.4 Proton NMR of E17

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4.2 Results of Central Nervous System Activity Study

4.2.1 Result of acute toxicity test of n-butanol fraction of methanolic extract of *Alysicarpus ovalifolius* (Schum&Thonn)

Stock solution: 100mg/ml 10mg/ml 1mg/ml

The route of administration is intraperitoneal and the duration of study was for 24 hours and the LD_{50}

result for the n-butanol fraction was 177.48mg/kg.

Acute toxicity value for n-butanol was 177.48mg/kg

Table 4.2 Effect of n-butanol fraction n-BF on diazepam induced sleep in mice

Treatment (mg/kg)	Mean onset of sleep (min.)	Mean duration of sleep (min)
Normal saline	6.6± 1.1	29.±07.2
Extract (50)	2.3 ± 0.3^{b}	154.3 ± 17.1^{c}
Extract (25)	2.8 ± 0.4^{b}	93.6 ± 2.6^{c}
Extract (12.5)	4.8±0.7	$66.\pm10.7^{b}$

‘

Table 4.3 Effect of the n-butanol fraction of methanolic extract of *Alysicarpus ovalifolius* on exploratory behavior

Treatment (mg/kg)	No. of head dips in 5min.
Normal saline	18.8±3.3
Extract (50)	14.8±1.7
Extract (25)	21.3±1.8
Extract (12.5)	17.6±2.0
Diazepam (2)	10.8±1.4[a]

Data presented as mean ± standard error of mean, student t –test, n=6. The values a, b and c as superscript are statistically significant at $p \leq 0.01$ and 0.001 respectively.

‘

CHAPTER FIVE

DISCUSSION

The dried powder of methanol extract (500g) of *Alysicarpus ovalifolius* plant was extracted with petroleum ether using the soxhlet apparatus to defat it thereby removing the non-polar constituents. The percentage yield of the petroleum fraction was found to be 0.4%.This percentage yield is very low and poor; this may be due to the fact that there are very few non polar constituents present in the extract. The percentage yield of the methanol extract was found to be 6.1%. Based on this result, the methanol extract has more phytochemical content than the petroleum ether extract. The result of the percentage yield of the other fractions are as follow; 0.16% PEF, 0.51% EAF and 1.11% n-BF. The phytochemical screening of the two extracts revealed the presence of cardiac gylcosides, steroids and triterpenes in petroleum ether extract while saponins, flavonoids, cardiac glycosides, steroids and triterpenes were present in the methanolic extract.

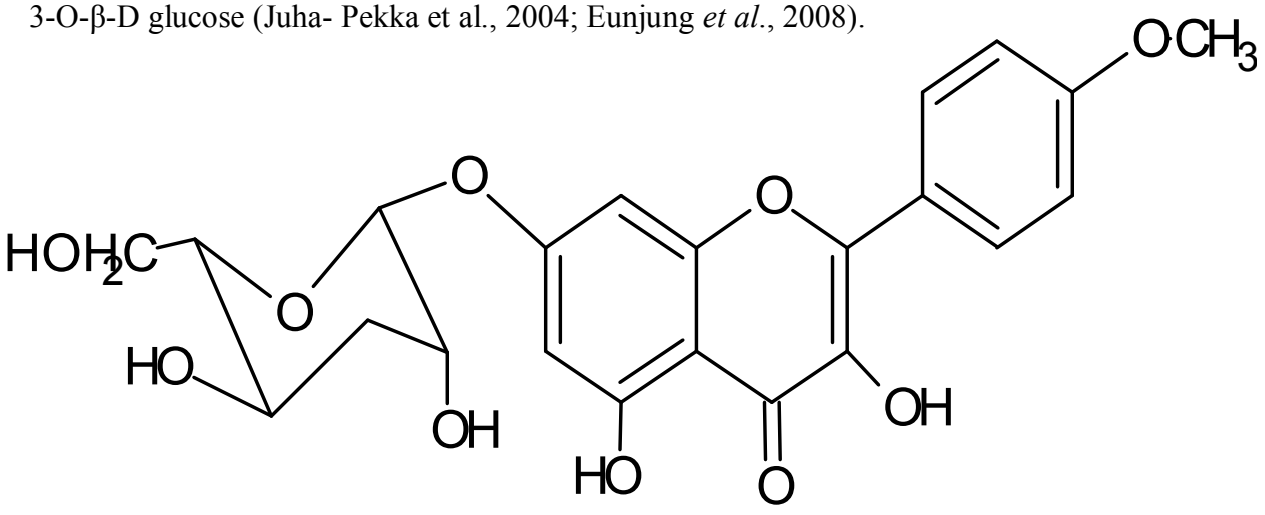
COMPOUND D7

Column Chromatography separation of n-butanol fraction led to isolation of a compound that was positive for shinoda and sodium hydroxide test (yellow colour change) which suggest presence of flavones, bio flavones or flavononol nucleus. The result is further supported by proton NMR of the isolated compound run in DMSO- d_6. The broad singlet at δ 13.75 (1H s) and δ8.53 were assigned to the C-5 and C-3 chelated hydroxyl proton respectively (Dipti and Singh 1991).The signal at δ3.74 was assigned to methoxy group, in flavonoids with few exceptions, signals appear in the region around 3.5- 4.1ppm.The 7-O glycosyl have chemical shift at 4.65(1H d J 9.52), the large coupling constant indicates β linkage of the glycosyl moiety, glucose

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commonly forms β –linkage with high coupling constant (Yaqing *et al*., 2007; Hideyuki *et al*., 2001)

D7 was obtained as a bright yellow powder with Rf value of 0.4 (n-butanol: aceticacid: water; 4:1:5 upper layer).The characteristic bright yellow colour change positive for shinoda and sodium hydroxide test for flavonoids. (Paolo *et al*., 2001; Xu *et al.,*). ^{1}H NMR (400MHz, DMSO-d_6) δ13.75 (1H,s -5-OH) and δ8.53(1H,s -3 OH), (Dipti and Singh, 1991) ,δ6.85 (2H d - 3^1 5^1-H J 8.32Hz), δ6.36(1H,s -8H), δ3.46 (1H m – 3^2 5^2 6^2 H), δ3.74 (3H s – 4^1 OME). Diagnostic peaks to identify flavonoid derivative with OH group on C3- C5 –C7 appear at sharp peaks at δ13.75(1H s 5-OH) and δ8.53(1H,s-3OH).The signals at 6.85(1H 3^1 5^1) are the protons on 3^1 5^1 while the signals at δ6.65(s 1H) and s 1H) are proton signals on C-3and C-8 typical of flavones skeleton. The singlet at 3.74(4-OCH3) is evidence for one methoxy group. The sharp doublet signal at δ6.21 and δ6.43 is assigned to 6-H and 8-H (Ruben and Wagner, 2005) .Being easily assigned by consideration of symmetry H2^1 and 6^1 occur at sharp doublet at δ8.20 ppm. Based on the reasons above the compound was named 3, 5, dihydroxy - 4^1 – methoxy – 7 – O – β glycosyl flavones. The chemical formula for compound D7 is $C_{19}H_{22}O_{1o}$ and the molecular weight is 410. Compound exhibited ^{1}H NMR /^{13}CNMR chemical shift identical to Kaempferide-3-O-β-D glucose (Juha- Pekka et al., 2004; Eunjung *et al*., 2008).

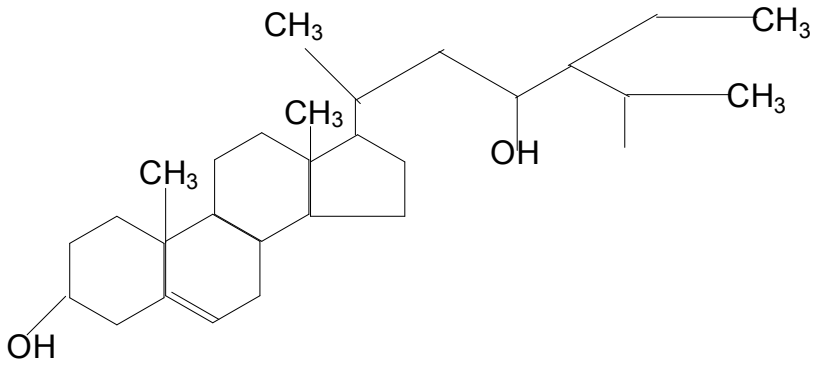


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3, 5, dihydroxy, 4^1 methoxy, 7 – O – β glycosyl flavone

COMPOUND E17

E17 was obtained as white crystals with Rf value of 0.92 (ethyl acetate: methanol: water 3:2:1). H-NMR (400MHZ DMSO-d_6) of the isolated compound showed signals at δ5.38 (1H,s H.6) signifying oleifinic proton. The signal at δ3.45 show the presence of OH group for proton adjacent to alcohol, δ3.45 (1H,s – H3) (Xu and Zeng, 2000). δ2.05 (H3 and H2) methine proton a (Lopez *et al.,*2008), δ0.9 (H – 18, H19) which is characteristic of 5-ene – 3 β – hydroxyl sterol, δ1. 60, δ0.90, δ0.77 are methyl protons, these are series of proton signals within the region of δ1.0 – δ1.8 which are attributed to resonance of overlapping of methylenes and methines, a characteristics framework of steroids (Yun – Song *et al*., 2006). E17 was compared with Taraxerol (Ogihara *et al.,* 1987) and spectra values are similar.Hence, E17 was characterized as a steroid (3-β – cholest – 5- ene 3, 21 -diol ($C_{30}H_{55}O_2$)



3-β – cholest – 5 ene – 3, 21 – diol

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5.2 PHARMACOLOGICAL STUDIES

The acute toxicity study suggest the n- butanol extract of *Alysicarpus ovalifolius* (LD50)was 177.48mg/kg, suggesting that it is not toxic as the greater the number of doses causing the mice to “react”without killing any of them, the more effective and non toxic, the drug (Campbell and Richter, 1967). The drug (diazepam) induced- sleep test showed that the n-butanol fraction at 50mg/kg, 25mg/kg and 12.5mg/kg significantly increased the duration of sleep compared to the control while the onset of sleep was reduced significantly by the 50mg/kg and 25mg/kg extracts. All the extracts at all the doses used, significantly ($p \leq 0.01$ and $p \leq 0.001$) increased the duration of sleep, thereby suggesting that the fraction has the ability to induce sleep. How best to measure exploratory behavior in mice remains a contentious issue. Many unconditioned test such as the open field potentially confuse general loco motor activity with exploration. The hole –board apparatus appears to avoid this confusion, as head dipping in holes in the floor is assumed to be a valid measure of the subject’s attraction towards novelty (neophilia), (Boissier and Simon 1962). The result showed that head dip initially decreased across repeated exposures then increased .Again the large proportion of repeats suggest that the drug possess stimulant activity rather than exploratory activity.(Boissier and Simon, 1962).From the experiment above, diazepam reduced the exploratory activities of the mice while there was significant increase in activity especially when the 25mg/kg dose of the extract was administered. The result support the fact that the n-butanol fraction of *Alysicarpus ovalifolius* may possess some stimulant activity.

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CHAPTER SIX

6.0 Conclusion and Recommendation

6.1 Conclusion

The phytochemical screening revealed the presence of cardiac glycosides, steroids and triterpenes in petroleum ether extract while the methanolic extract shows the presence of cardiac glycosides, saponins, flavonoids, steroids and triterpenes. The two compounds isolated from then-butanol fraction of the methanolic extract revealed a glycosidic flavone and a steroid based on chemical tests, proton nuclear magnetic resonance and comparative literature review. From the result of the pharmacological studies, the acute toxicity test result showed that the n-butanol fraction is not toxic. The onset of sleep and duration of sleep were significantly affected and the observed stimulant effect could be attributed to the flavonoid present in the n-butanol fraction.

6.2 Recommendation

From the research work carried out it is recommended that extensive work should be done to isolate more compounds and confirm their structures, also to investigate more constituents from other fractions of the plant.. Toxicity study should be carried out on the different fractions. The research has shown that the extract of *Alysicarpus ovalifolius* isolated contains flavonoids and that it has some stimulant activity and this may due to the flavonoid isolated**.**

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APPENDIX

Appendix 1: Result for Phytochemical testing of the petroleum ether and methanolic extracts

Constituents	Test	Observation	Inference	
Anthraquinones	Borntrager’s	No color change	-	-
Cardiac glycosides	Keller – killiani	Colour change	+	+
	Salkowski	Reddish brown	+	+
	Legal test	Deep red colour	+	+
Saponins	Frothing	Frothing observed	-	+
Steroids and Triterpenes	Lieberman Burchard	Colour change	+	+
Flavonoids	Shinoda test	Orange colour	-	+
	Sodium hydroxide	Yellow colour	-	+
Tannins	Ferric chloride	No colour change	-	-
Alkaloids	Mayer’s test	No colour change	-	-
	Dragendoff ’s	No colour change	-	-
	Wagner’s test	No colour change	-	-

+ = Presence

-= Absent

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Appendix II: Result for Acute toxicity testing of the n-butanol fraction

First Phase.

S/N	Weight (g)	Dose (mg/kg)	Percentage of survival.
1	28	1000	
2	29	1000	0
3	29	1000	
4	30	100	
5	30	100	100
6	28	100	
7	31	10	
8	27	10	100
9	27	10	
Second phase			
1	28	140	0
2	30	225	100
3	29	370	100
4	29	600	100

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Appendix III: LD50 Calculation

LD_{50} for n- butanol fraction = $\sqrt{\text{highest non} - \text{lethal dose x lowest lethal dose}}$

= $\sqrt{140\ X\ 225}$ = 177.48mg/kg