PHYTOCHEMICAL AND ANTIFUNGAL STUDIES OF METHANOL LEAF EXTRACT OF *TAPINANTHUS GLOBIFERUS* (A.Rich) Tiegh GROWING ON *VITEX DONIANA*

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AUGUST, 2018

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ZARIA

AUGUST, 2018

DECLARATION

I declare that the Dissertation entitle ”PHYTOCHEMICAL AND ANTIFUNGAL STUDIES METHANOL LEAF EXTRACT OF *TAPINANTHUS GLOBIFERUS* (A.RICH) TIEGH GROWING ON *VITEX DONIANA*” has been performed by me in the Department of Pharmaceutical and Medicinal Chemistry, Ahmadu Bello University, Zaria. The information derived from the literature has been duly acknowledged in the text and a list of references was provided. No part of this Dissertation was previously presented for another diploma or degree at this or any other institution.

Hassan Abubakar	_____	_____
Name of student	signature	Date

CERTIFICATION

This dissertation entitled "PHYTOCHEMICAL AND ANTIFUNGAL STUDIES OF *TAPINANTHUS GLOBIFERUS* (A.RICH) TIEGH GROWING ON *VITEX DONIANA*" by Hassan ABUBAKAR 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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DEDICATION

This work is dedicated to my parents and well-wishers.

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All praise, total submission and obedient are due to Allah (S.W.A). The Lord of the universe. I thank him for seeing me through this work.

My profound gratitude goes to my supervisors Prof A. M. Musa and Dr. M. I. Abdullahi for their encouragement, patient, sound criticisms, absolute guidance and devoting their time, energy and resources for the success of this work. This work could not have been possible without their ample support.

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ABSTRACT

Tapinanthus globiferus (A.Rich) (Loranthacae)is a semi-parasitic plant growing on several plant sepecie such as *Vitex doniana, Vitellaria paradoxa, Kola*, *Citrus,Combretum, Acacia, Aloe* and *Terminalia*. Itis used in ethno-medicine for the treatment of fungal infection, itching, hypertension, ulcers, epilepsy, diabetes and cancer. The leaves of the plant was subjected to maceration using methanol to afford a methanol extract which was further subjected to partitioning with n-hexane, chloroform, ethylacetate and n-butanol. Preliminary phytochemical screening on methanol leaf extract and its fractions (ethylacetate and n-butanol fractions) was carried out according to standard procedures and the result revealed the presence of flavonoids, saponins, steroids, cardiac glycosides, tannins, alkaloids and carbohydrate. Chromatographic studies using silica gel column followed by gel filtration chromatography led to the isolation of two flavonoids viz 2-(3',4'-dihydroxyphenyl)–3,5,7-trihydroxy-4*H*-chromane-4-one and 2-(2,4-dihydroxyphenyl)-5,7-dihydroxy-3-(3-methylhexyl)-4*H*-chromen-4-one. The two structure of compounds were established using spectroscopic analysis. Preliminary antifungal screening was carried out using agar well diffusion method. Four clinical fungal isolates, *Candida albicans, Trychophyton mentagrophytes, Trychophyton rubrum* and *Aspergillus niger* were used for the study. Four concentrations of exract/fractions at 100 mg/mL, 50 mg/mL, 25 mg/mL and 12.5 mg/mL and that of standard drug (fluconazole 0.05 mg/mL) were used. The mean zone of inhibition of methanol leaf extract, ethylacetate and n-butanol fractions range from 9.83 to 27.83 mm while that of the fluconazole range from 18.49 to 26.16 mm. The minimum inhibitory concentration (MIC) was determined using broth micro-dilution method, the MIC range from 0.39 mg/mL to 25.0 mg/mL. Minimum fungicidal concentration (MFC) range from 0.39 to 25 mg/mL against the test organisms. The result of this study validated the use of the plant in ethno-medicine to treat pathogenic and opportunistic fungal infections and that the isolated compounds might serve as lead compounds with better antifungal potential.

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Abbreviations and Acronyms

DMSO:	Dimethyl sulfoxide
SDA:	Sabouruad dextrose agar
VLC:	Vacuum liquid chromatography
TLC:	Thin layer chromatography
CME:	Crude methanol extract
HEX:	n-Hexane
EtOAc:	ethylacetate
H_2O:	Water
MIC:	Minimum inhibitory concentration
MFC:	Minimum fungicidal concentration
ANOVA:	Analysis of variance
EAF:	Ethylacetate fraction
BT:	n-butanol fraction
HF:	Hexane fraction
CF:	Chloroform fraction
CD_3OD:	deuterated methanol
IR:	Infra-red
^{1}H-NMR:	Proton nuclear magnetic resonance
^{13}C-NMR:	Carbon-13 nuclear magnetic resonance
HMBC:	Heteronuclear multiple bond correlation
HSQC:	Heteronuclear single quantum correlation
COSY:	Correlation spectroscopy
DEPT:	Distortionless enhancement by polarization transfer
ERG11:	Ergosterol biosynthetic inhibitor enzyme.
UPC2:	Upcoupling protein 2
GOF:	Germline gain-of- function

CHAPTER ONE

1.0 INTRODUCTION

1.1 Natural Products

Over the years, natural product have contributed in providing humanity with the means of cure and prevention of diseases. Natural products discovered so far have played a vital role in improving the human health and have been the drugs of choice with their most striking feature being their structural diversity that is still largely untapped (Ciddi, 2012).

Natural product are the most successful source of potential drugleads (Haefner, 2003; Butler, 2004; Cragg and Newman, 2005; Mishra and Tiwari, 2011; Rey-ladino *et al.*,2011). The plant-derived drug used directly or as precursor includes Morphine (I), the first commercial pure natural product introduced for therapeutic use marketed by Merck in 1826 was obtained from *Papaver somniferum*.Quinine (II), an antimalarial agent isolated from the bark of *Cinchona succirubra* tree, a potent antimalarial drug named Artemisinin (III) was also isolated from plant *Artemisia annua* as a remedy against the multidrug resistant strains of *Plasmodium* spp.The first semi-synthetic pure drug aspirin (IV) was introduced by Bayer in 1899 based on a natural product *salicin* isolated from *Salix alba*. Other semisynthetic compounds such as artemether dihydroartemisinin, chloroquine, 4-aminoquinoline and 8-aminoquinoline were synthesized with a view to optimizing the pharmacokinetic principal of the molecules as potent antimalarial agents in widespread use across the world.

III

III **IV**

1.2 Traditional Medicine

The medicinalplant has been the most affordable and easily accessible source of treatment in the primary healthcare system of resource poor communities (Haile and Delenasaw, 2007). Traditional medicine is a health practices, knowledge and beliefs incorporating plants, animals and mineral based medicines, spiritual therapies, manual techniques and exercise, applied singularly or in combination to treat, diagnose and prevent illnesses or maintain well-being (NNMDA, 2008; WHO 2005). Farnsworth *et al*. (1991) reported that about 80 percent of people world-wide still rely on plant-based traditional medicines for some aspect of their primary health care. This has been attributed to their affordability and accessibility.

The knowledge associated with traditional medicine has promoted further investigations of medicinal plants as potential source of medicines, and has led to the isolation of many chemical compoundswhich 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 (V) derived from foxglove (*Digtialis purpurea*), Paclitaxel (Taxol®) (VI) 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).

1.3 Mistletoe

Mistletoes are group of multipurpose plant used by traditional herbalists to treat different diseases in both urban and rural communities especially in the sub-Saharan Africa (Adesina *et al*, 2013). Mistletoe is commonly known as- bird lime, all heal, devil's fuge (Adesina *et al*, 2013). It is a general term for woody shoot parasites in several plant families especially Loranthaceae and Viscaceae (Burkill, 1985 and Polhill and Wiens, 1998); most genera of African mistletoes belong to the family Loranthaceae (Polhill and Wiens, 1998). Mistletoes of the Loranthaceae and Viscaceae are hemiparasitic plants and their preparations in the form of extracts, infusions, tinctures or tea bags are widely used in various cultures in almost every continent to treat or manage various health problems including hypertension, *diabetes mellitus*, inflammatory conditions, irregular menstruations, menopause, epilepsy, arthritis, cancer, etc (Adesina *et al.,* 2013). Mistletoe is commonly found growing on tree crops like cocoa, kolanut and coffee. Mistletoe can also growon citrus trees like orange (*Citrus sp*.) and guava(*Psidium guajava* L.) (Ogunmefun *et al.,* 2015).

1.4 Fungi

Fungi are a group of microorganisms which live as saprophytes in the soil and on dead organic matter or as parasites of plants and animals including man. They can be found in the soil, decaying plants, air and water, and as part of the microbial flora within the body or on the skin of man (Olumayowa, 2014). There are many species of fungi that causes skin infections in man. These are mainly *Dermatophytes* (*Trichophyton* specie, *Epidermophyton* specieand *Microsporum* specie), *Malassezia furfur and Candida* specie. *Aspergillus* specie, *Trichothecium roseum, Cladosporium* specie, *Fusarium* specie, *Curvularia* specie, *Penicillium* specie, *Epidermetaphyton* specie, *Drechslera* specie and *Alternaria* specie also cause skin, hair or nail infections (Gupta Gupta, 2013).

Skin diseases and their complications are a significant burden on the health system of many nations. The most frequent fungal pathogens being *Candida*, *Aspergillus*, *Pneumocystis*, and *Cryptococcus* spp. It is estimated that these fungal species cause at least 1.4 million deaths worldwide per year (Brown *et al.,* 2012). The World Health Organization's report on the global burden of disease indicated that skin diseases were associated with mortality rates of 20,000 in Sub-Saharan Africa in 2001 (WHO, 2005). Malnutrition, poor environmental sanitation, lack of portable water supply are the contributing factor to the burden of skin disease In Africa and Nigeria inclusive (Mahé *et al.*, 1998; Doe *et al.*, 2001; Adebola, 2004).

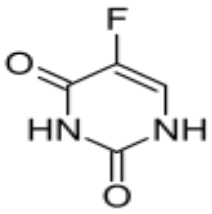
1.4.1 Antifungal Agents

Despite extensive research dedicated to the development of new therapeutic strategies, there are only a limited number of available drugs to fight against invasive fungal infections (Patrick*et al.,* 2012).

About four molecular classes of drugs that target three distinct fungal metabolic pathways are currently used in clinical practice to treat essentially topical and systemic fungal infections. These include: fluoropyrimidine analogs, polyenes, azoles, and echinocandins. Other classes, such as morpholines and allylamines are only used as topical agents due to either poor efficacy, severe adverse effects when administered systemically (Patrick*et al.,* 2012).

1.4.1.1 Fluoropyrimidines

Fluoropyrimidines, of which only 5-fluorocytosine (5-FC) (VII) and 5-fluorouracil (5-FU) are used in human medicine, these are synthetic structural analogs of the DNA nucleotide cytosine (Patrick*et al.,* 2012). 5-FC possesses a broad spectrum of activity, it is active against *Candida* and *Cryptococcus* genera.5FC activity on *phialophora,Cladosporium* and *Aspergillus* genera is much less limited.



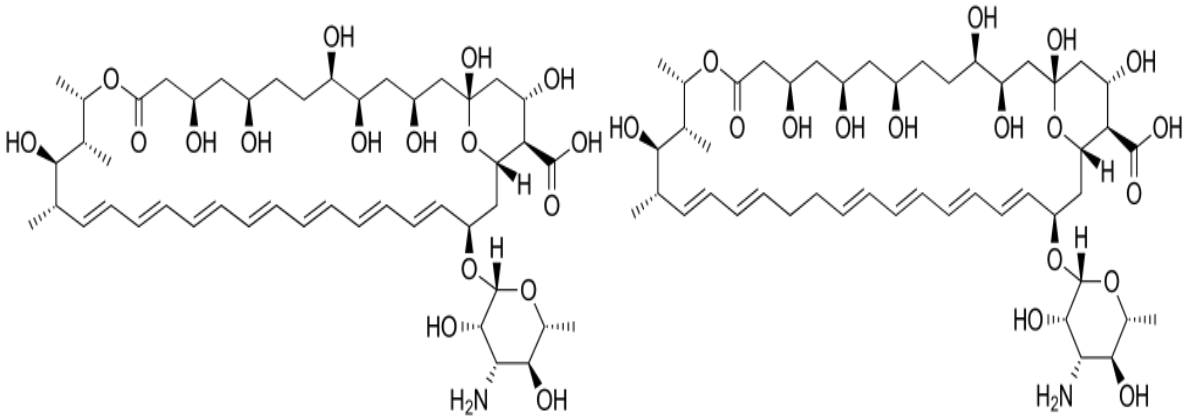
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1.4.1.2Polyenes

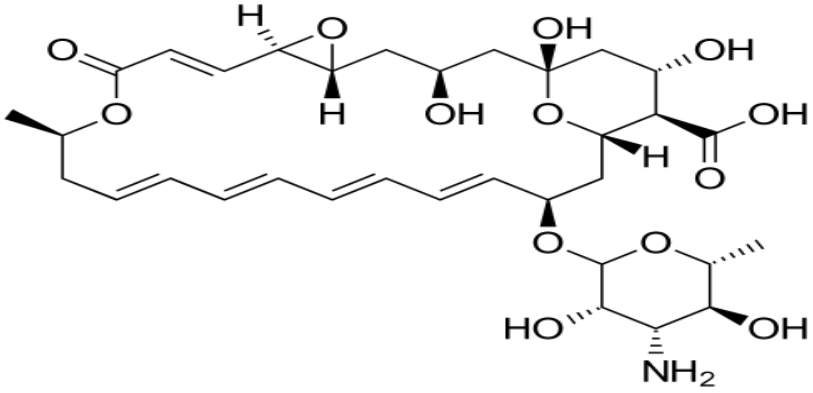
Polyenes are cyclic amphiphilic organic molecules known as macrolides. Most of them consist of 20 to 40 carbons macrolactone ring conjugated with a d-mycosamine group. Their amphiphilic properties are due to the presence of several conjugated double bonds on the hydrophobic side of the macrolactone ring, and to the presence of several hydroxyl residues on the opposite, hydrophilic side (Lemke *et al.,* 2005). More than 200 molecules belonging to the chemical class of polyenes have been reported to have antifungal activity, most of them

being produced by *Streptomyces* bacteria. However, only three possess a toxicity within tolerable limit allowing their use in clinical practice:Amphotericin B(VIII),Nystatin (IX)and

Natamycine(X) (Patrick *et al*., 2012)



(VIII) (IX)



(X)

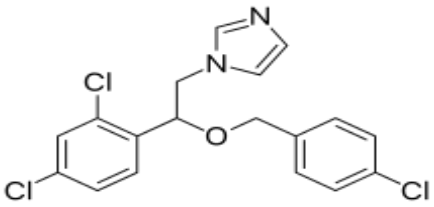
1.4.1.3Azoles

Azoles are cyclic organic molecules that are group into two on the basis of the number of nitrogen atoms present in their azole ring which include; imidazolethatcontain two nitrogen atoms, and the triazoles whichcontain three nitrogen atoms (Maertens, 2004).

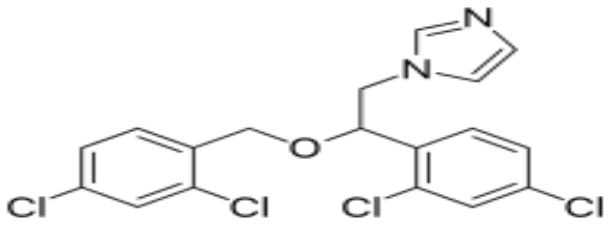
Azoles are group of antifungals that are commonly used in clinical practice, and are the most studied antifungal agents by the scientific community regarding their mode of action, their pharmacological properties and the resistance mechanisms developed by microorganisms(Patrick*et al.*, 2012).

1.4.1.3.1Imidazoles group

Imidazoles are group of azole antifungal agents that are used in systemic and nonsystemic candidiasis. It disrupt the function of membrane bound enzymes by inhibiting ergosterol synthesis. They include: ketoconazole (XI), Econazole (XII) and Miconazole (XIII)among others.



(XI) (XII)

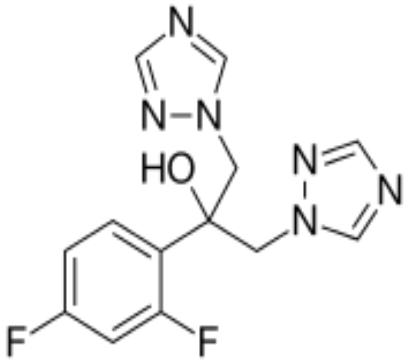


(XIII)

1.4.1.3.2 Triazoles group

Triazoles are group heterocyclic compounds with broad spectrum antifungal activity especially against candidiasis and other fungal infection.They include: Itriconozol (XIV), Fluconazole (XV) and Voriconazol (XVI) among others.

(XIV)

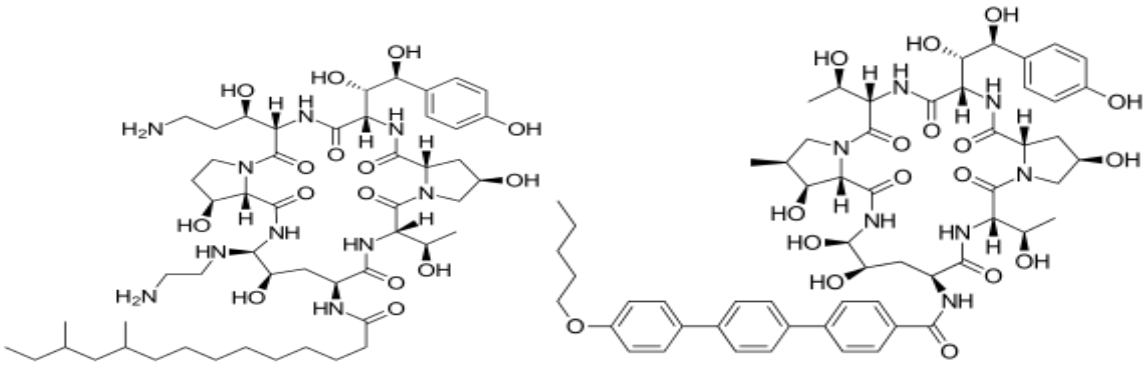


(XV)(XVI)

1.4.1.4Echinocandins

Echinocandins are synthetic derivatives of lipopeptides naturally produced by several fungal species, namely: *Aspergillus rugulovalvus* which synthesizes caspofungin B (XV), *Zalerion arboricola*which synthesized pneumocandin B and *Papularia sphaerosperma* synthesized*papulacandin*. Echinocandins is the only new class of antifungals drugs made available for clinicians to fight invasive fungal infections for the past 15 years

(Denning, 2002). Anidulafungin (XVI) has proven to be effective against oesophageal candidiasis and other invasive candidiasis (Pfaller *et al.*, 2005)



(XVII) (XVIII)

1.4.1.5 Other Antifungal Agents

These are:*(i) Allylamines group(ii) Thiocarbamate group*

1.4.1.5.1 Allylamines group

Butenafine (XIX) and terbinafine (XX) belong to allylamine antifungal agents. Theyare used for the treatment of dermatophytic infection, i.e *Tinea*infection.

(XIX) (XX)

1.4.1.5.2 Thiocarbamate group

These groups includes: Tolnaftate,is a synthetic thiocarbamate used as an antifungal agent to treat fungal conditions such as jock itch, athlete's foot and ring worm.

(XXI)

1.4.2Mechanism of action of antifungal agent

Understanding the mechanisms of action of different antimicrobial agents is an important prerequisite to understanding mechanisms of resistance. In fact, in many cases an elucidation of resistance mechanisms has allowed or enhanced thespecific mechanisms of action of antifungal agents (Mahmoud and Louis, 1999). Three general mechanisms of action for the antifungal agents have been reported (Bennett, 2011). These includes:cell membrane distruption, inhibition of cell division and inhibition of cell wall formation.

1.4.2.1 Inhibition of cell wall formation

An *in vitro*antifungal studies have shown that drugs have been developed to interfere with various steps in fungal cell wall synthesis with good antifungal effect (Urbina *et al*., 2000). But development of these agents into useful drugs has proven very difficult. Inhibitors of β-glucan synthesis have been developed.

1.4.2.2 Cell membrane disruption

Cell membrane disruption occur either by (1)inhibiting ergosterol biosynthesis as observed in azole antifungal agents (White *et al*., 1998). (ii) Binding to the sterol there by forming pores, causing the membrane to leak as with polyene antifungals. Ergosterol is similar to mammalian cholesterol. Ergosterol differs from mammalian sterols due to the presence of two conjugated double bonds (Heeres *et al*., 1979).

1.4.2.3 Inhibition of cell division

Nucleoside antifungal agents affect cell division by targeting the microtubule effects in forming the mitotic spindle. They can also act by inhibiting DNA transcription.5FU also is converted to 5-fluorodeoxyuridine monophosphate, a potent inhibitor of thymidylate synthase, an enzyme involved in DNA synthesis and nuclear division (Diasio*et al*., 1978). Thus, 5FC acts by interfering with pyrimidine metabolism, as well as RNA, DNA, and protein synthesis in the fungal cell.

1.4.3 Antifungalresistance

The term "resistance" is used to describe a relative insensitivity of a microbe to an antimicrobial drug as tested *in vitro* and compared with other isolates of the same species(Juergen and David, 2003). Antifungal resistance is the therapeutic concentration at which the growth of the pathogen is unaffected by antifungal agent (Anand*et al.*, 2014). The study of resistance to antifungal agents has lagged behind that of antibacterial resistance for a number of reasons. However, the reason being that fungal diseases were not recognized as important pathogens until relatively recently (Anaissie and Bodey, 1989; Wey *et al*., 1988). The use of antifungal drugs in the therapy of fungal diseases can lead to the development of antifungal resistance. Resistance has been described for virtually all antifungal agents in diverse pathogens, including *Candida* and *Aspergillus* species. The

majority of resistance mechanisms have also been elucidated at the molecular level in these pathogens (Dominique**,** 2016**)**.

1.4.4 Mechanism of antifungal resistance

Microorganisms develop mechanisms to counteract the fungicidal or fungistatic effects of all antifungals classes that are based on three major mechanisms, namely, (i) Reducing the accumulation of the drug within the fungal cell, (ii) Decreasing the affinity of the drug for its target, and (iii) Modifications of metabolism to counterbalance the drug effect (Patrick *et al*., 2012).

1.4.4.1 Reducing the accumulation of the drug within the fungal cell

Reducing the accumulation of the drug within the fungal cell involved the decrease of effective drug concentration with specific mechanisms which includes increase drug efflux, increased number of target, drug sequestration of extracellular and intracellular origins and poor pro-drug conversion (Dominique, 2016).

i.Increase drug efflux

Drug intracellular concentrations can be decreased by active efflux.It is known that drug resistance can be mediated by the activity of several efflux transport systems, including ATP-binding cassette (ABC) transporters and transporters of the major facilitator superfamily (MFS). The analysis of fungal pathogen genomes has identified varying numbers of ABC transporters and MFS transporters with different topologies (Dominique, 2016)

ii. The increased in drug target.

By increasing the number of drug targets, the effective drug concentration needs to be also increased to saturate all target molecules, which results in drug resistance (Dominique, 2016). *ERG11* upregulation has been associated with azole resistance in*C.albicans*. This transcriptional regulation is mediated by a zinc cluster finger transcription factor

called *UPC2*. As in the case of other drug resistance transcriptional regulators, GOF mutations in *UPC2* have been described and result in upregulation of various genes, among which is *ERG11* (Dunkel *et al*., 2008)

iii. The drug is sequestered in extra- or intracellular compartments.

Fungal pathogens have the ability to sequester drugs within extracellular compartments. Several fungal pathogens, including *Candida* and *Aspergillus* spp., are implicated in biofilms production in specific growth conditions (Ramage *et al*., 2005). Biofilms are multicellular structures in which cells form a dense network that is covered by the so-called matrix. The matrix is composed of different elements in *C. albicans* biofilms, including several cell wall polymers. Biofilm formation is known to be associated with resistance to several drugs, including azoles, polyenes, and pyrimidine analogs (Desai *et al*., 2014).

iv. A pro-drug is poorly converted to an active drug.

Poor drug metabolization as a principle of antifungal resistance is also observed when 5-FC resistance occurs. 5-FC is a pro-drug, which is metabolized by cells into fluorinated pyrimidine analogs, thus inhibiting nucleic acid and protein biosynthesis. After import into cells, cytosine deaminase converts 5-FC into 5-fluorouridine (5-FU) and therefore the deficiency of this step decreases further processing and toxicity of the drug (Dominique, 2016). Mutations in cytosine deaminase in *C. albicans* (*FCA1*) (Hope *et al*., 2004) and *C. glabrata* (*FCY1*) have been reported, resulting in 5FC resistance (Vandeputte*et al*., 2011).

1.4.4.2.Decreasing the affinity of the drug for its target

Another mechanism by which fungal pathogens are able to develop resistance is a decrease in antifungal affinity for their respective targets, without a major decrease in target activity. Such is the case for azole drugs, in which a decreased affinity between azole and a mutated

lanosterol 14α-demethylase, can lead to resistance. A point mutation in the ERG11 gene that codes for lanosterol 14α-demethylase leads to the complete inhibition of the binding capacity of the azole drug to its target (Vanden *et al*., 1990).While target site alteration is far from being the most significant mechanism of resistance to azole drugs, it is the only known mechanism by which fungal pathogens are able to develop resistance to echinocandin drugs (Patrick *et al.,* 2012)

1.4.4.3.Modifications of metabolism to counterbalance the drug effect

Some yeasts and filamentous fungi are able to grow in elevated echinocandin concentrations much higher than the MICs (Perlin, 2007). This phenomenon, called paradoxical effect, is due to the metabolic adaptation of microorganism and is mediated by the cell wall integrity signalization pathway. This response is the direct consequence of the β (1-3)-glucans synthesis inhibition and the subsequent cell wall composition modifications, upon echinocandin administration (Stevens *et al*., 2006).

Development of resistance occurs by selection of naturally occurring resistant cells, present in small numbers in the population. These naturally resistant cells produce modified sterols that bind nystatin with lower affinity. The rate of growth in the presence of nystatin is therefore dependent upon the normal growth rate in the absence of nystatin and the rate at which nystatin causes cell membrane damage. This rate is presumed to be a function of the affinity of nystatin for the membrane sterols: the greater the nystatin-sterol affinity, the greater the rate of membrane damage (Fryberg, 1974). The development of inducible resistance (induced by adaptation mechanism) in a strain of *C. albicans* was accompanied by a decrease in the ergosterol content of the cells. This decrease in ergosterol content was due to inhibition of ergosterol synthesis not due to enzymatic degradation of preformed ergosterol (Capek *et al.,*1974). Nystatin degradation has been reported by an induced enzyme system elicited by dermatophytic fungi (Capek and Simek, 1971)

1.5 Statement of Research Problem

Fungal infection is responsible for high morbidity and mortality especially in patients whose immune system is compromised (Arunaloke, 2011). It is estimated that fungal species cause at least 1.4 million death worldwide per year (Brown *et al.,* 2012). About 11.8% of the Nigerian population is estimated to suffer from different types of fungal infections each year with1.5 million Nigerian women suffered from virginal thrush, 15.5 million children suffered from *Tenia capitis,* 253,000 suffered from oral candidiasis and 144,000 oesophageal candidiasis (Oladele and Denning, 2014).

Several classes of antifungal drugs are expensive, majority have been compromised by resistance and are beyond the reach of poor in underdeveloped countries such as Nigeria.

Therefore, there is need for cheaper and more effective alternative sources of antifungal agent.

1.6 Justification of the Study

Fungal diseases especially dermatophytosis have been reported in different cities and communities in Nigeria (Nweze and Okafor, 2004).

Tapinanthus globiferus mistletoe is used in ethno-medicine for management of fungal infection without validation.

To the best of our knowledge, no work has been reported on the isolation, characterization and antifungal activity of *Tapinanthus globiferus* growing on *Vitex doniana.*

1.7 Aim and Objectives

The aim of the work was to isolate some of the bioactive compounds and validate the claim for the use of the plant as an antifungal agent.

The objectives are:

To carry out preliminary phytochemical screening on the methanol extract and some fractions of *Tapinanthus globiferus* growing on *Vitex doniana* using standard procedure.

To isolate and characterize the bioactive compound(s) using chromatographic and spectroscopic techniques.

To determine the antifungal activity of the plant extract using agar well dilution diffusion and broth micro dilution methods.

1.8Statement of Research Hypothesis

The methanol extract of *Tapinanthus globiferus* contains bioactive compound with no antifungal activity.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 The Plant, *Tapinanthus globiferus*

Tapinanthus globiferus is a semi-parasite with glabrous pendulous stems up to 1.2 m long with roots that mostly grows on the branches of a large number of tree species including *Vitellaria paradoxa, Kola*, *Citrus,Combretum, Acacia,Aloe* and *Terminalia* as host trees (Waterberg *et al.*, 1989). The plant, *tapinanthus globiferu*belongs to the familyloranthaceae (Polhill and Wiens, 1998).Seven genera have been reported for loranthaceae family, they are:*Helixanthera, Berhautia, Englerina, Globimetula, Agelanthus, Tapinanthus* and *Phragmanthera* with at least five dozen species recognized in West Africa (Burkill, 1985)

2.1.2 Habitat

Tapinanthus globiferus is widely distributed throughout tropical and subtropical region of Western and Eastern African including Nigeria



Plate I:*Tapinanthus globiferus*

2.1.3 Taxonomy

The six taxonomic levels of the plant are as follows:

Kingdom: *Plantae*

Phylum:*Tracheophyte*

Class:*magnoliopsida*

Order:*Santalales*

Family*:* *Loranthaceae*

Genus: *Tapinanthus*

Species: *Globiferus*

2.1.4 Common names

Tapinanthus globiferus is commonly known as mistletoe (English), *Kauchi* (Hausa), *Eme-emi afomo* (Yoruba), and *Osisi/Okwuma osa* (Igbo) in Nigeria.

2.1.5 Botanical and morphological feature

Tapinanthus globiferus is a woody, spreading shrub with blackish, smooth stems made rough by the presence of lenticels. The leaves are opposite but sometimes alternate. The leaf length varies from 7-15(20) cm while the leaf width could be 3-10(15) cm. The leaves are thick, ovate, obtuse, rounded to cuneate at base, petiolate. Petiole length up to 2cm long and grooved axially. Nerves pinnate with barely prominent and irregular lateral nerves. The inflorescence is a sub-sessile fascicle with up to 6 flowers. The flower is bisexual with a red corolla tube up to 2cm long and a swollen base that is greenish in color. Calyx forming a short tube enclosing the corolla tube. The stamens are five alternating with the petals and partly fused to the petals. The unattached portion of the filament curls up as soon as the petal lobes open. The fruits are one-seeded, globose and green when immature (Bassy, 2012).

2.1.6 Ethno-Medicinal Uses of *Tapinanthus globiferus*

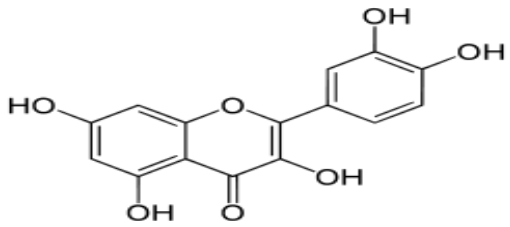
It is used in ethnomedicine to treat itching (Burkill, 2000), tumour (Haile and Delensaw, 2007) and removal of placenta after parturition (Sher and Alyemini, 2011). It is also used to treat diseases such as hypertension, ulcers, epilepsy, diabeties, weakness of vision and promoting muscular relaxation before delivery (Bassay, 2012).

2.1.7 Pharmacological Action of species of the Genus *Tapinanthus*

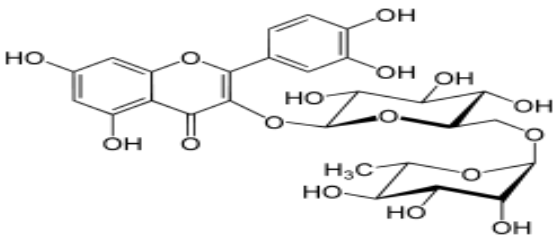
Tapinanthus dodoneifolius has been reported to have anxiolytic and antidepressant effects with a sedative side effect(Foyet *et al*.,2014).*Tapinanthus sessilifolius* has been exhibit antimalarial activity (Okpakoand Ajaiyeoba, 2004), anti-inflammatory and anti-oxidant properties(Adekunle *et al*.,2012)**.** *Tapinanthus globiferus*extract exhibited antitrypanosomal activity (Abedo *et al.,* 2013) and anticonvulsant activity (Abubakar *et al.,* 2016).

2.1.8 The Phytochemistry of *Tapinanthus globiferus* and related species

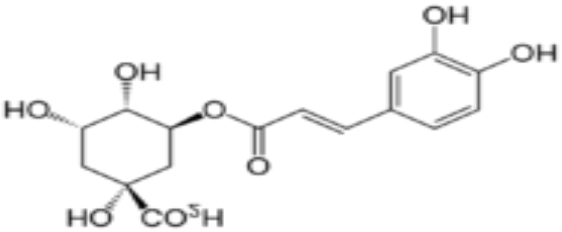
Previous phytochemical screening on *Tapinanthus globiferus* revealed the presence of alkaloids, tannins, saponins, flavonoids, carbohydrate cardiac glycosides and steroids (Abubakar *et al.,* 2016). So far there is no report of isolation and characterization of compounds from *Tapinanthus globiferus* growing on *Vitex doniana* but Yetunde*et al.* (2014) reported the identification and quantification of two flavonoids and three phenolic acids from *Tapinanthus globiferus* growing on *Zanthoxylum zonthoxyloides* namely; quercetin, (XXII) rutin, (XXIII) chlorogenic acid (XXIV),galic acids (XXV) and caffeic acid (XXVI)



(XXII)



(XXIII)



(XXIV)

(XXV)

(XXVI)

2.2 Natural product derived antifungal agents

A number of compounds isolated from plants such as dimethyl pyrrole, hydroxydihydrocornin-aglycones, indole derivatives, are reported to have antifungal activities (Schultes, 1987).

A number of studies have been reported on the antifungal activity of phenolic compounds from natural sources in recent years. The site(s) and number of hydroxyl groups on the phenol group are thought to be related to their relative toxicity to microorganisms, with evidence that increased hydroxylation results in increased toxicity (Tasleem *et al.,* 2009).

Amentoflavone (XXVII) isolated from *Selaginella tamariscina* exhibited potent antifungal activity against several pathogenic fungal strains and has a very low hemolytic effect on human erythrocytes (Jung *et al.*, 2006). The four compounds eupomatenoid-3 (XXVIII), eupomatenoid-5 (XXIX), conocarpan (XXX), and orientin (XXXI), isolated from *Piper solmsianum* exhibited antifungal action against the tested dermatophytes tested (De Campos *et al*.,2005).

(XXVII) (XXVIII)

(XXIX)(XXX)

(XXXI)

CHAPTER THREE

3.0 Materials and Methods

3.1 Materials

The solvents/reagents used are of general purpose grade and were distilled prior to use, they include methanol, n- butanol, ethyl acetate, chloroform, n-hexane and dimethyl sulfoxide

(DMSO). Silica gel (60- 120 mesh), Sephadex LH-20, Sabouruad dextrose (SDA) agar and broth, fluconazole powder. Thermoelectric UV spectrometer. Ohaus digital weighing balance (Champ 11 CH15R, Ohaus Corporation, Pinebrook NJ, USA), Metler balance (Model P162 supplied by Gallenhamp). Bruker AVANCE III NMR (600MHz) spectrometer at the University of KwaZulu-Natal, Durban. South Africa for 1D and 2D-NMR spectroscopy. 96 well Microtitre plate, single and multi-channels micro pipette, Chromatographic tanks, Vacuum Liquid Chromatographic column and low pressure column (75cm by 3.5cm). Disposables petri dishes. Precoated plates (TLC Silica gel 60 F_{254}, Germany) and capillary tubes and fluconazole powder (Cat No. F8929, Sigma Aldrich, U.S.A.)

3.2 Methods

3.2.1 Collection, Identification and Preparation of Plant Material

The plant sample was collected from Dange Shuni Local Government of Sokoto State, Nigeria in December 2016. It was identified and authenticated by Namadi Sanusi of the Herbarium Section, Department of Botany, Ahmadu Bello University Zaria, with a voucher number 900107. The plant material was air dried, pulverized, labelled and stored in a polythene bag for further use.

3.2.2 Extraction and Partitioning

Two thousand grams (2000 g) of the pulverized plant leaves was macerated with 3 litres of methanol with occasional agitation for 3 days, the extract was filtered and the solvent was removed using rotary vacuum evaporator, the extraction was repeated two more times to obtain more extract which was referred to as methanol leaf extract (MLE). Methanol leaf extract (120 g) was suspended in 400 mL of distilled water which was then filtered and partitioned with n-hexane (600 mL), chloroform (800 mL), ethyl acetate (1100 mL) and n-butanol (1400 mL) solvents

3.2.3 Preliminary Phytochemical Screening

Phytochemical screening was carried out on the crude methanol extract in accordance with the standard procedures to test the presence of secondary metabolites as below:

3.2.3.1 Test for carbohydrates (Molisch's test)

A small portion of the extract was dissolved 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 sec. The solution was allowed to stand for 5 min, the formation of a persistent froth which lasted for 45 min 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 were added to the solution of aqueous extract, yellow coloration indicates the presence of flavonoids (Trease and Evans, 1996).

III. Ferric chloride test

The extract was boiled with water and filtered. To 2 mL of the filtrate, 2 drops of freshly prepared ferric chloride solution was added. A dark green coloration indicated the presence of phenolic hydroxyl group (Trease and Evans, 1996).

3.2.3.4 Test for tannins

I. Lead sub-acetate test

To a small portion of the extract, 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/steriod

I. Salkowki's Test

A small portion of the extract was dissolved in 2mL of chloroform, 3 drops of concentrated sulphuric acid (Salkowki's reagent) were 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. Steroid/triterpene (Liebermann-Burchard's Test)

To the portion of the extract equal volume 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).

3.2.3.6 Test for alkaloids

I. Dragendoff's Test

The extract (0.2g) 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, reddish precipitate indicates the presence of alkaloids (Trease and Evans, 1996).

II. Mayer's Test

To an acidic solution of the extract (2 mL) 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 5mL chloroform,shaken and filtered. To the filtrare, 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 extract was dissolved in 1mL 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 CHROMATOGRAPHY PROCEDURES

3.3.1 Thin Layer Chromatographic Analysis.

A precoated TLC plates was used to carry out thin layer chromatography by one way ascending technique. Capillary tubes were used to manually apply the sample on the TLC

plate and the chromatogram was developed in an air tight chromatographic tank at room temperature with appropriate solvent systems. The spots were visualized under UV spectrophotometer (254-366nm) and (10% Sulphuric acid) as spray reagent followed by heating in an oven at 105^{o}C for about 5 min. The various TLC solvents system used to develop the plates include:

3.3.1.1 TLC solvent systems of crude methanol extract

a. Hexane: Chloroform (9:1)
b. Chloroform: methanol (9:1)
c. Ethylacetate: Chloroform: Methanol: Water (15:8:4:1)

3.3.1.2 TLC solvent systems for ethyl acetate fraction

a. Ethylacetate (100%)
b. Chloroform: methanol (9:1)
c. Ethylacetate: Chloroform: Methanol: Water (15:8:4:1)

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 were used. Wet packing sample loading method was used for silica gel column chromatography.

3.3.3 Gel Filtration Chromatography

In gel filtration chromatography, Sephadex LH-20 was used as stationary phase and was suspended in an eluting solvent (methanol) and left for 24 hr prior to use. The suspended Sephadex was then poured into a column and allowed to set. The sample was dissolved in a small volume of the eluting solvent (methanol) and introduced into the column.

3.3.4 Vacuum liquid Chromatography of Ethylacetate Fraction

Ethylacetate fraction (2 g) was subjected to vacuum liquid chromatography using gradient elution technique; starting with mixtures of (1) hexane: ethyl acetate 30:70 (400 mL) (2) 20:80 (400 mL) hexane: ethyl acetate, (3) 10:90 (400 mL) hexane: ethyl acetate and (4) ethyl acetate 100% (400 mL). A total of 60 collections of 20 mL each were made. The collections were pooled together on the basis of their TLC profiles (solvent system 15:8:4:1) to obtain 4 pooled fractions coded A-D.

Fraction D (0.34 g)was further subjected to low pressure column chromatography using gradient elution technique; starting with (1) chloroform: methanol 90:10 (400 mL), (2) 85:15 chloroform: methanol (400 mL), the column was finally washed with (3) chloroform: methanol 80:20. A total of 89 collections were made. The collections were pooled together on the basis of their TLC profiles into 10 pooled fractions coded D1-D10.

Fraction D4 was subjected to gel filtration, The elution was done using methanol 100% as eluting solvent and 30 collections of 1mL each were made and pooled into five fractions coded D4a-D4e. Base on the TLC profile. Repeated gel filtration of D4e afforded a yellow amorphous compound coded H3. Compound H3 was subjected to physicochemical test and spectroscopic analysis.

3.3.5 Column ChromatographicSeparationof Ethylacetate Fraction

Ethyl acetate fraction (4 g) was subjected to column chromatography using gradient elution technique; starting with Hexane (1) 100% (300 mL) followed by (2) 1:1 hexane: ethyl acetate (400 mL), (3) 1:2 (200mL), (4) 1:3 (600mL).Column was finally washed with hexane: ethyl acetate (5) 1:4. A total of 76 collections of 20 mL each were made. The collections were pooled together on the basis of their TLC profile to obtain 4 pooled fractions coded E1-E7.

Fraction E3 was subjected to gel filtration using methanol as eluting solvent, 15 collections of 1mL each were made and pooled into four fractions coded E3a and E3d. E3b was further subjected to purification using Sephadex LH-20 to afford a single homogeneous spot in different solvent system coded H1 which was subjected to physicochemical test and spectroscopic analysis.

3.4 Antifungal studies on methanol leaf extract and some fractions

3.4.1 Test organisms

Four (4) clinical fungal isolates obtained from the Department of Clinical Microbiology Usmanu Danfodiyo University Teaching Hospital, Sokoto were used for the study.Theyare*Candida albicasn, Aspergillus niger, Trychophyton rubrum* and *Trychophyton mentagrophyte*.

3.4.2 Preparation of test organisms

Test organisms were sub cultured and grown on 10 mL Sabouraud dextrose agar slants and was eventually kept in the refrigerator at $2 – 8^0$C.

3.4.3 Preparation of reference antifungal agents

Stock solutions (5 mg/mL) of fluconazole was prepared by dissolving 50 mg of its powder in 10 mL dimethyl sulfoxide (DMSO) from which 0.05 mg/mL (50 µg/mL) working concentration was prepared.

3.4.4 Preparation of plant extract/fractions

Stock concentrations of 100 mg/mL was prepared with 10% dimethyl sulfoxide (DMSO) by dissolving 0.5 g each of the methanol extract and fractions (ethylacetate and n-butanol) in 5mL 10% DMSO and two-fold serial dilution was carried out to obtain three more concentrations of 50, 25 and 12.5 mg/mL.

3.4.5 Preparation of Culture Media

The sabouraud dextrose agar (SDA) and broth as growth media were weighed and prepared with distilled water according to the manufacturer's specifications. SDA was gently heated to aid its dissolution, it was dispensed into sterile petri dishes and allowed to cool and solidify. These were kept aseptically until ready for use.

3.4.6Determination of Antifungal Activity of *Tapinanthus globiferus*

3.4.6.1 Cultivation and Standardization of Test Fungi

Culture of the *Candida albican* was suspended into sterile Sabouraud dextrose liquid medium. It was standardized by inoculating in normal saline to compare its turbidity to 0.5 McFarland standard which is approximately 1.0×10^6 CFU/mL. *Trichophyton* spp.and *Aspergillus niger*. were harvested from 6 day old Sabouraud dextrose agar (SDA) slant cultures, the spore suspension was standardized to 1.0×10^6 spores / mL by using spectrophotometer at 530 nm.

3.4.6.2 Antifungal screening of Tapinanthus globiferus

The antifungal activities of crude methanol extract, ethyl acetate and butanol fractions were determined according to the method by Olowosulu *et al.* (2005). Antifungal screening of the methanol leaf extract and the fractions was determined using agar wall diffusion method. A Sabouraud Dextrose agar (SDA) as the growth medium was prepared according to Manufacturer's instructions and autoclaved at 121^oC for 15 min, the media was transferred into sterile petri dishes and allowed to cool and solidify. Wells were punched into the solidified SDA plates using a sterile cork borer of 8 mm diameter. 0.1 mL of the inoculum

was seeded and spread evenly over the surface of the sterilized media using a sterile cotton swab, the wells were filled separately with 200 µL solution of the graded concentration of extract/fractions and 0.05 mg/mL Fluconazole which served as positive control, 10%DMSO was used as negative controls, plate were incubated at 27 ^{o}C for 48-72 hr, zone of inhibition was measured using transparent ruler. The experiment was carried out in triplicates.

3.4.6.3 Determination of minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) was determined using a 96 wells microplate method, as previously described byKhatoon *et al*. (2014) Methanol leaf extract and fractions were diluted serially from well-1 to well-10. 100 µL of Sabouraud Dextrose broth was added into each micro well of the microtitre plate. 100 µL of methanol extract and fractions were added into different well-1 to make 200 µL total volume. 100µL of the mixture extract/fractions and media (SDA) were serially diluted from well 1-10, well 11 (extract blank)served as negativecontrol and well-12 (media + inoculum) as positive controls. 100 µL of the fungal spore suspension approximately (2×10^6 spores mL^{-1}) was added to each well except well-11 of the microplate. The microplates was allowed to stand for 30 min, the absorbance was taken before incubation. The plate was incubated for 48 hr at 27 °C and the absorbance was taken after 48 hr. The MIC readings were performed by visual comparison and spectrophotometrically with a microplate reader at 595 nm.

3.4.6.4 Minimum Fungicidal Concentration (MFC)

A 20 µL of each well that showed no visible growth after MIC determinationwas sub-cultured onto the solid media (SDA) and incubated at 27C for 48 hr. The lowest concentration of the extract that does not yield any fungal growth on the solid medium used is the MFC (Espinel-Ingroff *et al*., 2002).

3.4.7 Statistical Analysis

The results obtained were expressed as mean ± standard error of mean and it was analyzedfor significant using analysis of variance (ANOVA), Pvalue *(P<0.05)*.

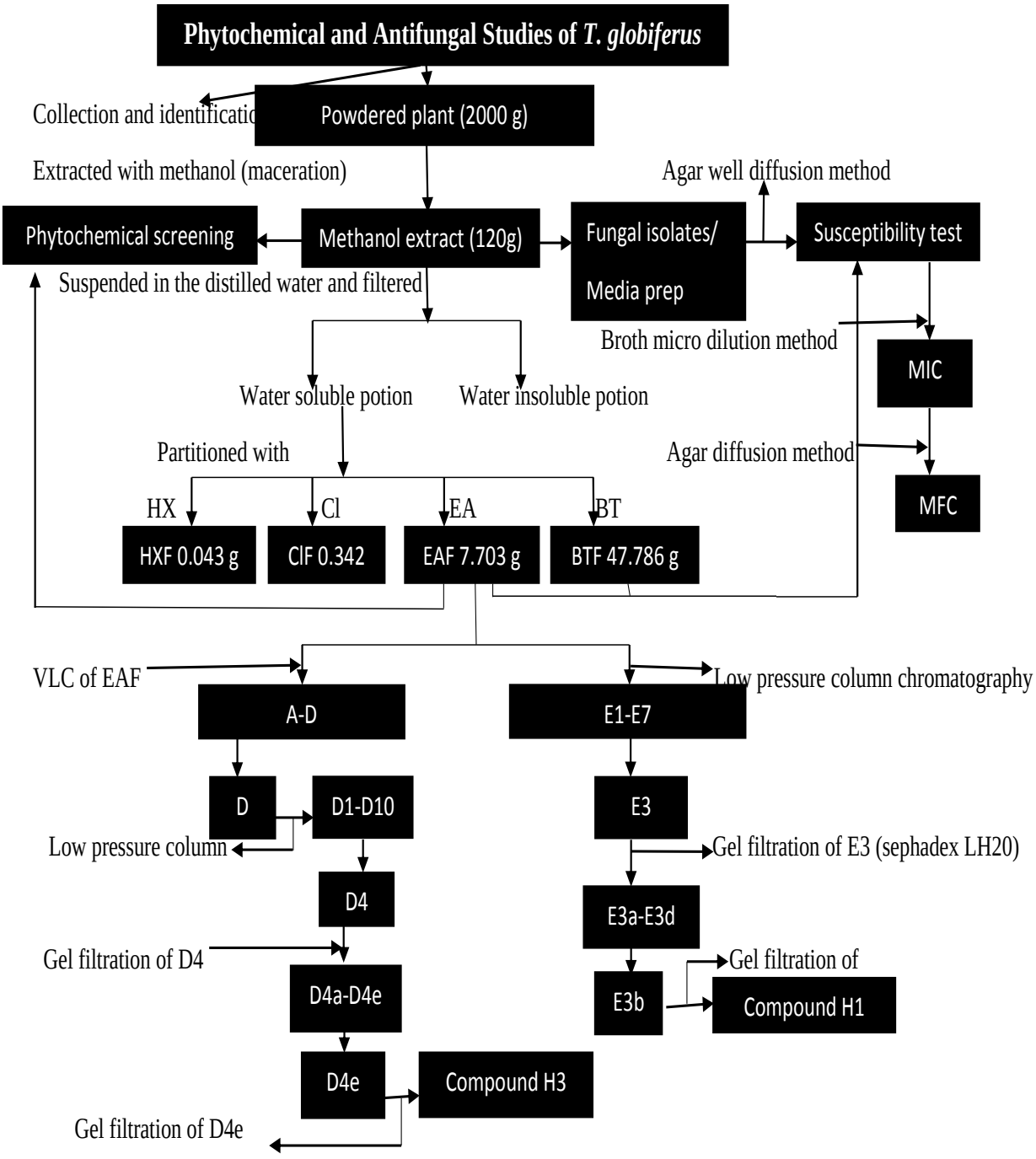


Figure 3.1: Schematic diagram of research methodology

CHAPTER FOUR

4.0 Results

4.1 Extraction and Fractionation Yield

The extraction of *Tapinanthus globiferus* (2000 g)afforded a yield of 140g (7%) of the crude methanol extract. The percent yield from the partitioned crude methanol extract (120g) are presented in Table 4.1

Table 4.1: Results of % Yield of Partitioned Fractions from 120g Crude Methanol Extract

Solvent	Weight (g)	Yield (%)	Colour
Hexane	0.043	0.033	greenish
Chloroform	0.342	0.258	light brown
Ethyl acetate	7.703	6.419	brownish
Butanol	47.786	39.822	reddish brown
Water insoluble	63.731	53.109	pale greenish

4.2 Phytochemical Studies

4.2.1 Phytochemical Screening ofMethanol Extract/fraction of *Tapinanthus globiferus*

Preliminary Phytochemical screening of the methanol extract, ethylacetate and n-butanol fractions revealed the presence of carbohydrates, flavonoids, tannins, saponins, steroids/triterpenes, alkaloids and flavonoids (Table 4.2).

Table 4.2: Phytochemical screening of the Methanol Extract (M.E), ethyl acetate fraction (EAF) and n-butanol fraction (BTF) of *Tapinanthus globiferus*

Constituents	test	M.E	EA F	BTF
Carbohydrates	Molisch	+	+	+
Anthraquinones	Bontrager	-	-	-
Steroid/Terpenes	Liebermann-Burchard	+	-	-
Glycoside	Keller- Killiani	+	+	+
Saponins	Frothing	+	+	+
Tannins	Ferric chloride	+	+	+
	Lead acetate	+	+	+
Flavonoids	Shinoda	+	+	+
Alkaloids	Ferric chloride	+	+	+
	Dragendoff	+	-	-
	Mayer	+	-	-

- = absent; + = present

4.2.2Chromatographic Separation

4.2.2.1 Thin-Layer Chromatography of methanol leaf extract (MLE)

The thin layer chromatographic analysis of methanol leaf extracts using hexane: ethylacetate (9:1), chloroform: methanol (9:1) and ethylacetate: chloroform: methanol: water (15:5:4:1)as solvent systemsafforded 8, 5 and 10 spots are shown in Plates I-III respectively.



Plate II: HX: EA (9:1)Plate III: $CHCl_3$:MeOH (9:1)

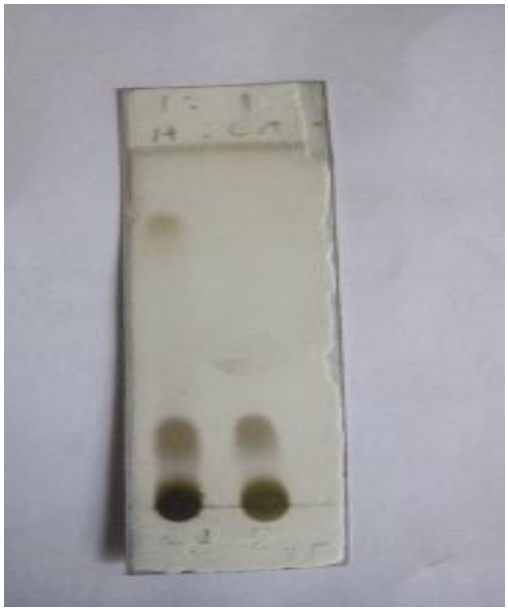


Plate IV: EA:$CHCl_3$:MeOH:H_2O

Plate II, III & IV: TLC profile of methanol leaf extract (MLE)

4.2.2.2 Thin-Layer Chromatography of ethylacetate fraction (EAF)

The thin layer chromatographic analysis of ethylacetate fraction using hexane: ethylacetate (1:1), ethylacetate (100%) and ethylacetate: chloroform: methanol: water (15:5:4:1) as solvent systems afforded 3,5 and 6 spots as shown in plates IV-VI respectively.



PlateV: HX: EA (1:1)



Plate VI: EA (100%)



Plate VII: EA:CHCl3:MeOH:H_2O (15:8:4:1)

Plate V, VI& VII: TLC profile of ethylacetate fraction (EAF)

4.2.2.3Thin-Layer Chromatography of n-butanol fraction fraction (BF)

The thin layer chromatographic analysis of n-butanol fraction using chloroform: methanol (9:1) as solvent systems afforded 5 spots as shown in plates VII.



Plate VIII: $CHCl_3$:MeOH (9:1)

Plate VIII: TLC profile of n-butanol fraction

Table 4.3 Result of TLC Profiles ofMethanol Extract for Various Solvent System

Extract	solvent system	No. of Bands	R_f values
Methanol extract	$CHCl_3$:MeOH 9:1	5	0.09,0.23,0.33,0.51,0.93
	HEX:EA9:1	8	0.18, 0.43, 0.57, 0.67, 0.82, 0.89, 0.94, 0.97
	EA:CHCl3:MeOH:H_2O 15:8:4:1	10	0.12, 0.24, 0.32, 0.44, 0.50, 0.56, 0.64, 0.72, 0.80, 0.96

Table 4.4 Result of TLC Profiles ethylacetate using Various Solvent System

Extract	Solvent system	No. of Bands	Rf values
Ethylacetate fraction	1:1	3	0.19. 0.32, 0.75
	100%	5	0.09, 0.26, 0.38, 0.47, 0.87
	15:8:4:1	6	0.13, 0.22, 0.27, 0.36, 0.58, 0.80

Table 4.5 Result of TLC Profiles of n-butanol fraction

Extract	Solvent system	No. of Bands	Rf values
n-butanol	9:1	5	0.08, 0.15, 0.27, 0.38, 0.46

4.2.3Column Chromatographic separation of Ethyacetate fraction of methanol leaf extract

4.2.5.1 Column chromatographic separation of ethylacetate fraction (EAF)

Column chromatographic analysis of ethylacetate fraction of the methanol leaf extract carried out using different solvent combinations afforded four major fractions (Table 4.8).

Table 4.6Column Chromatographic collections from Ethylacetate Fraction

S/N	Solvent system	No. of Eluates (25 mL each)	Code
1	Hexane (100%)	1-2	
1	Hexane: Ethylacetate (1:1)	3-6	E1
2	Hexane: Ethylacetate (1:1)	7-8	E2
3	Hexane: Ethylacetate (1:1)	9-22	**E3**
4	Hexane: Ethylacetate (1:1)	23-28	E4
5	Hexane: Ethylacetate (1:2)	29-40	E5
6	Hexane: Ethylacetate (1:3)	41-61	E6
7	Hexane: Ethylacetate (1:4)	62-76	E7

4.2.3.1 TLC Profile of Ethyl Acetate Fraction from Column Chromatography

TLC profile of column collections from ethylacetate fraction conducted using hexane: ethylacetate (1:1), (1:2), (1:3) and (1:4) as solvent systems revealed various spots after spraying with 10% H_2SO_4 followed by heating at 105 ^oC (Plate IX-XIII)

Plate IX-XIII shows the TLC Profile of column chromatographic of ethyl acetate fraction

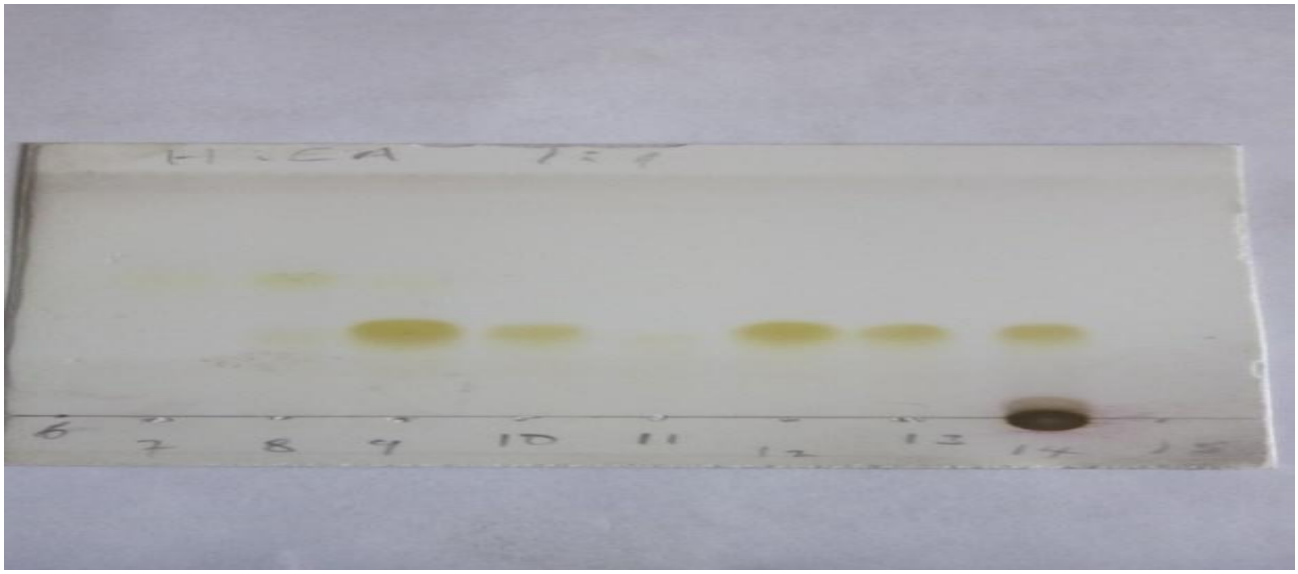


Plate IX: TLC profile of various fractions of EAF using HEX:EA (1:1)

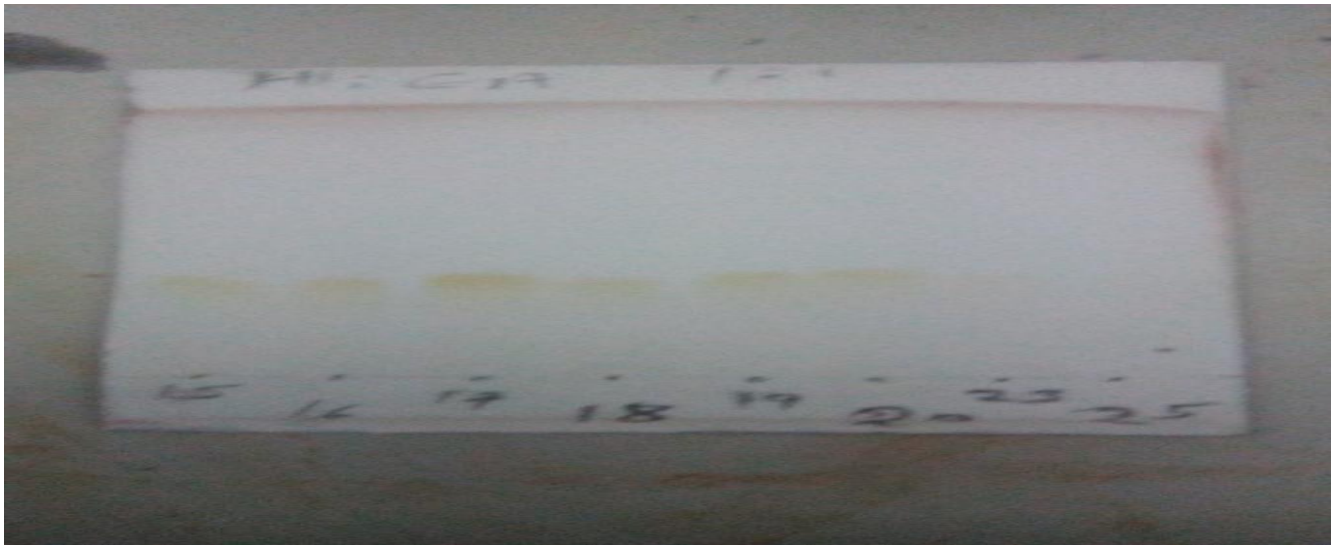


Plate X: TLC profile of various fractions of EAF using HEX: EA (1:1)

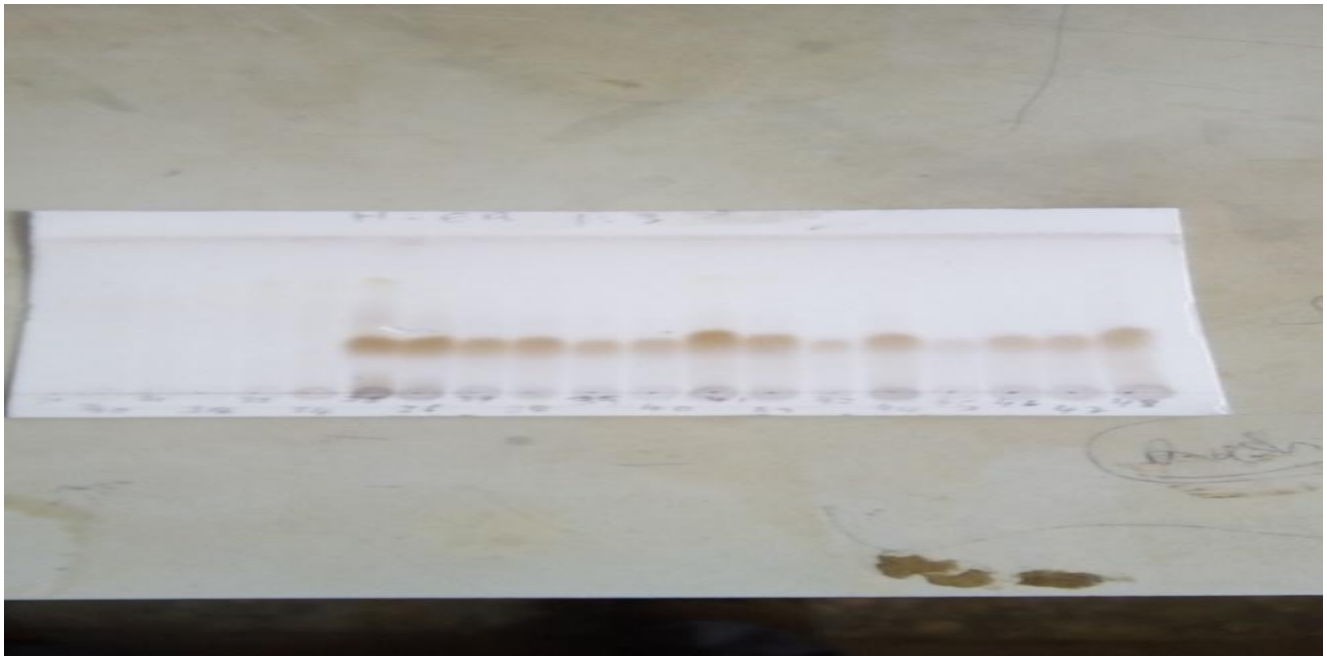


Plate XI: TLC profile of various fractions of EAF using HEX: EA (1:2)

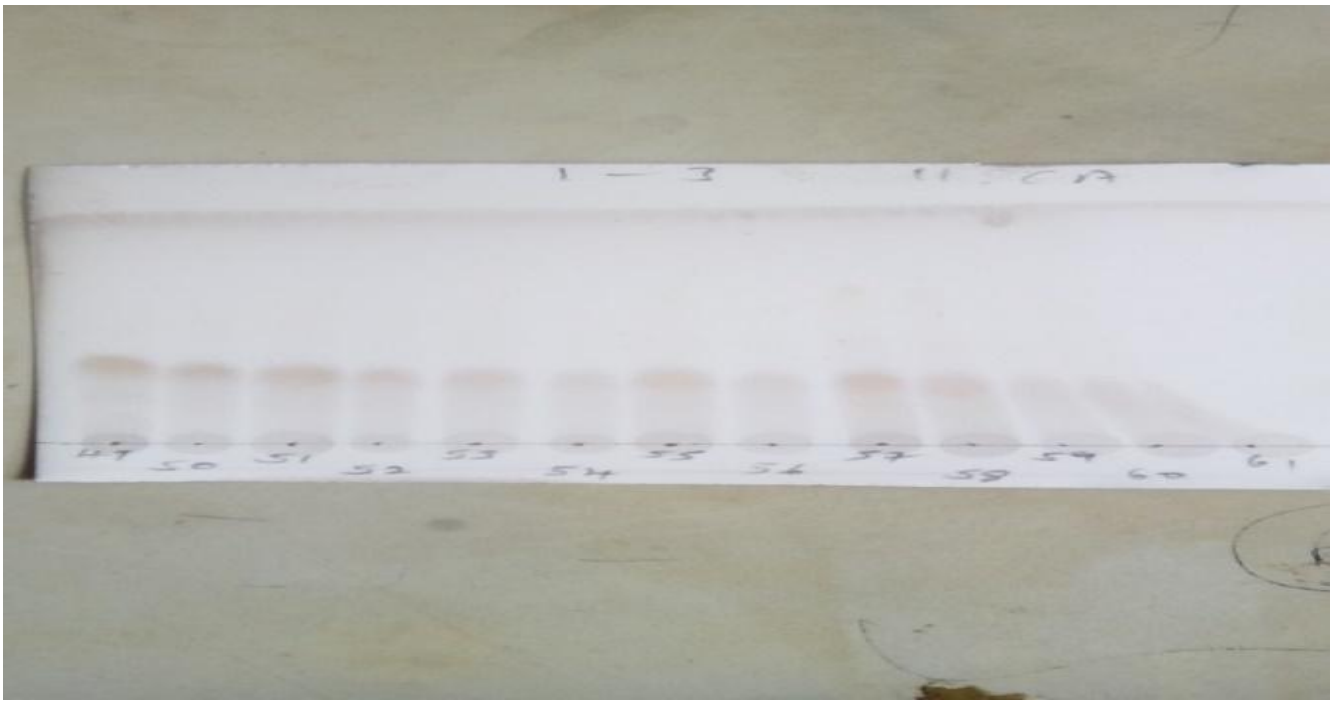


Plate XII: TLC profile of various fractions of EAF using HEX: EA (1:3)

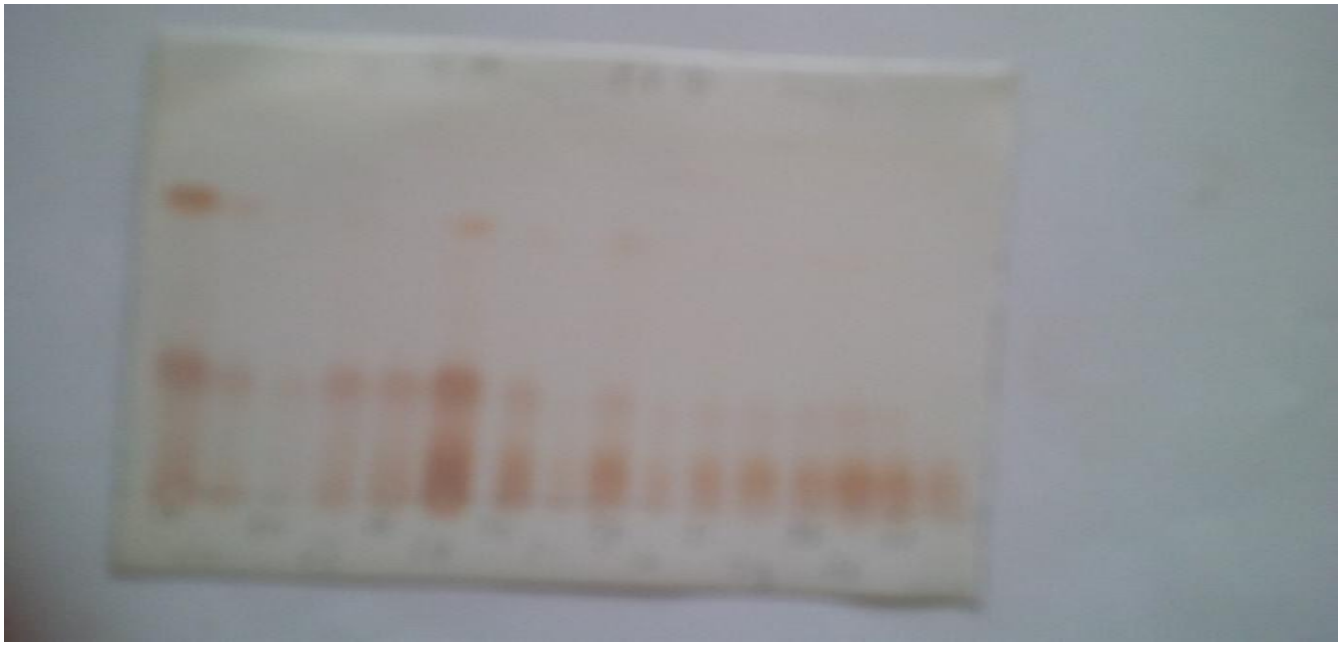


Plate XIII: TLC profile of various fractions of EAF using HEX: EA (1:2)

4.2.4 Gel Filtration of E3 fraction fromethyl acetate fraction (EAF)

4.2.4.1 Gel filtration chromatography of E3

Sephadex LH-20 column collections of fraction **E3** gave four (4) major fraction and further purification of fractions **E3b** gave a yellow amorphous solid coded H1

Table 4.7: Gel filtration chromatography (Sephadex LH-20) of E3

S/N	No. of Eluates	Code
	E3	
1	1 - 6	E3a
2	7 - 8	**E3b**
3	9 - 10	E3c
4	11 - 15	E3d

*4.2.4.2 TLC profile of gel filtration chromatography of **E3***

Thin layer chromatographic analysis of the collection made for the gel filtration chromatography of **E3** using hexane: ethylacetate (1:1) as solvent system revealed a number of single spots and some spots with little impurities which base on their TLC profile were pooled into two fractions coded **E3a**-**E3d**.

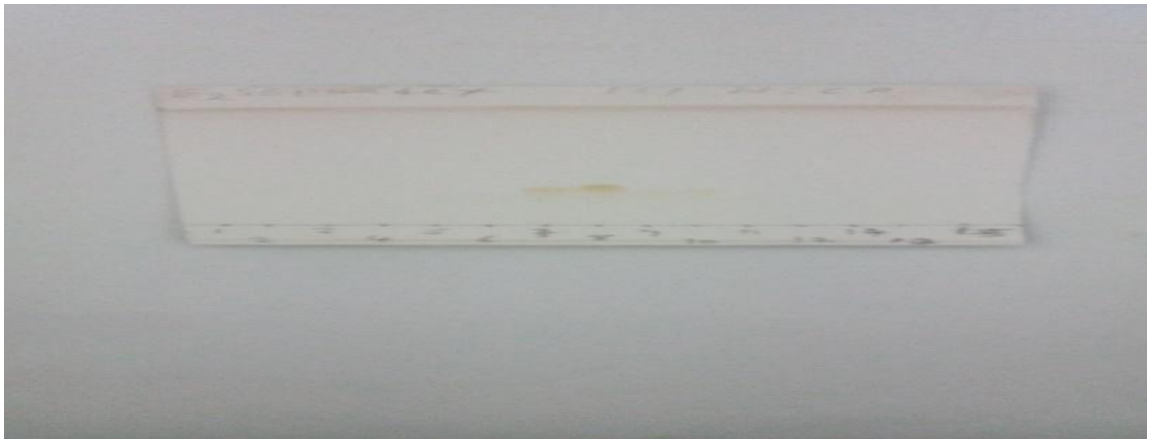


Plate XIV: TLC Profile of gel filtration of E3 HX: EA 1:1

4.2.5Purificationof E3b

4.2.5.1 Gel filtration chromatography of fraction E3b

Repeated gel filtration chromatography of fraction **E3b** obtained from fraction **E3** of the ethylacetate fraction of methanol leaf extract led to the isolation of yellow amorphous compound coded H1 (10 mg) (Table 4.10).

4.2.5.2TLC profile of H1 using five different solvent systems.

Compound H1 gave a single homogeneous spot on TLC plate using three different solvent systems (Table 4.11) and plate (XV, XVI and XVII).



Plate XV: TLC of H1 HX: EA 1:3 **Plate XVI: TLC of HX: EA 1:1** **Plate XVII: TLC of HX: EA 1:4**

Table 4.8: $R_f$values of H1

Solvent System	Colour of spot after heating	R_f Value
HX: EtOAc (1:3)	Yellowish	0.61
HX: EtOAc (1:1)	Yellowish	0.32
HX: EtOAc (1:4)	Yellowish	0.67

4.3Physicochemical properties of compound H1

4.3.1 Solubility Profile of H1

Compound H1 was found to be sparingly soluble in ethylacetate and completely soluble in methanol.

4.3.2 Melting point of H1

The uncorrected melting point of H1 was found to be between 245-246^{O}C.

4.3.3 Chemical Test on H1

H1 gave positive test to both shinoda and ferric chloride tests

4.4 Spectral Analysis of H1

4.4.1 Infrared Spectrum of **H1**

The IR spectrum in (KBr) showed absorption at 3360cm^{-I} is possibly due to O-H stretching, 3010 cm^{-I} is due to C-H stretching, a carbonyl C=O stretching around 1660cm^{-I}, C=C bending around 1480 cm^{-1} and a sharp C-O stretching around 1050cm^{-1} due to ether linkage in the chromen nucleus (Singh and Singh, 2016)

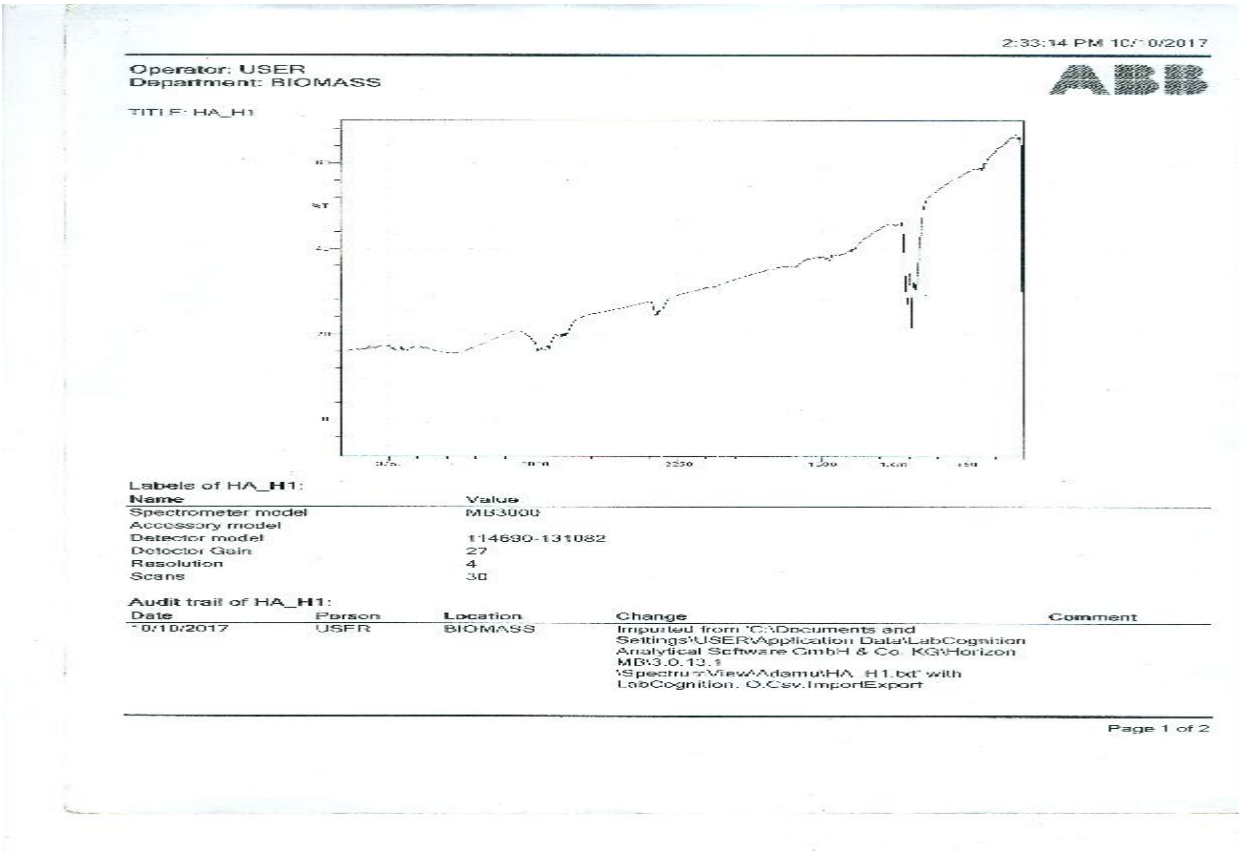


Figure 4.1 Infrared spectrum of compound H1 in (KBr)

4.4.2 Proton Nuclear Magnetic Resonance (^{1}H NMR) Analysis of **H1**

^{1}H-NMR spectrum of compound H1 revealed resonances at δ 6.21 (1H, d, J=1.8 Hz, H-6), δ 6.42 (1H, d, 1.5 Hz, H-8), δ 6.92 (1H, d, 8.5 Hz, H-6'), δ 7.67 (1H, d, J= 8.1 Hz, H-3') and a broad singlet at δ 7.76 (1H, s, H-3'). It also revealed some aliphatic signals at δ 0.89, δ 0.97, δ 1.36,δ 1.62,δ 2.05,and δ 2.21.

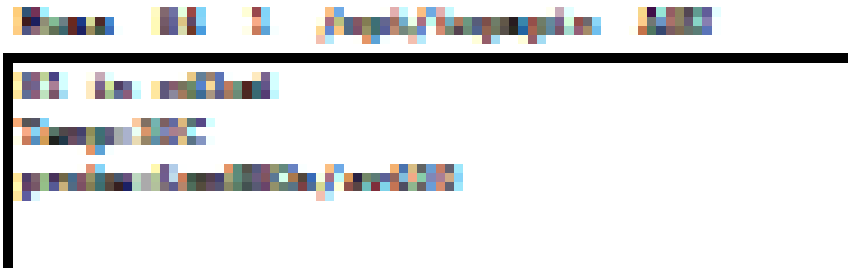


Figure 4.2 ^{1}H-NMR spectrum of H1 in CD3OD

4.4.3 ^{13}C NMR Spectral Analysis of **H1**

The ^{13}C-NMR (δ ppm, 600MHz, CD_3OD) analysis revealed signals at 175.9 (C-4), 164.72 (C-7), 161.2 (C-5), 156.8 (C-9), 146.7 (C-2), 147.4 (C-4'), 144.8 (C-6'), 120.3 (C-3), 122.8 (C-1'), 120.3 (C-3') 114.9 (C-2'), 114.6 (C-5'), 103.2 (C-10'), 97.90 (C-6), 93.1 (C-8). Other resonances were observed at δc 35.1, δc 31.6, δc 29.3, δc 28.9, δc 22.3, δc 13 in the ^{13}C-NMR of H1.



Figure 4.3:^{13}C NMR of Compound H1 in CD_3OD

4.4.4 The DEPT spectra revealed the presence of ten (10) quaternary carbons viz: C-2, C-3, C-4, C-5, C-7, C-9, C-10, C-1', C-4' and C-6' six (6) methine carbons viz: C-6, C-8, C-2', C-3',C-5'and C-3'', Four (4) methylene viz: C-1'', C-2'', C-4'',C-5'' and two methyl carbons viz: C-6'' and C-1'''.

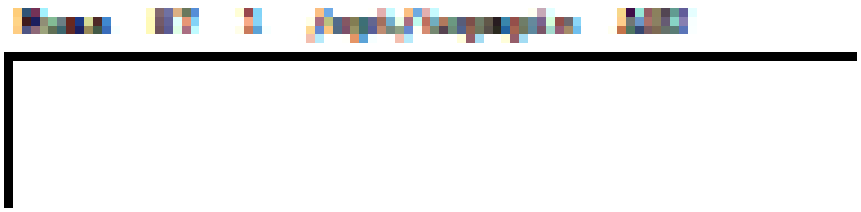


Figure 4.5: DEPT spectrum of H1 in CD_3OD

4.4.5^{1}H-^{1}H COSY Spectral Analysis of H1

The ^{1}H-^{1}H COSY revealed the correlation between the following aromatic protons: H-8 (6.42) # H-6 (6.21), H-5’ (6.92) # H-6’ (7.67) and correlations between aliphatic protons: H-1’’ (2.21) # H-2’’ (1.62), H-2’’ (1.62) # H-3’’(1.36), H-3’’(1.36) # H-4’’(2.05), H-3’’(1.36) # 1’’’(0.93).



Figure 4.5 HSQC spectrum of H1 in CD_3OD

4.4.6 HSQC Analysis of **H1**

The various attachment of protons to their respective carbons were confirmed from the Heteronuclear Single Quantum Correlation spectrum (HSQC). The established attachments include the following: C (114.63) # 7.76, C (120.31) # 7.67, C (114.86) # 6.92, C (93.10) # 6.21, C (97.90) # 6.42, C(28.91) # 1.36, and C(29.32) # 1.34. Figure 4.7



Figure 4.6 HSQC spectrum of H1 in CD3OD

4.4.7 HMBC Spectral Analysis of **H1**

HMBC spectrum established the linkages between protons and carbons through the various correlation. Correlation between proton at δ 6.21 (H-6) and carbons at δ 93.1 C-8, 103. C-10, 161.1 C-5, 164.2 C-5. Proton at δ 6.42 (C-8) was found correlating with carbons at δ 97.9 C-6, δ 103.1 C-10, δ 156.9 C-9, δ 164.2 C-7. Proton at δ 6.92 (C-6') correlate with carbons at δ 122.8 C-1', 144.8 C-6', 146.7 C-2, δ 147.4 C-4', proton at δ 7.76 (C-3') correlate with the carbon at δ 122.8 C-3'. In the aliphatic region proton at δ0.97 correlates with the carbons at δ 31.6 C-2'' and 25.5 C-4''



Figure 4.7 HMBC spectrum of H1 in CD3OD

Figure 4.8: Structure of H1 showing 2D correlation

Table 4.9: Summary of 1D and 2D Spectral Data for Compound H1 (CD3OD, 600MHz)

Position	δ ^{13}C	δ ^{1}H, J (Hz)	DEPT	COSY	HMBC
2	146.7		C		
3	100.0		C		
4	176.0		C		
5	161.1		C		
6	97.9	6.21 (1H, d, J=1.56)	CH	H-6	C-8, 5,7, 10
7	164.2		C		
8	93.1	6.42 (1H, d, J=1.50)	CH	H-8	C-6, 7, 9, 10
9	156.9		C		
10	103.1		C		
1’	122.8		C		
2’	147.3		CH	H-2’	C-1’
3’	114.4		C		
4’	144.8		C		
5’	114.6	7.76 (br s, 1H)	CH	H-5’	C-3’
6’	114.8	6.92(1H, d, J=8.50)	CH	H-6’	C-1’, 2, 4’6’.
1’’	35.1		CH_2		
2’’	31.6		CH_2		
3’’	28.9		CH	H-3’’	C-5’’
4’’	25. 5		CH_2		
5’’	29.3		CH_2	H-5’’	C-3’’
6’’	13.0		CH_3		
1’’’	22.3		CH_3	H-1’’’	C-2’’,4’’

4.5Isolation of H3

4.5.1 Vacuum liquid chromatographic separation of ethylacetate fraction

Vacuum liquid chromatographic analysis of ethylacetate fraction of methanol leaf extract carried out using different solvent combinations afforded four major fractions (Table 4.12)

Table 4.10Vacuum liquid chromatography of ethylacetate fraction

N/S	No of Eluates	Number of spots
18-14**A**		
2	15-20	B
321-32C		
4	33-60 D	

4.5.2 TLC profile of VLC collection from ethylcetate fractionof methanol leaf extract

TLC analysis of VLC collection from ethylacetate fraction using ethylacetate: chloroform: methanol: water as solvent system revealed a number of spots when sprayed with 10% sulphuric acid followed by heating at 105 ^{o}Cfor five minutes (Plate XVIII)

Plate XVIII: TLC profile of various fractions of EAF using E:C:M:W (15:8:4:1)

4.5.3 Chromatographic separation of fraction D from VLC collection of ethylacetate fraction.

Column chromatographic analysis of fraction D of ethylacetate fraction from VLC conducted using different solvent combinations affordedten pooled fractions (Table 4.13) and their TLC profile are shown on plates XVIII-XX.

Table 4.11 Column chromatography of fraction D of ethylacetate from VLC

S/N	Solvent System	No of euates (30 mL)	Code
1	Chloroform: methanol (90:10)	1-3	D1
2	Chloroform: methanol(85:15)	4-5	D2
3	Chloroform: methanol(85:15)	6-7	D3
4	Chloroform: methanol(85:15)	8-16	**D4**
5	Chloroform: methanol(85:15)	17-33	D5
6	Chloroform: methanol(85:15)	34-38	D6
7	Chloroform: methanol(85:15)	39-43	D7
8	Chloroform: methanol (85:15)	44-51	D8
9	Chloroform: methanol (85:15)	52-62	D9
10	Chloroform: methanol (80:20)	63-89	D10

4.5.3.1 TLC Profile of fraction C from Column collections for ethlyacetate fraction of methanol leaf extract

TLC profile of column collections for ethylacetate fraction carried out using chloroform: methanol of fraction C using solvent system ethyl acetate: methanol (90:10), (85:15) and (80:20) as solvent systems revealed various spots after spraying with 10% H_2SO_4 followed by heating at 105 ^oC (Plate XIX- XXI).

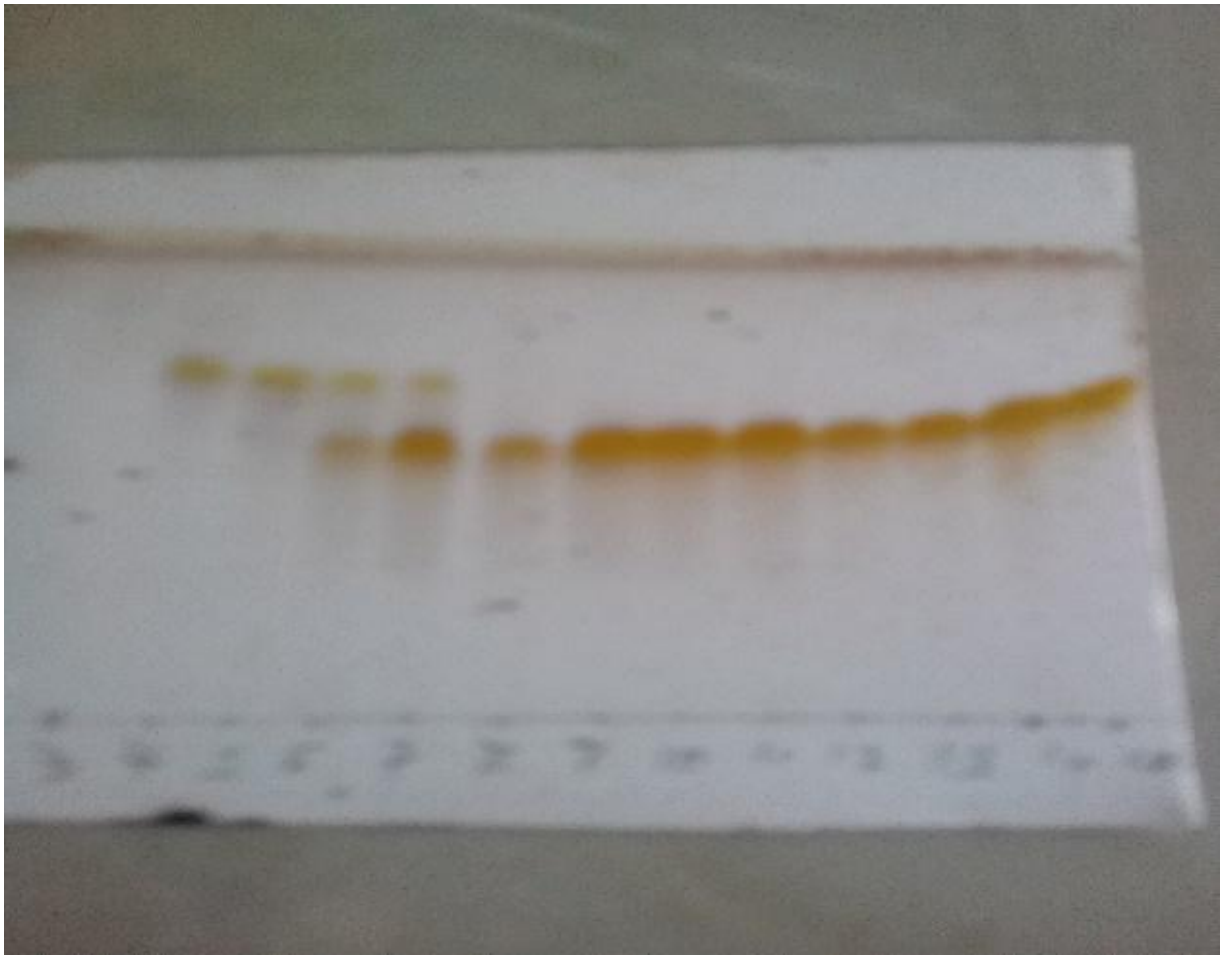


Plate XIX: TLC profile of various fractions of EAF using$CHCl_3$: MeOH (85:15)



Plate XX: TLC profile of various fractions of EAF using $CHCl_3$: MeOH(85:15)

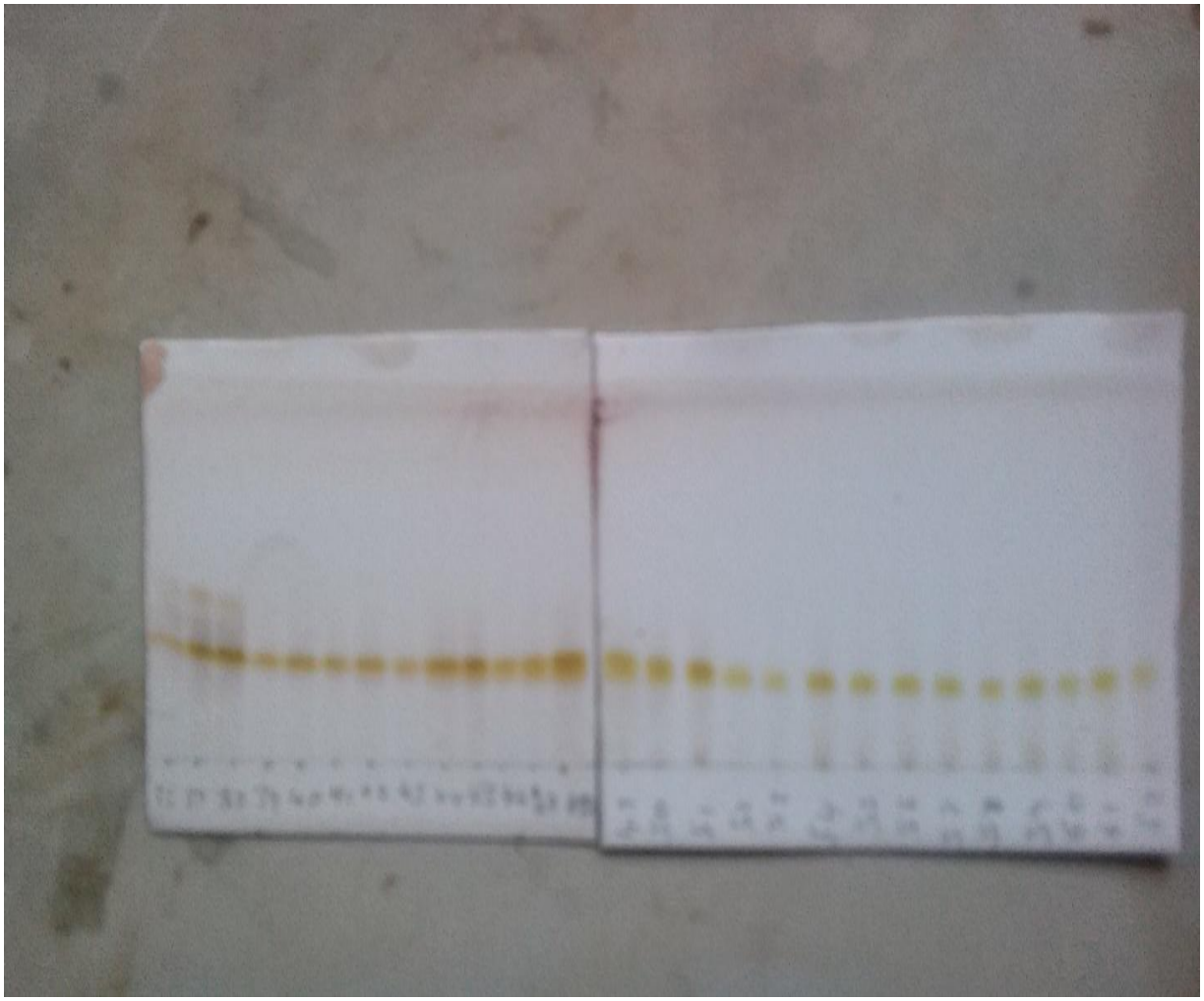


Plate XXI: TLC profile of various fractions of EAF using $CHCl_3$: MeOH (85:15)

4.5.4 Gel Filtration of D4 and D4e fraction from ethyl acetate fraction (EAF)

*4.5.4.1 Gel filtration chromatography of **D4**and **D4e***

Sephadex LH-20 column collections of fraction **D4** gave five (5) major fraction **D4a**-**D4e**and further purification of fractions D4e gave a yellow amorphous solid coded H3

Table 4.12: Gel filtration chromatography (Sephadex LH-20) of D4 and D4e

S/N	No of Eluates	Code
	D4	
1	1-2	D4a
2	3-6	D4b
3	7-14	D4c
4	15-17	D4d
5	18-30	**D4e**
	D4e	
	1-15	**D4e**

4.5.5 TLC profile of gel filtration chromatography of D4 and D4e

Thin layer chromatographic analysis of the collections made for the gel filtration chromatography of **D4**. Further purification of D4 gave a single spot**D4e**usingethylacetate: chloroform: methanol: water (15: 8: 4: 1) and chloroform: methanol (85: 10) as solvent systems.

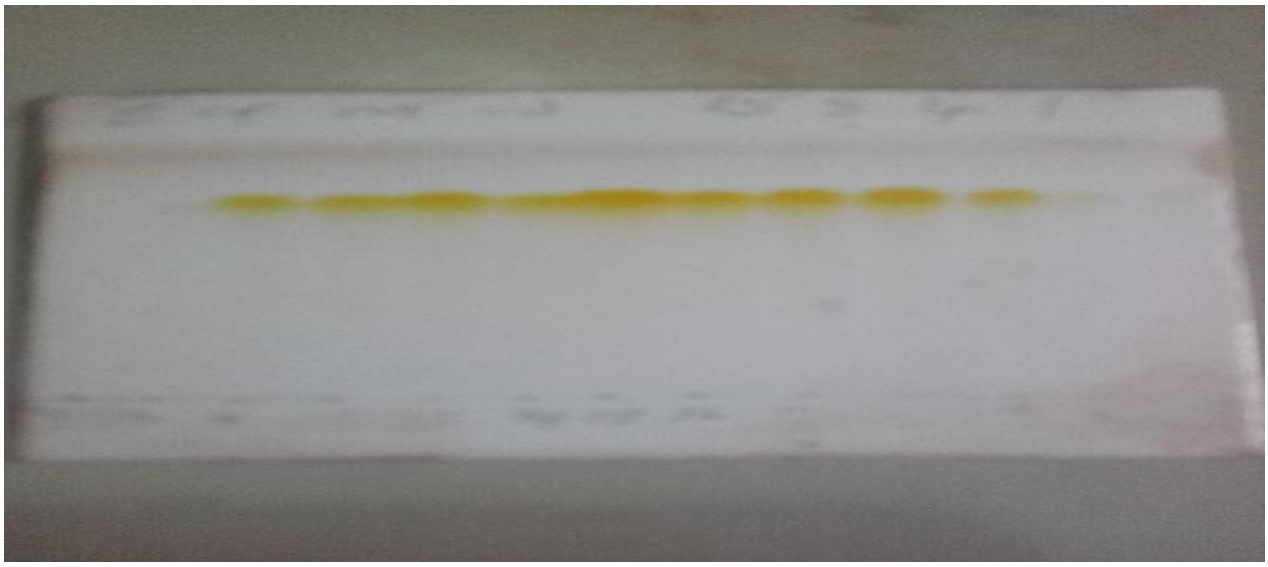


Plate XXII: TLC profile of gel filtration chromatography of D4e E:C:M:W (15:8:4:1)

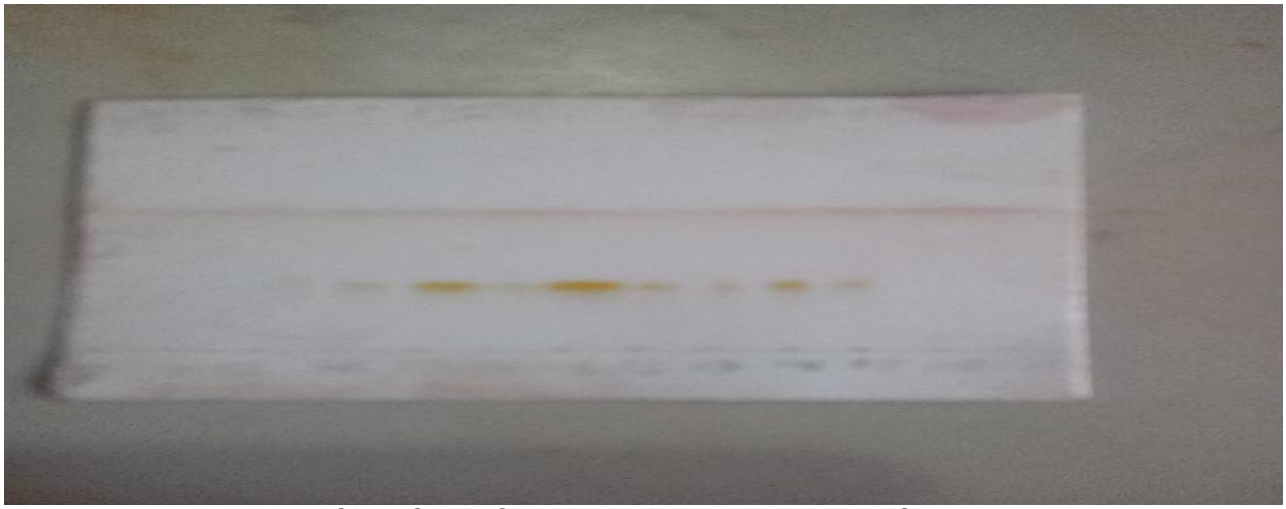


Plate XXIII: TLC profile of gel filtration chromatography of D4e $CHCl_3$: MeOH (85:15)

4.5.6 Purification of D4e

*4.5.6.1*Repeated gel filtration chromatography of fraction **D4e** obtained from fraction **D4** of the ethylacetate fraction of methanol leaf extract led to the isolation of yellow amorphous compound coded H3 (12 mg), (Table 4.15)

4.5.7 TLC profile of H3 using five different solvent systems

Compound H3 gave a single homogeneous spot on TLC plate using three different solvent systems (Table 4.11) and Plate (XXIV and XXV).

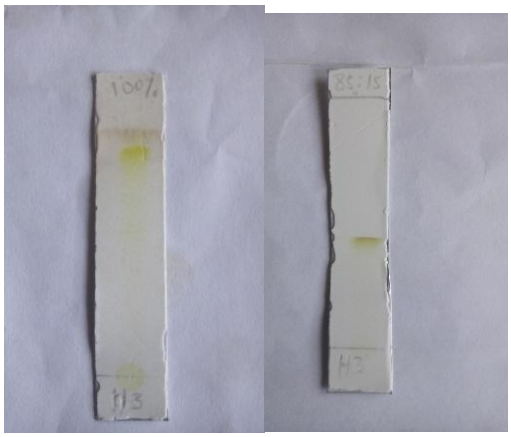


Plate XXIV: EA 100%Plate XXV: CHCl3: MeOH (85:15)

Plates XXIV- XXV: TLC profile of H3

Table 4.13: R_f Values of H3

Solvent System	No.of spots	Colour of spot after heating	R_f Value
EtOAc: 100%	1	Yellowish	0.87
$CHCl_3$:MeOH (85:15)	1	Yellowish	0.34

4.6. Physicochemical properties of compound H3

4.6.1 Solubility Profile of H3

Compound H1 was found to be sparingly soluble in ethylacetate and completely soluble in methanol.

4.6.2 Melting point of H3

The uncorrected melting point of H1 was found to be between 314-315^{O}C.

4.6.3 Chemical Test on H3

H1 gave positive test to both shinoda and ferric chloride tests

4.7 Spectral Analysis of H3

4.7.1 Infrared Spectrum of **H3**

The IR spectrum of compound H3 showed a broad absorption around 3420 cm^{-1} due to O-H stretching, an aromatic C-H stretching around 2950 cm^{-1}, a carbonyl stretching around 1680cm^{-1} due to ketonic group, C=C absorption around 1480 cm-1 and a sharp C-O stretching around 1050 cm^{-1} due to ether linkage (Singh and Singh, 2016)

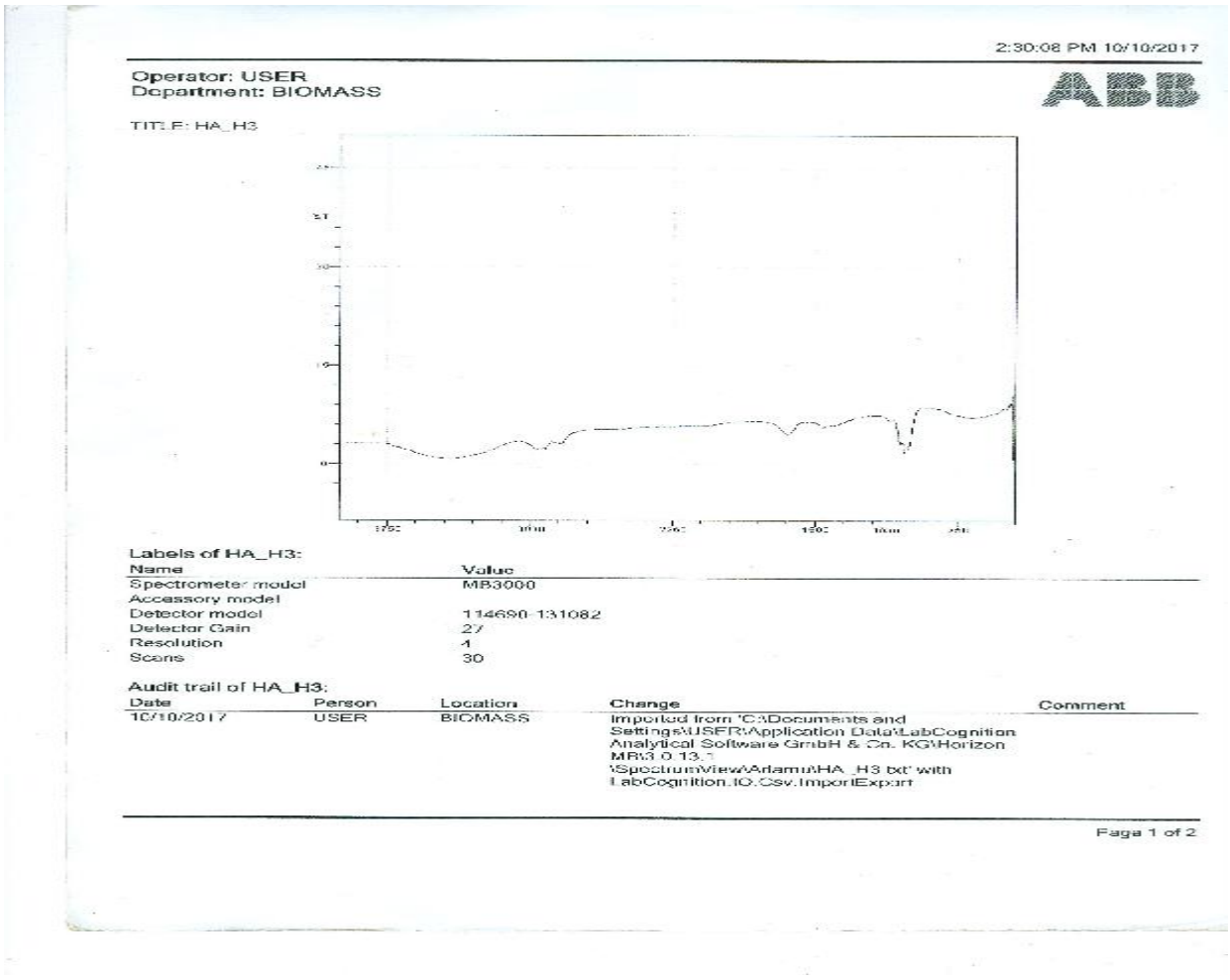


Figure. 4.9 shows the IR spectrum of H3 in (KBr)

4.7.2 Proton Nuclear Magnetic Resonance (^{1}H NMR) Analysis of **H3**

The ^{1}HNMR spectrum of H3 in CD3OD (600MHz) revealed signal at aromatic proton region;δ 6.42 (1H, d, J= 1.50 Hz) and δ 6.21 (1H, d, J= 1.56 Hz) assignable to H- 6 and H-8 respectively while an ABX system of 1,3',4' trisubstituted benzene ring B was observed via protons at δ 7.76 (1H, d, J= 1.38 Hz, H-2'), δ 7.67 (1H, dd, J= 1.7 Hz, 8.4 Hz, H-6') and δ 6.92 (1H, d, J= 8.4 Hz, H-5') (Figures 4.10).

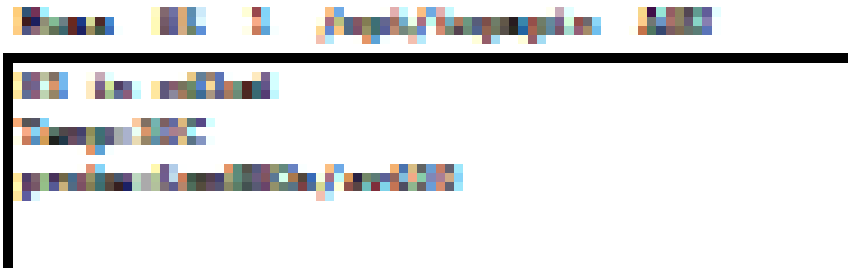


Figure 4.10^{1}H-NMR spectrum of H3 in CD_3OD (6.21-7.76 ppm)

4.7.3 ^{13}C NMR Spectral Analysis of **H3**

The ^{13}C-NMR (δ ppm, 600MHz, CD_3OD) analysis revealed signals at 175.9 (C-4), 164.72 (C-7), 161.2 (C-5), 156.8 (C-9), 146.7 (C-2), 147.4 (C-4'), 144.8 (C-3'), 135.8 (C-3), 122.8

(C-1’), 120.3 (C-6’) 114.9 (C-5’), 114.6 (C-2’), 103.2 (C-10’), 97.90 (C-6), 93.1 (C-8)(figure 4.11)

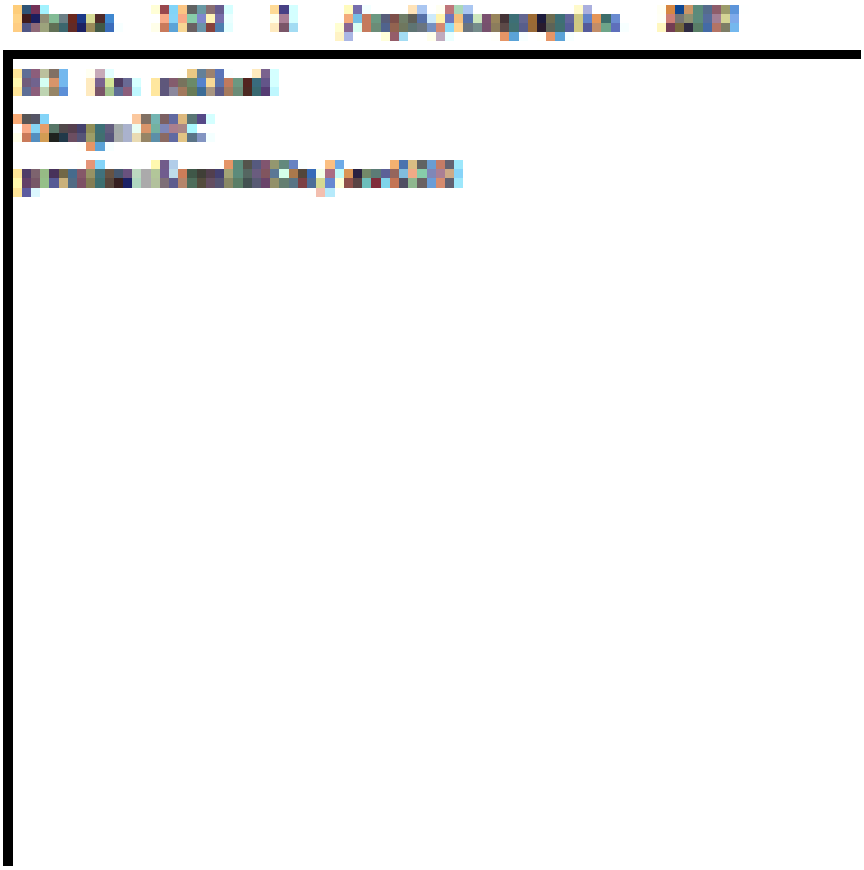


Figure 4.11 shows ^{13}C NMR spectrum of H3 in CD3OD

4.7.4 DEPT NMR Spectral Analysis of **H3**

The DEPT and ^{13}C NMR spectra revealed the presence of ten (10) quaternary carbons viz: C-2, C-3, C-4, C-5, C-7, C-9, C-10, C-1', C-3'and C-4', five methine (5) viz: C-6, C-8, C-2', C-5' and C-6'.



Figure 4.12 DEPT spectrum of H3 in CD_3OD

4.7.5 ^{1}H-^{1}H COSY Spectral Analysis of H1

The 1H-1H COSY revealed the correlation between the following aromatic protons: H-8 (6.42) # H-6 (6.21), H-5’ (6.92) # H-6’ (7.67).



Figure 4.13 ^{1}H-^{1}H COSY NMR spectrum of H3 in CD_3OD

4.7.6 HSQC Analysis of **H3**

The various attachment of protons to their respective carbons were confirmed from the Heteronuclear Single Quantum Correlation spectrum (HSQC). The established attachments among others include the following: C 114.63 # 7.76, C 120.31 # 7.67, C 114.86 # 6.92, C 93.10 # 6.21, C 97.90 # 6.42. Figure 4.



Figure 4.14 HSQC spectrum of H1 in CD_3OD

4.7.7 HMBC Spectral Analysis of **H3**

Heteronuclear multiple bond correlation spectroscopy (HMBC) established the linkages between protons and carbons through the various correlation; proton at δ_H 6.21 (H-6) and carbons at δc 93.1 C-8, 103.1 C-10, δ 161.1 C-5, 164 C-7, between proton at δ 6.42 (H-8) and carbons at δ 97.9 C-6, 103.1 C-10, 156.9 C-9, 164.2 C-7 of ring A; correlation between proton at δ 6.92 (H-5'), and carbons at δ 122.8 C-1', 144.8 C-3', δ 147.4 C-4'; Proton at δ 7.67 (H-6') is correlating with carbons at δ 114.6 C-2', 144.7 C-3' while proton resonating at δ 7.76 (H-2') is correlating with carbons at δ 120.3 C-6', δ 144.8 C-4', δ 146.7 C-2 of ring B.

Figure 4.15: HMBC spectrum of H3 in CD_3OD

Figure 4.16 Structure of H1 showing 2D correlation

Table 4.14 Comparison of ^{1}H-NMR and ^{13}C-NMR Data of Compound H3 with That of the Literature

POSITION	^{1}H-NMR OF H3	^{1}H-NMR (Mabry *et al.*, 1970)	^{13}C-NMR OF H3	^{13}C-NMR (Mabry *et al.*, 1970)
2			146.66	147.39
3			135.82	135.84
4			175.95	175.96
5			161.10	161.12
6	6.21 (d, 1.56 Hz)	6.21 (d, 1.98 Hz)	97.88	99.00
7			164.21	164.20
8	6.42 (d, 1.50 Hz)	6.42 (d, 1.98 Hz)	93.10	94.00
9			156.86	156.86
10			103.13	103.13
1’			122.77	122.76
2’	7.76 (d, 1.38 Hz,)	7.76 (d, 2.04 Hz)	114.63	114.84
3’			144.83	146.64
4’			147.38	144.61
5’	6.92 (d, 8.4 Hz)	6.91 (d, 8.34 Hz)	114.86	114.61
6’	7.67(dd,1.7, 8.4 Hz)	7.66 (dd, 2.1, 8.4 Hz)	120.31	120.29

4.8 Antimicrobial Studies

4.8.1. Susceptibility studies

The result of susceptibility tests showed that an increase in concentration of the extract and fractions of *Tapinanthus globiferus* led to an increase in antifungal activity at the graded concentrations (100, 50, 25 and 12.5 mg/mL).The standard drug, fluconazole exhibited good activity (26.16±0.44) against *C. albican* at 0.05 mg/mL.

4.8.1.1 Antifungal Susceptibility test of Methanol leaf Extract Tapinanthus gloiferus against selected fungal species

Methanol leaf extract recorded mean zone of inhibition 27.83±0.16 mm. *C albican* was the most sensitive organism to methanol leaf extract at 100 mg/mL with mean zone of inhibition of 18.83±0.44 mm while *Aspergillus niger* was the least sensitive to extract at 100 mg /mL with mean zone of inhibition 14.46±0.29 mm.

Table 4.15: Antifungal Susceptibility test of Methanol leaf Extract *Tapinanthus gloiferus* against selected fungal species

Zone of inhibition (mm)			Fluconazole		
Organism	100 mg/mL	50 mg/mL	25 mg/mL	12.5 mg/mL	0.05 mg/mL
C. candida	18.83±0.44	17.16±0.16	14.16±0.44	11.66±0.33	25.16±0.44
T.mentagrophyte	15.83±0.16	12.83±0.16	11.50±0.28	8.00±0.00	21.00±0.57
T. rubrum	16.50±0.28	13.50±0.28	12.50±0.28	8.00±0.00	20.16±0.72
A. niger	14.46±0.29	11.50±0.28	8.00±0.00	8.00±0.00	18.83±0.44

Values are mean inhibition zone (mm) ± S.E of three replicates

4.8.1.2 MIC and MFC of methanol leaf extract of Tapinanthus globiferus against selected fungal species

The minimum inhibitory concentration (MIC) range for methanol leaf extract (CME) was 6.25 mg/mL to 25 mg/mL CME recorded mild activity against *C. albican* and *T. rebrum* both at 6.25 mg/mL and *T. mentagrophyte* at 12.5 mg/mL. It showed the least activity against *A. niger* at 25.0 mg/mL while it showed fungistatic activity against *C. albican*, and *A. niger* at 12.5 mg/mL and 25.0 mg/mL while exhibiting fungicidal activity against *T. mentagrophyte* and *T. rebrum* respectively.

Table 4.16: MIC and MFC of methanol leaf extract of *Tapinanthus globiferus* against selected fungal species.

Concentration of crude methanol extract (mg/ml)		
Organism	MIC mg/mL	MFC mg/mL
C. albicans	6.25	12.5$^{\eta}$
T.mentagrophyte	12.5	12.5*
T. rubrum	6.25	6.25*
A.niger	25.0	25.0$^{\eta}$

Key: * = fungicidal effect, η = fungistatic effect

4.8.1.3 Antifungal Susceptibility test of ethylacetate fraction Tapinanthus gloiferus against some selected fungal species.

The result of the susceptibility test showed that ethylacetate exhibiting the highest mean zone of inhibition (27.83±0.16 mm). Ethyl acetate fraction showed good mean zone of inhibition (22.00±0.57 mm) at 25 mg/mL in which there is no significant different in activity as compared to that of standard drug with mean zone of inhibition (22.16±0.27 mm) at 0.05 mg /mL.

Table 4.17: Antifungal Susceptibility test of ethylacetate fraction *Tapinanthus gloiferus* against some selected fungal species.

Zone of inhibition (mm)				Fluconazole	
Organism	100 mg/mL	50 mg/mL	25 mg/mL	12.5 mg/mL	0.05 mg/mL
C. albicans	27.83±0.16	22.33±0.16	18.83±0.44	15.00±0.57	26.16±0.44
T.mentagrophyte	27.16±0.44	24.16±0.16	17.83±0.16	8.00±0.00	20.00±0.57
T. rubrum	27.00±0.57	24.50±0.28	22.00±0.57	8.00±0.00	22.16±0.72
A. niger	17.33±0.88	15.50±0.2	13.16±0.44	11.50±0.28	18.93±0.24

Values are mean inhibition zone (mm) ± S.E of three replicates

4.8.1.4 MIC and MFC of ethylacetatefraction of Tapinanthus globiferus against some selected fungal species

Ethyl acetate fraction exhibited MIC range from 3.13 mg/mL to 25.0 mg/mL. It recorded lowest MIC against *T. rebrum* at 3.13 mg/mL and *C. albican* at 12.5 mg/mL while exhibiting higher MIC against *T. mentagrophyte* and *A. niger* at 25.0 mg/mL respectively. ethylacetate fraction showed fungicidal activity against *T. rebrum, C. albican* and *T. mentagrophyte* but exhibiting fungistatic activity against *A. niger* at 25.0 mg/mL.

Table 4.18: MIC and MFC of Ethylacetatefraction of *Tapinanthus globiferus* against some selected fungal species

Concentration of ethyl acetate fraction (mg/mL) MIC MFC		
Organisim	MIC mg/mL	MFC mg/mL
C. albican	12.5	12.5*
T.mentagrophyte	25.0	25.0*
T. rebrum	3.13	3.13*
A. niger	12.5.0	12.5η

Key: * = fungicidal effect, η = fungistatic effect

4.8.1.5 Antifungal Susceptibility test of n-butanol fraction Tapinanthus globiferus against some selected fungal species

Susceptibility result showed that*C. albican* was the most susceptible to n-butanol fraction at 100 mg/mL (24.50±0.28 mm) while *T. mentagrophyte* was the least sensitive with mean zone of inhibition (15.50±0.44 mm). It showed a better zone of inhibition (24.50±0.28mm) over standard drug (24.16±0.16 mm) against *C. albican* at 100 mg/mL.

Table 4.19: Antifungal Susceptibility test of n-butanol fraction *Tapinanthus globiferus* against some selected fungal species

Zone of inhibition (mm)					Fluconazole
Organism	100 mg/mL	50 mg/mL	25 mg/mL	12.5 mg/mL	0.05 mg/mL
C. albican	24.50±0.28	23.66±0.33	21.00±0.57	19.33±0.33	24.16±0.16
T.mentagrophyte	15.50±0.28	10.33±0.16	8.00±0.00	8.00±0.00	22.00±0.53
T. rubrum	15.85±0.44	13.33±0.33	9.38±0.33	8.00±0.00	21.26±0.72
A. niger	18.83±0.29	17.16±0.16	13.16±0.28	10.66±0.33	18.49±0.16

Values are mean inhibition zone (mm) ± S.E three replicates

4.8.1.6 MIC and MFC of butanol fraction of Tapinanthus globiferus against selected fungal species.

The MIC range for n-butanol fraction was 0.39 mg/mL to 12.5 mg/mL. This fraction recorded the highest activity against all the test organisms with lowest MIC of 0.39 mg/mL against *C. albican*, 3.13 mg/mL *A. niger*, 6.25 mg/mL *T. rebrum* and 12.5 mg/mL *T. mentagrophyte*. The fraction was found to be fungicidal against all the test organisms.

Table 4.20: MIC and MFC of n-butanol fraction of *Tapinanthus globiferus* against selected fungal species

Concentration of n-butanol fraction (mg/ml)		
-Organism	MIC mg/ml	MFC mg/ml
C. albican	0.39	0.39*
T.mentagrophyte	12.5	12.5*
T. rebrum	6.25	6.25*
A. niger	3.13	3.13*

Key: Key: * = fungicidal effect, η = fungistatic effect

CHAPTER FIVE

5.0 Discussions

5.1 Preliminary Phytochemical Studies and Spectra Analysis

The preliminary phytochemical screening of the methanol leaf extract of *Tapinanthus globiferus* growing on *Vitex donina* revealed the presence of saponins, tannins, alkaloids, cardiac glycosides, carbohydrates, steroids and flavonoids. The presence of these phytochemical constituents in *Tapinanthus globiferus* growing on other plants have been reported (Abubakar *et al.*, 2016). Preliminary phytochemical screening of ethyl acetate fraction revealed the presence of flavonoids, tannins, saponins and glycosides. These phytochemicals are thought to be responsible for different pharmacological and physiological activities of plant extract (Cragg and Newman, 2005).

Chromatographic separation of ethyl acetate fractionof *Tapinanthus globiferus* by gel filtration chromatography led to the isolation of two yellow amorphous compounds coded H1 and H3.

The IR spectrum of H1 showed absorption band at 3350cm^{-I} which is due to O-H stretching, 3010cm^{-I} is due to C-H stretching, a carbonyl stretching around 1660 cm^{-I} due to C=O, C=C bending around 1480cm^{-1} and a sharp C-O stretching around 1050cm^{-1} due to ether linkage in the chromen nucleus typical of flavonoids (Singh and Singh, 2015)

^{1}H-NMR spectrum of compound H1 exhibited 1,2,3,5-tetrasubtituted benzene ringA and 1',4',6'- trisubtituted benzene ring B via the protons at δ 6.42 (1H, d, J=1.5 Hz, H-8), δ 6.21 (1H, d, 1.8 Hz, H-6) for ring A and via other protons at δ 6.92 (1H, d, 8.5 Hz, H-6'), δ 7.67

(1H, d, J= 8.1 Hz, H-5') and a broad singlet at δ 7.76 (1H, s, H-3'). Proton ^{1}H-NMR also revealed some aliphatic signals at 0.89, 0.97, 1.36, 1.62, 2.05, and 2.21,these signals are typical of aliphatic side chain.

^{1}H-^{1}H-COSY correlation established via cross linking of protons that lie adjacent to each other across the diagonal showed correlation of protons at δ 6.21 and δ 6.42 ppm,the proton at δ 6.97 and 7.76 ppm.Correlations between protons as observed in ^{1}H-^{1}H Cosy of compound H1 between H-1''' (2.21) # H-2''(1.62), H-2'' (1.62) # H-3''(1.36), H-3''(1.36) # H-4''(2.05), H-3''(1.36) # 1'''(0.93) further indicated the presence of aliphatic side chain as part of the structure.

The ^{13}C-NMR (600 MHz, CD_3OD) and DEPT experiment of compound H1 revealed the presence of 15- carbon atoms, seven aromatic carbon signals at δ 103.1 (C-10), δ 97.9 (C-6), δ 93.1 (C-8), δ 122.8 (C-1'), δ 120.3 (C-6'), δ 114.9 (C-5'), and δ 114.6 (C-2') respectively. Six quaternary oxygenated aromatic carbon atoms at δ 164.2 (C-7), δ 161.1 (C-5) δ 156.9 (C-9), δ 147.7 (C-4'), δ 144.8 (C-3'), δ 146.7 and a down field signal due to carbonyl carbon resonating at δ 175.9, the absence of resonance at δ 135.8 (C-3)as observed in flavonol and thepresence of signal at δ 120.3 further suggest that the compound is a flavone base. .Aliphatic resonances at δc 35.1, δc 31.6, δc 29.3, δc 28.9, δc 26.7, δc 25.5, δc 22.3, δc 13.0 further confirmed the presence of aliphatic side chain of flavone type.

The HSQC spectrum established direct correlation between protons and their respective carbons. The proton at δ_H 6.21 correlates with the carbon at δc 97.8, proton at δ_H 6.42 correlates with the carbon at 93.1, proton at δ_H 6.92 correlates with the carbon at δc 114.9, proton at δ_H 7.67 correlates with the carbon at δc 120.3, proton at δ_H 7.76 correlates with the carbon at δc 114.6. HSQC attachment between C (28.91) # 1.36, and C (29.32) # 1.34 further indicated the presence of aliphatic side chain.

The connectivity between various fragments was established through Homo Nuclear Multiple Bond Correlation Spectroscopy (HMBC). It established a long range correlation between the proton at δ 6.21 (H-6) with the carbons at δ 93.1 (C-8), 103. (C-10), δ 161.1 (C-5), δ 164.2 (C-5). Proton at δ 6.42 (C-8) correlates with carbons at δ 97.9 (C-6), δ 103.1 (C-10), δ 156.9 (C-9), δ 164.2 (C-7). Proton at δ 6.92 (C-2') correlates with carbons at δ 122.8 (C-1'), δ 144.8 (C-4'), δ 146.7 (C-2) andδ 147.3 (C-6'), these correlation further confirmed the position of hydroxyl groups at (C-4') and (C-6').Proton at δ 7.76 (C-5') correlate with the carbon at δ 122.3 (C-3').Lack of carbon signal at δ_C101.8 and proton at δ_H 6.76and the presence of signal at 120.3 suggest that the aliphatic chain is attached to the flavone nucleus at C-3 position. Based on the spectral data and comparison with the existing literature (Chun-Nan *et al*., 2003), compound H1 was proposed to be 2-(2,4-dihydroxyphenyl)-5,7-dihydroxy-3-(3-methylhexyl)-4H-chromen-4-one with the following structure.

2-(2,4-dihydroxyphenyl)-5,7-dihydroxy-3-(3-methylhexyl)-4*H*-chromen-4-one

The uncorrected melting point of H3 was found to be between 315-316^{o}C indicating that the compound melt at a temperature similar to that reported by Sani*et al*. (2015). The positive

shinoda test and ferric chloride test implies that the compound is a flavonoid with free hydroxyl group (Silva *et al*., 1998).

The IR spectrum of H3 showed a broad absorption around 3350 cm^{-1} due to O-H stretching and, an aromatic C-H stretching was observed at 2950 cm^{-1},a carbonyl stretching at 1680cm^{-1}due to ketonic group, an aromatic C=C bending at 1480cm^{-1} and a sharp C-O stretching around 1050cm-1 due to ether linkage in the chromene nucleus typical of flavonoids (Singh and Singh, 2016).

The ^{1}H-NMR spectrum of H3showed the presence of 1,2,3,5- tetrasubstituted benzene ring A via the protons at δ 6.42 (1H, d, J= 1.50 Hz) and δ 6.21 (1H, d, J= 1.56 Hz) assignable to H- 6 and H-8 respectively and 1', 2',4' trisubstituted benzene ring B via protons at δ 7.76 (1H, d, J= 1.38 Hz, H-8'), δ 7.67 (1H, dd, J= 1.7 Hz, 8.4 Hz, H-6') and δ 6.92 (1H, d, J= 8.4 Hz, H-5') that are typical of flavonol nucleus (Mabry *et al*., 1970).

The^{1}H-^{1}H-COSY established the correlation between protons that are adjacent to each other, proton at δ 6.21 correlates with the proton at 6.42 ppm while proton at δ 6.92 was found to be correlating with the proton at δ 7.76 ppm. These correlations further confirmed the position of the protons in the ^{1}H-NMR of H3 (Sani *et al.,* 2015).

The ^{13}C-NMR (600 MHz, CD3OD) and DEPT experiment of compound H3 revealed the presence of 15- carbon atoms, seven aromatic carbon signals at δ 103.1 (C-10), 97.9 (C-6), 93.1 (C-8), 122.8 (C-1'), 120.3 (C-6'), 114.9 (C-5'), and δ 114.6 (C-2') respectively. Seven quaternary oxygenated carbon atoms at δ 164.2 (C-7), 161.1 (C-5) 156.9 (C-9), 147.7 (C-4'), 144.8 (C-3'), 135.8 (C-3), 146.7 and a down field signal due to carbonyl carbon resonating at δ 175.9. The presence of resonance at 135.8 (C-3) is an indication that compound H3 is a flavonol based.(Mabry *et al*., 1970).

The HSQC (Hetronuclear single quantum correlation) spectrum of H3 was used to attach each proton to their respective carbon atoms.Proton at δ_H 6.21 correlates with the carbon at δ_C 97.9, proton at δ_H 6.42 correlates with carbon at δ_C 93.1, proton at δ_H 6.92 correlates with the carbon at δC 114.9, proton at δ_H 7.67 correlates with the carbon at δ_C 120.3, while proton at δ_H7.76 correlates with the carbon at δ_C 114.6, the direct attachment of these protons to their respective carbons suggest that compound H3 contains flavonoid nucleus typical of flavonol.

The connectivity between various fragments was established through Heteronuclear Multiple Bond Correlation Spectroscopy (HMBC). It established the correlation between proton at δH 6.21 (H-6) with the carbons at δc 93.1 (C-8), δ 103.1 (C-10), δ 161.1 (C-5), δ 164 (C-7) and proton at δ 6.42 (H-8) showed a long range correlation with the carbons at δ 97.9 (C-6), δ 103.1 (C-10), δ 156.9 (C-9), δ 164.2 (C-7) which further confirmed the 1,2,3,5-tetrasubstituted benzene ring A. correlation between proton at δ 6.92 (H-5') with the carbons at δ 122.8 (C-1'), δ 144.8 (C-3'), δ 147.4 (C-4'), Proton at δ 7.67 (H-6') is correlating with carbons at δ 114.6 (C-2'), δ144.7 (C-3') and proton at δ 7.76 (H-2') is correlating with carbons at δ 120.3 (C-6'), δ 144.8 (C-4'), δ 146.7 (C-2) further confirmed the 1,3',4'-trisubstituted benzene ring B. Based on the 1D and 2D NMR data of H3 and comparison with the reported literature (Sani *et al*., 2015 and Mabry *et al*., 1970), the structure of H3 was confirmed to be a flavonol (quecertin).

2-(3',4'-dihydroxyphenyl)–3,5,7- trihydroxy-4*H*-chromane-4-one

5.2 Antifungal studies

The methanol leaf extract and its fractions (ethylacetate and n-butanol) exhibited varying degree of antifungal activity against the test organisms. The ethylacetate fraction of *T. globiferus* recorded the highest antifungal activity while methanol leaf extract had the least activity. The highest activity observed by the ethylacetate fraction might be due to the concentration of moderately polar compounds such as flavonoids and their derivatives that have been reported to possess antifungal effect (Harborne *et al*., 1993). Of all the fungal isolates*C. albican, T. mentagrophyte* and *T. rubrum* were the most susceptible to ethylacetate fraction. The fungi*C. albicans, T. mentagrophytes* and *T. rubrum* are implicated in diseases such as candidiasis, *Tinea capitis, Tinea pedis, Tinea corporis, Tinea barbae* and *Tinea cruris* (Macura, 1993 and Moran *et al*., 1997).

The MIC of the extract/fraction is the lowest concentration that caused growth inhibition of more than 90% after 48 hr of incubation(Paola *et al*., 2010). Interestingly, n-butanol fraction with minimum zone of inhibition (24.50 mm) when compared to ethyl acetate fraction (27.16 mm), recorded the lowest MIC against*C. albican* suggesting that the n-butanol fraction might have better antifungal activity at lower concentration. Ethyl acetate fraction recorded the lowest MIC against *T. rubrum*compared to n-butanol fraction suggesting that bioactive principles capable of inhibiting the growth of *T. rubrum* lie in the ethylacetate fraction.

The minimum fungicidal concentration was regarded as the lowest extract/fraction concentration that did not yield any fungal growth on the solid medium used (Paola *et al*., 2010).

The n-butanol fraction exhibited fungicidal effect on all the test organisms (*C. albican, T. mentagrophytes, T. rubrum,* and *A. niger*) with the lowest MIC against *C. albicans* and *A.niger*suggesting that the n-butanol fraction is more active than ethylacetate fraction and contains compounds which may be used directly or as precursor for developing better antifungal agents while ethylacetate showed its fungicidal activity on all the organisms except on *A. niger*

Fungal species involving *C. albicans*and *A. niger* are the major causative agents of infections such as oral candidiasis, oesophageal candidiasis virginal thrush, lung diseases and otomycosis (Abarca *et al*., 1994; Schuster *et al*., 2002 and Oladele and Denning, 2014).

Pandima *et al.* (2010) reported that the fungicidal effect of extract may be due to the inhibition of fungal growth by interfering with the production of fungal protein, DNA replication and through various cellular metabolisms of the organisms. It has also been reported that the fungicidal effect of the plant extract could be as a result of the damage caused to the cell membrane (Himratul-Aznita et al., 2011).

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 Summary

The phytochemical screening of the methanol leaf extract and fraction of *Tapinanthus globiferus* revealed the presence of carbohydrates, cardiac glycosides, saponins, tannins and flavonoids. Chromatographic studies of the ethyl acetate fraction of the plant led to the isolation of two flavonoids; 2-(3',4'-dihydroxyphenyl) –3,5,7- trihydroxy-4*H*-chromane-4-one (quercetin) and 2-(2,4-dihydroxyphenyl)-5,7-dihydroxy-3-(3-methylhexyl)-4H-chromen-4-one. The crude extract and fractions were found to possess *in vitro* antifungal activity against the test organisms.

6.2 Conclusion

Chromatographic studies of ethylacetate fraction of *Tapinanthus globiferus* afforded two flavonoids 2-(3',4'-dihydroxyphenyl)–3,5,7- trihydroxy-4*H*-chromane-4-one (quercetin) and 2-(2,4-dihydroxyphenyl)-5,7-dihydroxy-3-(3-methylhexyl)-4H-chromen-4-one.

To the best of our knowledge, this is the first report of isolation of these compounds from *Tapinanthus globiferus* growing on *Vitex doniana.*

The plant has demonstrated antifungal activity validating the ethnomedicinal claim for the use of the plant in the treatment of fungal infections.

6.3 Recommendations

1. Antifungal activity of methanol leaf extract and its fractions should be carried out on other fungal species.

2. Activity guided isolation of more bioactive constituents should be should be carried out on the fractions of *T. globiferus.*

3. Possible mechanism of action of antifungal agents should be determine on *T. globiferus.*

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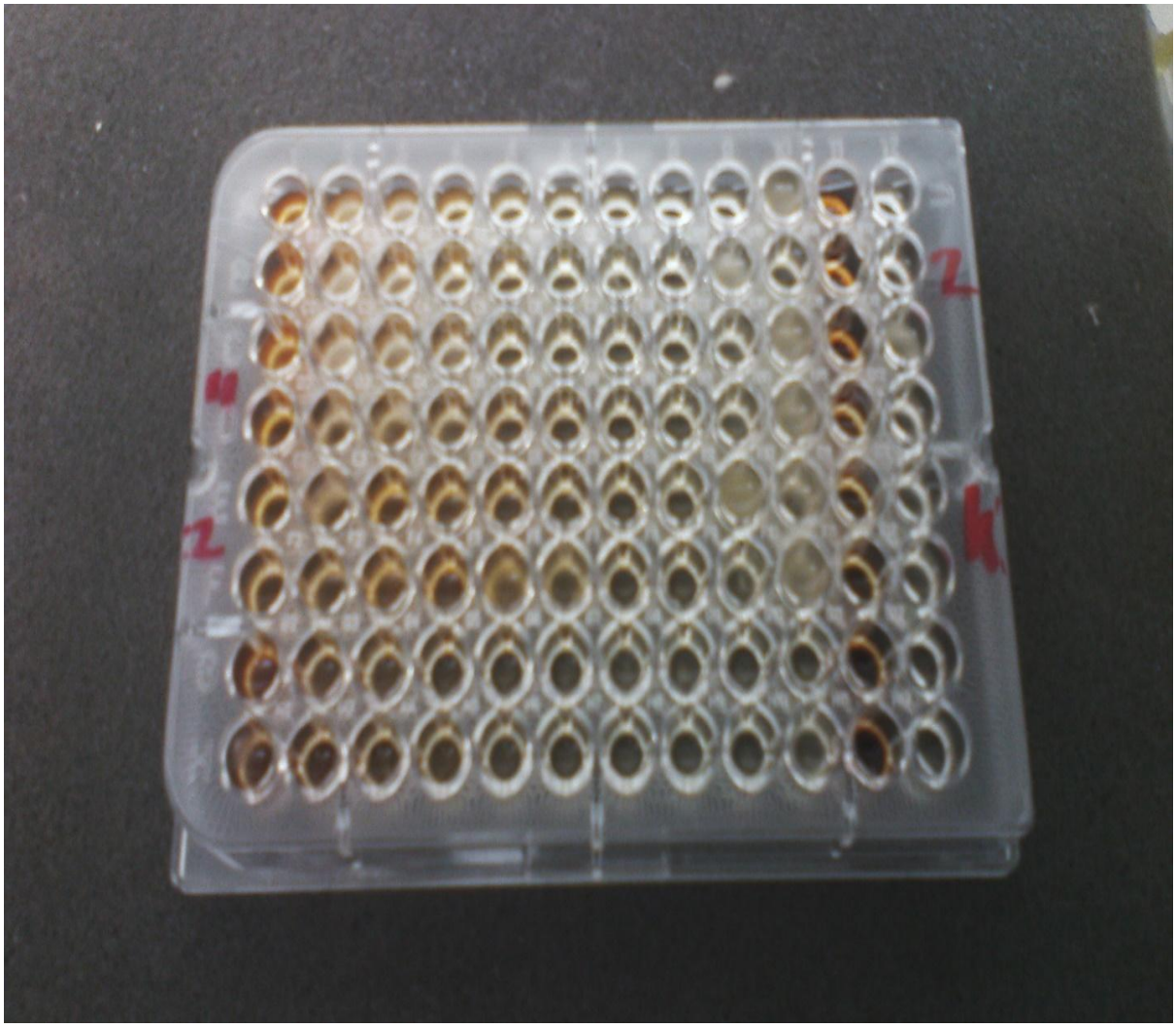
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APPENDIX

Appendix 1.0: 96 wells microtitre plate for MIC determination of crude methanol leaves extract, ethyl acetate and butanol fractions of *Tapinanthus globiferus* growing on *Vitex doniana* againstagainst some fungal species



Appendix 2.0: Showing the plates of antifungal susceptibility test of crude methanol leaves extract, ethyl acetate and butanol fractions of *Tapinanthus globiferus* growing on *Vitex doniana* against *Candida albicans.*

