PHYTOCHEMICAL EVALUATION OF *Elaeodendron buchananii* STEM BARK FOR MICROBIAL ACTIVITIES

BY

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ABSTRACT

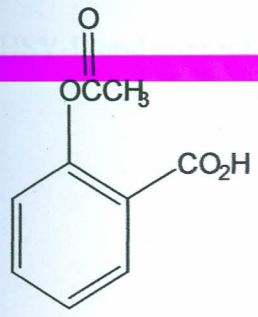
Public health is under serious threat due to wide array of emerging and re-emerging infections as a result of resistance of microorganisms to first line drugs. Despite the wide availability of clinically useful antifungal and antibacterial drugs, there is need for search for effective antimicrobials with new strategies to replace those with limited antimicrobial spectrum. Plants that are used by traditional healers in the management of ailments could be sources of safe and effective drugs. Many communities in Africa use *Elaeodendron buchananii* stem bark to manage fungal and bacterial infections. Despite its extensive use to manage bacterial and fungal infections, there is no phytochemical evaluation on the stem bark. Previous phytochemical analyses on its fruits and root bark revealed the presence of steroids and terpenoids. The objectives of this research were to investigate the lethality of the extracts, determine in vitro antibacterial and antifungal assays on crude extracts and isolates and characterize the active principles from stem bark of *E. buchananii*. The stem bark of *E. buchananii* was sequentially extracted using n-hexane, ethyl acetate and methanol. The extracts exhibited various degrees of activities, with ethyl acetate extract displaying highest lethality (LC_{50} of 2.784 µg /ml). The isolated compounds were purified by crystallization. Structural elucidation of isolates revealed two coumarins, two sterols and five triterpenoids characterized by MS, IR and NMR. The n-hexane extract yielded 3α-Acetylamyrin (**52**), stigmasterol (**53**) and 3-Oxofriedooleonane (**36**) while ethyl acetate extract gave coumarin (**54**), β-Sitosterol (**55**), 3α -Hydroxyfriedooleonane (**56**), umbelliferone (**57**), carnophyllol (**38**) and ursolic acid (**58**) in addition to those obtained from n-hexane extract. Methanol extract gave negligible isolates and were not analyzed. The ethyl acetate extract had strong

antibacterial activities than the conventional gentamycin used as positive control, especially against *Neisseria meningitidis* while methanol extract displayed strong antifungal activities especially against *Candida albicans*. The n-hexane extract showed mild antimicrobial activities. Coumarin (**54**) and umbelliferone (**57**) showed antifungal activities while 3-Oxofriedooleonane and carnophyllol exhibited antibacterial activities. Ursolic acid (**58**) displayed antibacterial and antifungal activities. These nine compounds have been isolated from this plant for the first time. The biological activities established in this study explain in part the use of *E. buchananii* stem bark in folk medicine for the management of bacterial and fungal related ailments. This study justifies the use of stem bark of *E. buchananii* in folk medicine for the management of bacterial and fungal ailments and recommends the conservation of the plant.

CHAPTER ONE

1.0 INTRODUCTION

In many developing countries, medical services are unavailable or unaffordable for the majority of the population (Iwu and Wooten, 2002). Traditional medicine continues to provide frontline pharmacotherapy for millions of people worldwide, particularly in Africa. Up to 80 % of Africa's population or more than half billion people visit traditional healers for some or all of their medical care needs annually (WHO, 2002). Higher plants are the main source of drug therapy in traditional medicines (Torssel, 1997). It is estimated that there are 250,000 to 500,000 species of plants on earth (Bornis, 1996), and that 14-28% of higher plant species are used medically (Farnsworth, 1991). Plants used traditionally in the management of various ailments could be good sources of new, safe, biodegradable and renewable antimicrobial drugs (Ampofo and Johnson-Romauld, 1987). Indeed, plants have always been considered to be alternative sources of new drugs and many drugs have been developed using structures of plant-derived compounds as templates (Philipson and Wright, 1991). The need for development of alternative medicines is being realized all over the world, as pathogens are increasingly becoming resistant to first line drugs. The future of conventional healing is probably not in alternative medicines but complimentary medicines, which bring together the best of different healing traditions (Plotkin, 2000). Natural products and their derivatives represent more than 50% of the drugs in clinical use in the world (Cowan, 1999). The transformations of traditional medicines into modern drugs have been widely adopted and examples include the antipyretic analgesic, aspirin (**1**) and artemisinin **(2).** Aspirin (**1**) was derived from salicylic acid **(3)** extracted from the bark of the willow tree (*Salix* species) used traditionally to treat fever and inflammation in many cultures worldwide for at least four millennia (Mahdi *et al.,* 2006).



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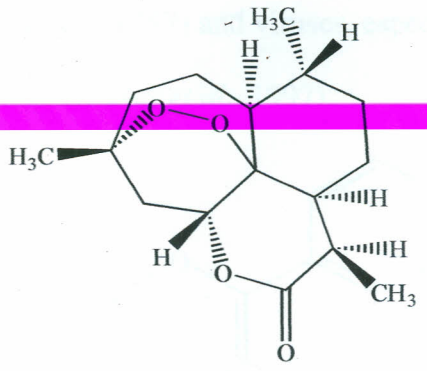

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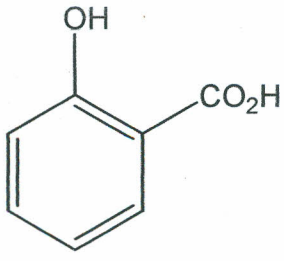

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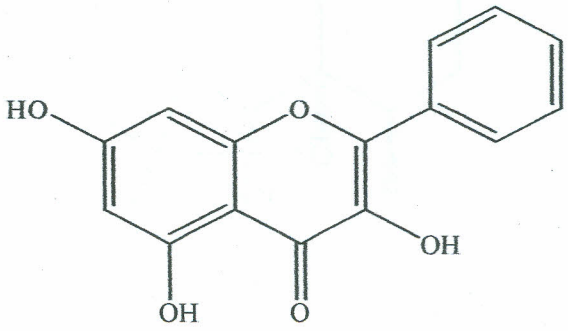
Artemisinin **(2)** was derived from *Artemisia annua* L; a shrub which was first documented for haemorrhoid treatment (Liu *et al.,* 2006). Its long historical use in the management of fever attributed to malaria prompted Chinese researchers to seek the active anti malarial principle, artemisinin which was isolated and its structure determined in the mid 1970s (Liu *et al.,* 2006). Combination therapies containing artemisinin are now considered the treatment of choice for malaria in Asia with growing adoption in Africa. Compounds derived from medicinal plant extracts are appealing for they have been "clinically" tested in their traditional milieu, in some cases over millennia (Schmidt *et al.,* 2007).

1.1 Background of the study

A number of compounds isolated from plants have proved effective against the four classes of infectious agents and in some cases they have proved to be more effective than the synthetic drugs (Duan *et al.,* 2000). For example galangin **(4)** (3, 5, 7-trihydroxyflavone) a compound derived from the perennial herb *Helichrysum aureonitens*; has shown activity against a variety of gram-positive

bacteria as well as fungi (Afoyalan and Meyer, 1997) and viruses, especially herpes simplex virus type one (HSV-I) and coxsackine B virus type 1 (Meyer *et al.*, 1997).



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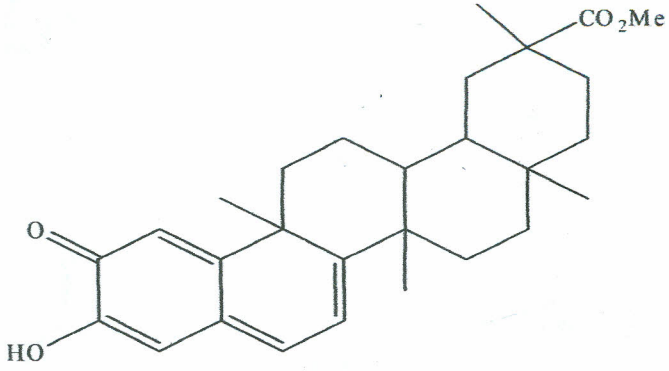
Celastrol (tripterin) (**5**) isolated from *Celastrus scandens* and *Trytpterigium wilfordii,* has been shown to have broad antimicrobil activities as an anti inflammatory compound in animal models of arthritis, lupus, amyotrophic lateral sclerosis and Alzheimer's disease (Sethi *et al.,* 2007).



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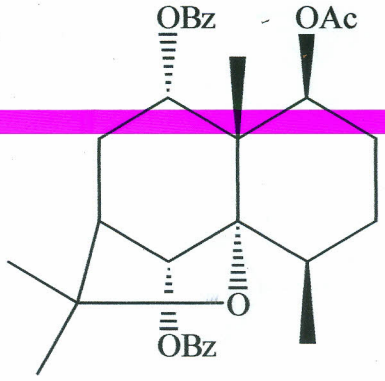
Multi-drug resistance (MDR) is a major problem in chemotherapy treatments. Some compounds isolated from natural sources have been found to reverse drug resistance. For example, celorbicol (**6**) isolated from *Celastrus orbiculatus* reversed either partially or totally, resistance to adriamycin (**7**), vinblastine (**8**) and paclitaxel (**9**) in the human nasopharynx carcinoma cells and the human breast cancer cells (Lee *et al.,* 1998). The compound was less toxic and of greater reversing power than other known reversing agents such as verapamil (**10**) that are currently approved for clinical use (Kim *et al.,* 1998).



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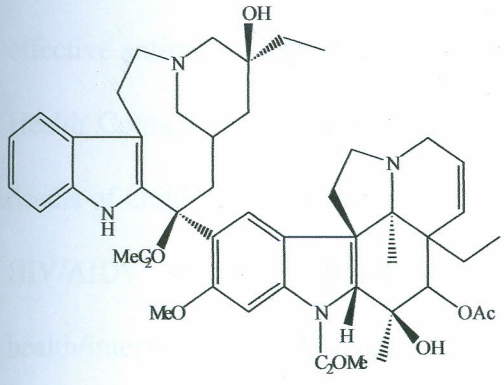

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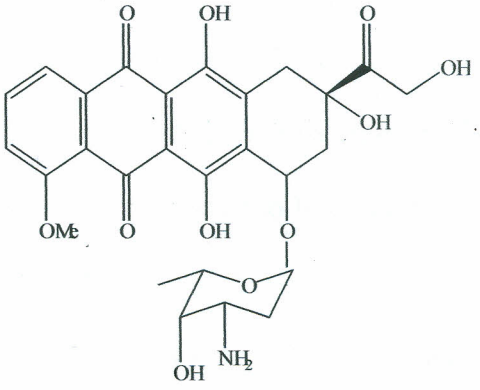

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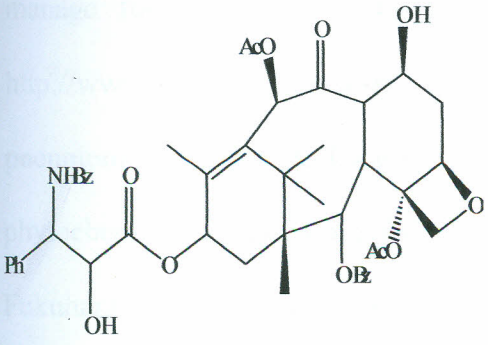

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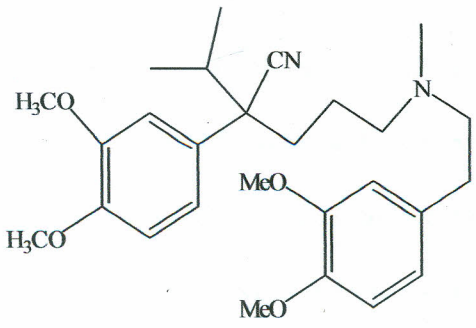

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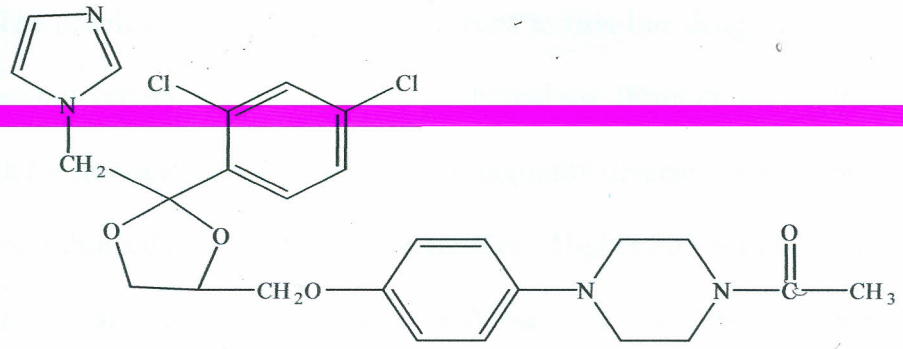
Some of the synthetic drugs on the other hand are known to have serious side effects on human health, for example the antifungal ketoconazole (**11**) has been shown to damage the liver (Fromtling, 1984).



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Pathogens are becoming resistant to first line drugs. There is need for research to come up with new effective antimicrobials in order to maintain a pool of effective drugs at all times. According to Global Health Council 2007 Annual Report, bacterial and fungal related infections claim higher number of deaths of children under five years annually (over 3 million) compared to both malaria (850,000) and HIV/AIDS (350,000) [Global Health Council (2007) http: //www.global health org/child health/interventions]. *Elaeodendron buchananii* (Loes) belongs to class *Magnoliopsida,* order *Celastrales* and family *Celastraceae.* The plant is widespread across Africa from north, to east, south and central Africa (Burkill, 1985). Many communities in Africa use *E. buchananii* stem bark to manage fungal infections such as candidaisis, onychomycosis [Vazquez, 2000; NCBI (2008) http://www.ncbi.nlm.gov/entrez] and bacterial infections such as diarrhea, wounds, coughs pneumonia, syphilis (Kokwaro, 1976; Bekalo *et al.*, 1996; Maundu and Tengnas, 2005). Previous phytochemical analyses on its fruits and root bark revealed steroids and terpenoids (Kubo and Fukuhara, 1990; Tsanuo *et al.*, 1993; Tsujino *et al.*, 1995). However, there has been no phytochemical evaluation of the stem bark to establish or justify the biological activities.

1.2 Statement of the problem

Infectious agents develop resistance to previously effective chemotherapeutics, thereby becoming the cause of wide array of emerging and re-emerging infections, which are serious threats to public health

(Vassil, 2005). The problem of resistance of pathogens to first line drugs has been due to the limited number and structural variety of antimicrobials in clinical use (Ram *et al.*, 2000). Consequently there is need for search for new and hopefully effective structurally diverse antimicrobial drugs to replace or augment the current drugs that are becoming ineffective. Higher number of deaths (over 3 million) of children under five years old annually due to both bacterial and fungal related infections [Global health Council (2007) http: //www.global health org/child health/interventions] compared to 850,000 from malaria which has been the number one infectious disease in the world (Griffith *et al.*, 2002). The inefectivity of the conventional medicines in managing certain disorders such as cancer, HIV/AIDS, together with the side effects of the synthetic drugs on patients, imply that more research must be carried out particularly on plants to come up with effective and safe drugs. Ethnomedicinal information indicates that *E. buchananii* stem bark has been used to manage fungal and bacterial infections (Vazquez, 2000; Bekalo *et al.*, 1996; Hamza *et al.*, 2006; [NCBI (2008) http://www.ncbi.nlm.gov/entrez]. It is necessary to undertake phytochemical analysis of *E. buchananii* stem bark to characterize any potential antimicrobial principles.

1.3 Justification of the research

Phytochemical evaluation and biological activities have been done on both the root bark and fruits of *E. buchananii* that led to the isolation of cytotoxic compound, mutangin (**13**), insect antifeedants, buchaninoside (**14**) and elabunin (**15**). Stem bark of E. *buchananii* is mainly used by traditional healers to manage bacterial and fungal related infections (Kokwaro, 1976; Vazquez, 2000; Hamza *et al.*, 2006). No reported work on phytochemical evaluation of stem bark.

1.4 Significance of the research

The research may identify antimicrobial compounds that can be of phytopharmaceutical value. The research findings may justify the conservation of the plant, and authenticate its ethnomedicinal uses.

1.5 Objectives of the study

1.5.1 General objective

To extract, isolate, characterize compounds and evaluate the possible antibacterial and antifungal extracts and compounds from the stem bark of *Elaeodendron buchananii.*

1.5.2 Specific objectives

1. To investigate the lethality of the extracts from the stem bark of *E. buchananii* using the brine shrimps, *Artemia salina.*
2. To determine in vitro antibacterial and antifungal assays on the crude extracts and isolates from *E. buchananii* stem bark.
3. To characterize the isolates from *E. buchananii* stem bark.

1.6 Hypothesis

The stem bark of *Elaeodendron buchananii* may exhibit brine shrimp lethality, antibacterial and antifungal activities and may result in isolation of antibacterial and antifungal compounds.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 General overview

Microorganisms belonging to all the four classes of infectious agents; parasites, bacteria, fungi and viruses develop resistance to previously effective chemotherapeutics, thereby becoming the cause of wide array of emerging and re-emerging infections, which are serious threats to public health (Vassil, 2005). An example is tuberculosis, which remains a global menace, affecting 7.5 million people and killing as many as 2.5 million people each year making it the leading cause of death of all infectious diseases [why files (2007) http: //why files.org/038 bad bugs/tb1]. Certain strains of *Mycobacterium tuberculosis* are resistant to a series of antibiotics [why files (2007) http: //why files.org/038 bad bugs/tb1]. Unless antibiotic resistance problems are detected as they emerge, and actions taken immediately to contain them, the society could be faced with previously treatable diseases that have become again untreatable, as in the days before antibiotics were developed.

There is medical threat presented by fungal diseases due to the persistent inability of chemotherapy to reduce their incidence and lethality (Nucci and Marr, 2005). Invasive fungal infections are frequent and severe in the settings of haematological malignancies and organ transplant, where they cause substantial mortality. Patients undergoing haematopoietic stem cell transplant appear to be particularly vulnerable to a variety of fungal pathogens, including zygomycetes and *Fusarium species*, with mortality exceeding 60% (Safdar, 2006). Uses of some antifungal drugs such as ketoconazole have serious side effects such as liver damage (Fromtling, 1984). The widespread over use of drugs has led to increasing chemical resistance of previously sensitive microorganisms (Geerts and Dorny, 1995) and the emergences of previously uncommon infections (Bloland, 2001). Despite the wide availability of clinically useful antifungal and antibacterial drugs continuing search for new effective agents remain indispensable because some of the major drugs have

Both bacteria and fungi related infections continue to pose challenges to public health especially to children under five years as illustrated by the Global Health Council 2007 Annual Report on major causes of child deaths [Global Health Council (2007) http://www.global health org/child health/interventions] in Table 2.1.

Table 2.1: Major causes of child deaths

Disease	Causative agent	Percentage of death under five	Number of deaths annually
Pneumonia	Bacteria	19	2 million
Diarrhoea	Bacteria, protozoa & fungi	17	1.8 million
Malaria	Protozoa	8	850,000
HIV/AIDS	Virus	3	350,000

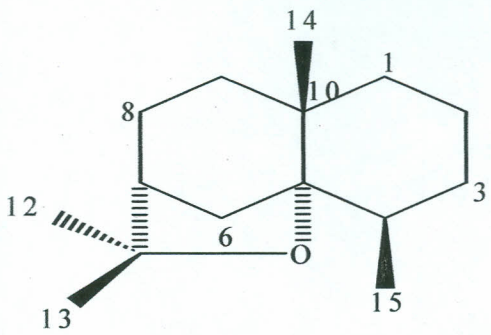
Source: [Global Health Council (2007) http: //www.global health org/child health/interventions]

Biologically active compounds from plants may inhibit microorganisms by a different mechanism than the currently used drugs and may have clinical value in the treatment of resistant strains. For this reason it is important to investigate plants as alternative source of medicine (Cox, 1994).

2.2 The Family *Celastraceae*

Elaeodendron buchananii (Loes) belongs to class *Magnoliopsida,* order *Celastrales* and family *Celastraceae.* The family is also commonly known as spindle or spike thorn tree. The *celastraceae* are trees and shrubs comprising approximately 800 genera and 1300 species worldwide (Duarte and Debur, 2005). Members of the family in Kenya include *Catha, Elaeodendron, Mystroxylon* and *Maytenus* (Noad and Birnie, 1992). Plants of this family are rich sources of hydroxylated sesquiterpene esters based on the dihydroagarofuran skeleton (**12**) and have attracted interest on

account of their cytotoxic, anti tumour, immunosuppressive and insect antifeedant activities (White *et al.*, 1997).



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2.3 The genus *Elaeodendron*

Elaeodendron is one of the largest genera in the *Celastraceae* family consisting of 147 species, which are evergreen or rare deciduous trees and shrubs. The African species are found in southern and eastern parts of the continent (Archer and Van-wyk, 1998). There are four species of *Elaeodenron* that occur in Kenya, *E. buchananii* (Loes), E. *schweinfurthianum* (Loes), *E. aquifolium* (Fiori) and *E. schlechteranum* (Loes); the latter three species are distributed in the coastal and adjoining zones (Beentje, 1994). Plants from this genus have been the focal point of several research investigations recording their ethnobotanical uses and many are being used in management of some ailments (Kokwaro, 1976; Keith, 1983; Noad and Birnie, 1992; Maundu and Tengnas, 2005; Gerhard, 2007).

2.4 *Elaeodendron buchananii* (Loes)

There are five synonyms for *E. buchananii* (Loes) that include: *E. keniensis* (Loes), *E. friesianum* (Loes), *E. stolizii* (Loes), *E. warneckei* (Loes) and *Cassine buchananii* (Loes) (Robson, 1966). *E. buchananii* is an African tree that grows to 20 m high with a strong, stout irregular trunk, dense evergreen foliage and a rounded or irregular crown. The bark is dark grey, smooth while young but fissured with age (Maundu and Tengnas, 2005) (Fig. 2.1).

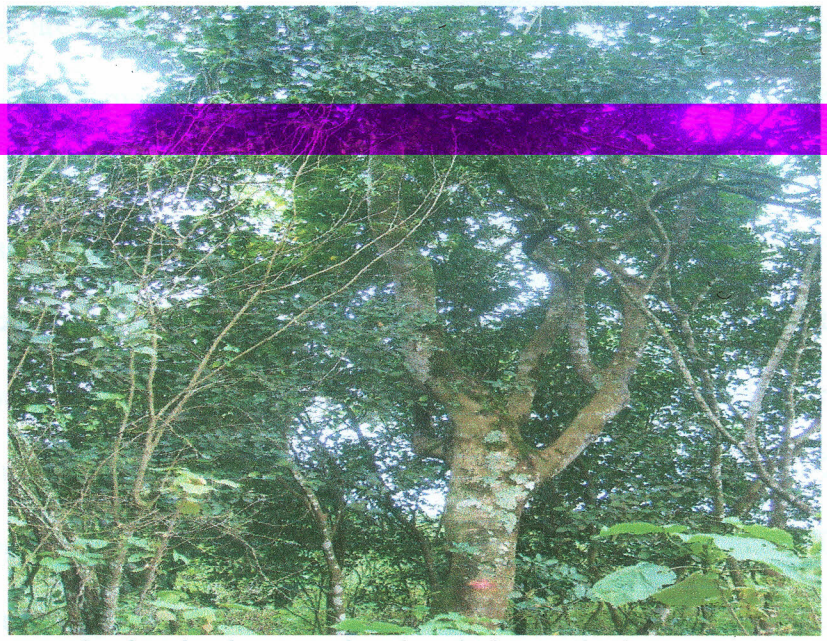


Fig. 2.1: Photograph of *E. buchananii* showing dark grey bark

Fig. 2.2: Green leathery leaves of *E. buchananii*

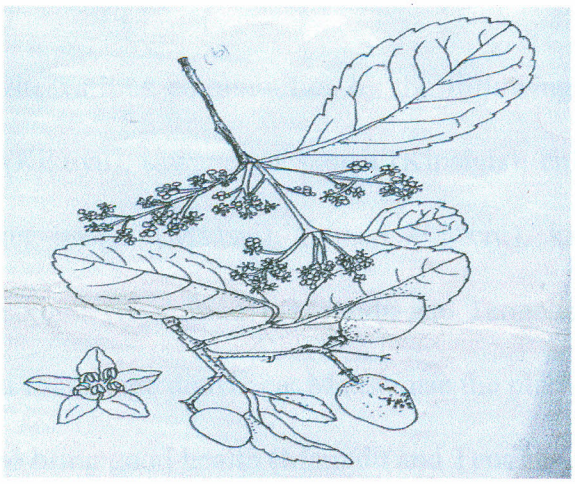


Fig. 2.3: Oval fruits of *E. buchananii* single or in clusters (Source: Maundu and Tingnas, 2005).

The leaves of *E. buchananii* are dark green, leathery up to 14 cm long, without hairs, widest in the middle or towards the tip with serrated margins (Fig. 2.2). The leaves are extremely poisonous to livestock. Ingestion of its leaves has resulted in many deaths to domestic stock with signs of dyspnoea, in coordination and diarrhoea in animals that live

for few days (Chhabra *et al.,* 1989). However, the leaves are surprisingly not poisonous to giraffes (Noad and Birnie, 1992; Verdcourt and Trump, 1969). Flowers are very small, yellowish, fragrant, arranged along branching stalk arising from the leaf axils to 10 cm long. Fruit is hard oval about 1-2 cm long, single or in clusters (Fig. 2.3), normally green turning yellow to brown (Maundu and Tingnas, 2005).

2.4.1 Ecology and distribution

E. buchananii is a tree of tropical Africa found in riverine woodland, wooded grassland, dry evergreen forest, forest remnants and sometimes on termite moulds in plateau deciduous woodlands at an altitude range of 100-1500m. The plant is widespread across Africa from north, to east, south and central Africa (Burkill, 1985), in countries including Zambia, Sierra Leone, Ethiopia, Angola, Malawi, Rwanda, Congo, Ghana, Sudan, Kenya, Tanzania, Uganda and United Republic of Togo (Robson, 1966). In Kenya *E. buchananii* is distributed in the dry upland forest and open woodland in Central, Rift Valley and Nyanza provinces and is also common in rocky riverine vegetation around Nairobi (Maundu and Tengnas, 2005).

2.4.2 Ethnomedicinal uses

E. buchananii is known amongst the various communities by the following common names: mulundu (Kamba), mutanga or mutimweru (Kikuyu), sawanet or saonet (Kipsigis), enkanda (Kisii), kumunyufwe or kumukulanyuni (Luhya), osoket osoget (Maasai), munati (Meru), kalkach (Orma), madanchui (Pokomo), sunwa (Sabaot) and mhakuma (Nyamwezi) (Maundu and Tengnas, 2005). An infusion of the inner bark is widely used by Maasai to manage diarrhoea. Maasai use the plant's wood in soup to add both colour and flavour and is believed to bring good health (Maundu and Tengnas,

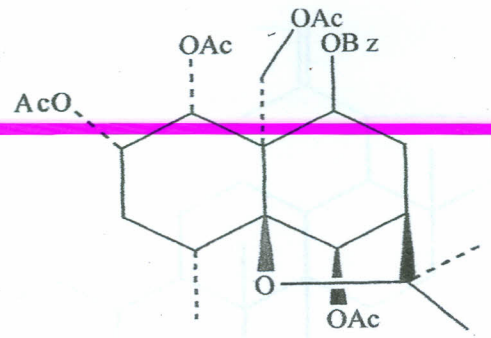
2005). An infusion of the inner bark is widely used by Maasai to manage diarrhoea. Maasai use the plant's wood in soup to add both colour and flavour and is believed to bring good health (Maundu and Tengnas, 2005). The Kipsigis use the plant as one of the ethnoveterinary medicines. A handful of pounded stem bark in one glass of water is applied on wounds once a day for five days on castrated animals. They also massage their bodies with the pounded stem bark in water once a day (Bekalo *et al.,* 1996). In Tanzania the stem bark of the *E. buchananii* is used for the management of candidaisis (Vazquez, 2000) and has been screened and found to exhibit strong antifungal activity (Hamza *et al.,* 2006) and recommended that a further investigation be done to isolate and identify the active compounds responsible for the antifungal activity. Other fungal infections managed by the plant's stem bark and seeds include groin, tinea cruris, tinea versiculor and onychomycosis [NCBI (2008) http://www.ncbi.nlm.gov/entrez]. Ash from burnt leaves is used to prepare local salt (Maundu and Tengnas, 2005). Chewing of the plant has been claimed to cure diarrhoea and its bark is used to manage stomach ailments (Kokwaro, 1976). Dried powdered roots are widely used in the management of wounds and the primary symptoms of syphilis (Kokwaro, 1976). A decoction or powder of the plant is mixed with milk and drunk by those with blood stained cough (Kokwaro, 1976). Most of the traditional healers in the communities (mentioned in section 2.4.2) use the stem bark of *E. buchananii* for the management of various ailments resulting in extensive debarking (Noad and Birnie, 1992; Maundu and Tengnas, 2005). Figures 2.4 (a) and 2.4 (b) show some of the trees of *E. buchananii* seriously debarked by traditional healers in Ngong Forest, Nairobi. Other uses of *E. buchananii* include; firewood, shade, mulching and soil conservation (Maundu and Tengnas, 2005).

Fig. 2.4a: Photograph showing debarked *E. buchananii* in the forest

Fig. 2.4b: Photograph showing a drying *E. buchananii* due to debarking

2.4.3 Phytochemistry of *E. buchananii*

Previous phytochemical studies of *E. buchananii* revealed four compounds. Mutangin (**13**), a dihydro agarofuranoid sesquiterpene was isolated from unripe fruits. The structure of this novel sesquiterpene of the eudesmane was elucidated as 1α, 2α, 6β-triacetoxy-9β 15-dibenzoyloxydihydroagarofuran (Tsanuo *et al.*, 1993). Buchaninoside (**14**), a steroidal compound was isolated from the chloroform extract of the unripe green fruits (Tsujino *et al.*, 1995).



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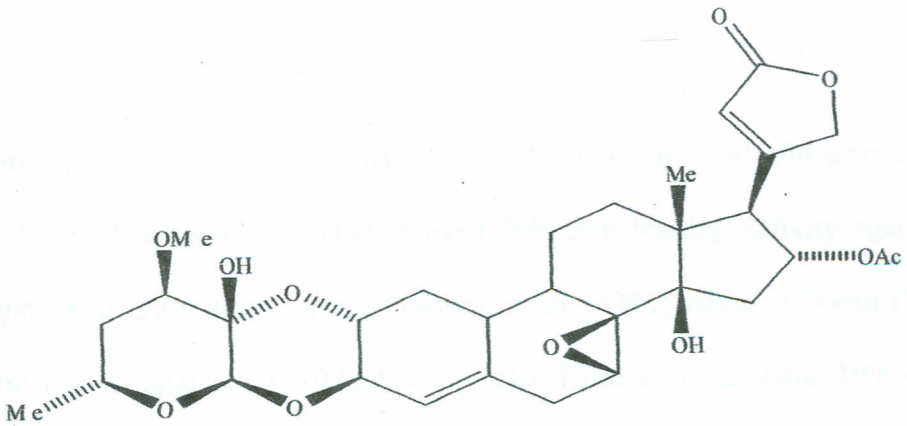

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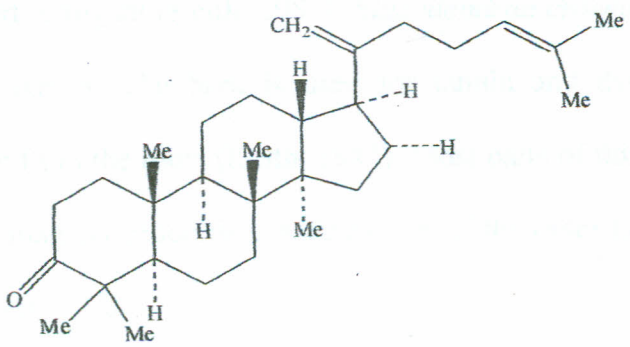
Elabunin (**15**), a cytotoxic dammarane triterpene and lupeol (**16**) have also been isolated from the root bark of the plant (Kubo and Fukuhara, 1990).



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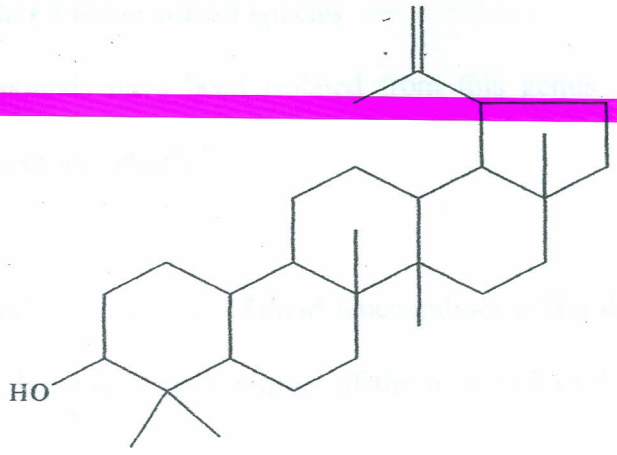

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Mutangin (**13**) exhibited moderate antifeeding activity against the *Lepidopteran chilopertellus* (Swin hoe) (Tsanuo *et al*., 1993). Buchaninoside (**14**) showed 70% anti feeding activity against larvae of *Spedoptera exempta* (Cut grass army worm) (Tsujino *et al*., 1995) while elabunin (**15**) exhibited moderate cytotoxic activity against L1210 leukaemia cells (Kubo and Fukuhara, 1990).

2.5 Other members of the genus of *Elaeodendron*, and their ethnomedicinal uses

Elaeodendron glaucum (Pers) is distributed in Bhutan, India, Nepal, Pakistan, Sri Lanka, Cambodia and Indonesia and is used in the management of pneumonia, snakebites, headache, swellings, emetics, fatality, and fumigant (Keith, 1983). *Elaeodendron croceum* (Thunb) occurs in the coastal forests of South Africa. The bark is used for tannin and dyeing and the Xhosa community prepares an emetic from the roots (Keith, 1983). Most parts of this plant are poisonous and valued for medicinal and magical properties. A decoction of the outer layer of the bark is an effective snakebite remedy (Keith, 1983).

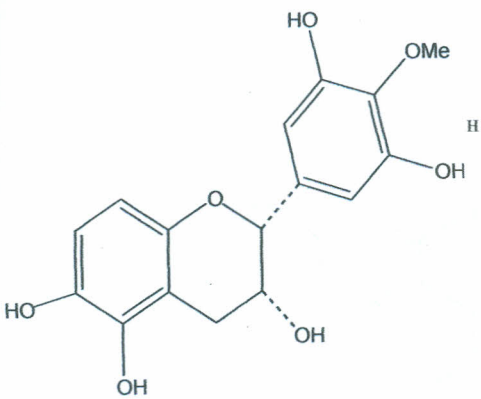
Elaeodendron transvalensis (Burtt) is used by Venda and Zulu people of South Africa who drink large quantities of the bark infusion as a general stomach conditioner and to relieve fevers. It is traditionally used to treat fungal infections, stomach disorders, stomach ulcers and venereal diseases (Keith, 1983). The extract of stem bark of *E. trasvalensis* has shown strong *in vitro* activity against most bacteria tested especially *S. aureus* and *E. coli* (Samie *et al*., 2005).

2.6 Pytochemistry of other *Elaeodendron* species

Various classes of compounds have been isolated from this genus. These include flavonoids, steroids and various types of terpenoids.

2.6.1 Flavonoids

Flavonoids ouratea-proanthocyanidin A (**17**) and leucoanthocyanidin derivative elaecyanidin (**18**) have been isolated from the ethyl acetate extract of the root bark of *E. balae* (Weeratunga *et al.*, 1985).



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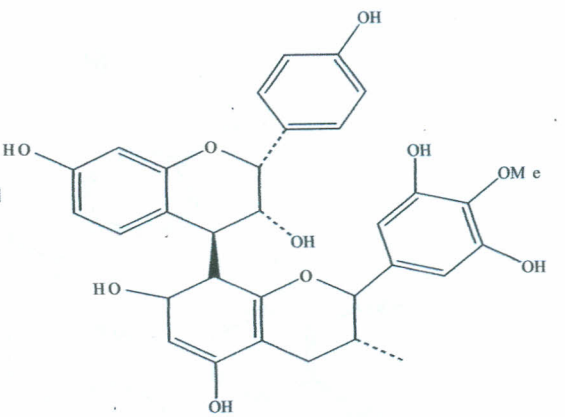
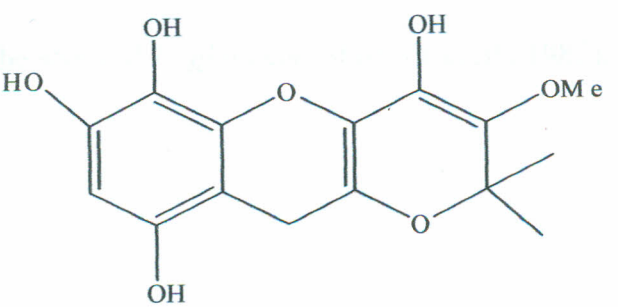


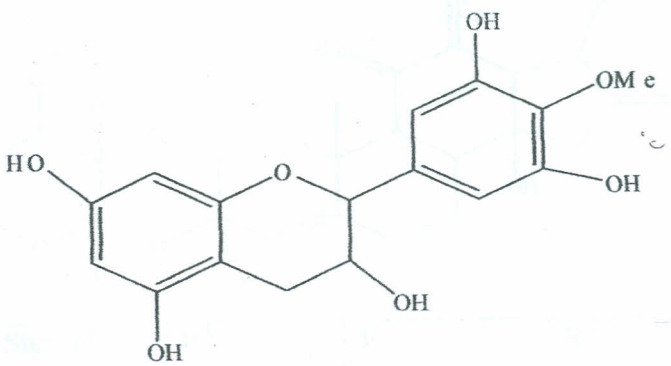
18

The root bark of *E. transvalensis* yielded (-)-11, 11-dimethyl-1, 3, 8, 10-tetrahydroxy-9-methoxypeltogynan (**19**) (Drewes *et al.*, 1991) as well as *E. croceum* (Drewes and Mashimbye, 1993).



19

Flavonoids (-)-4'-0-methyl epigallocatechin (**20**) and ourateaproanthocyanidin-nona-acetate (**21**) were isolated from *E. croceum* (Drewes and Mashimbye, 1993).



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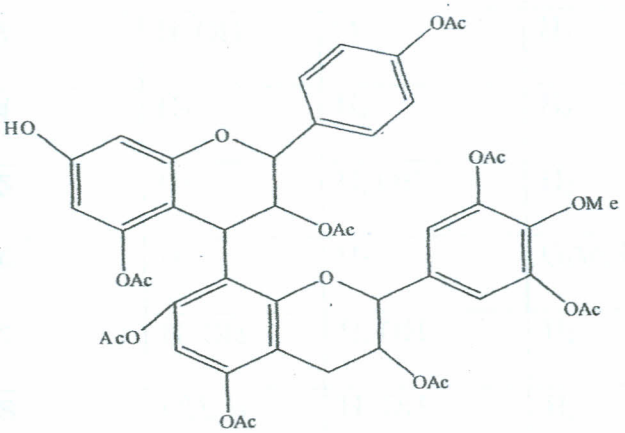
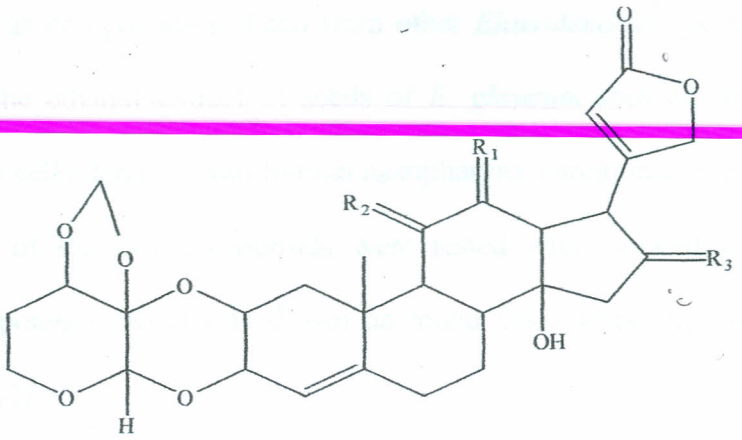


21

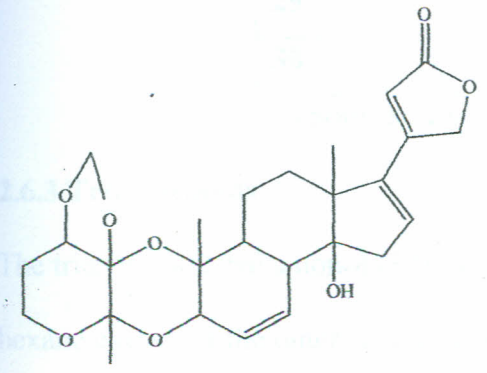
2.6.2 Steroids

The steroids; **22**, **23**, elaeodendroside A (**24**), D (**26**), E (**28**), H (**29**), I (**27**) and digitoxigen (**25**) have been isolated from the seeds of *E. glaucum* (Shimada *et al*., 1982).



Steroid	R^1	R^2	R^3
22	O	H, OH	H_2
23	H, OH	O	H_2
24	H_2	H_2	H_2
25	H_2	H, OH	H_2
26	H_2	H_2	OAc, H
27	H, OH	H, OH	H_2
28	OH, H	H, OH	H_2
29	O	O	H_2

A cardiac steroid, digitoxigen (**25**) was also isolated from *E. croceum* (Gerhard, 2007).



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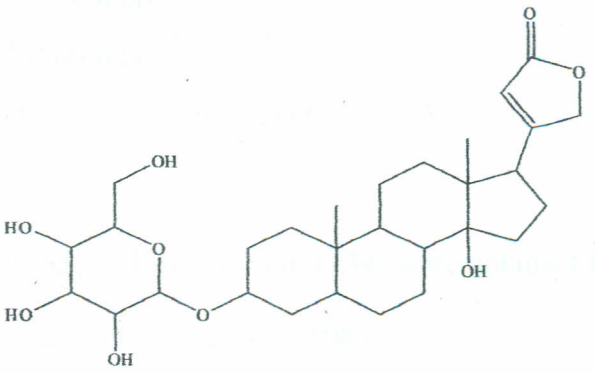


31

Crude extracts as well as compounds isolated from other *Elaeodendron* species exhibited varied biological activities. The ethanol extract of seeds of *E. glaucum* showed significant inhibitory activity *in vitro* against cells derived from human nasopharynx carcinoma (Kupchan *et al.*, 1977). The cardiac activities of the isolated steroids were tested with isolated frog heart (Straub`s preparation). Relative potency (RPa) was shown on molar basis while digitoxigen was taken as standard (1.0) (Table 2.2).

Elaeodendroside A had showed significant growth inhibitory activity *in vitro* against cells derived from human carcinoma of the nasopharynx (Kupchan *et al.*, 1977; Shimada *et al.*, 1985). An anti-HIV, digitoxigen-3-0-glycoside (**31**) was isolated from *E. croceum.* It was also known for its cardiac activity as well as cytotoxicity (Gerhard, 2007).

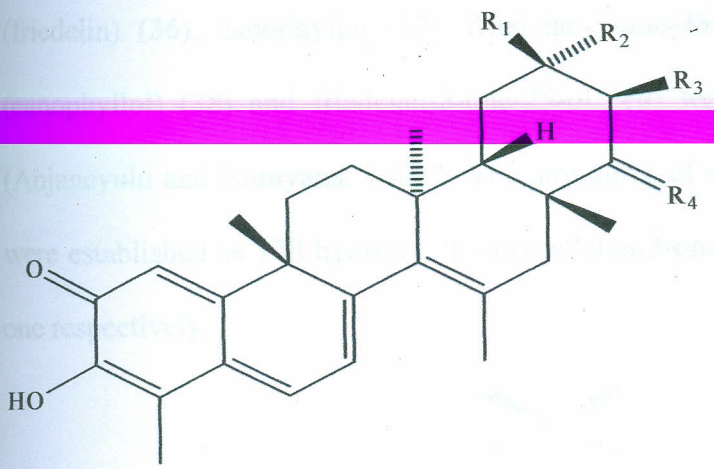
Table 2.2: Cardiac activities and cytotoxicity of the steroids

Compound	Cardiac activity (Rpa)	Cytotoxicity (ED_{50} µg/ml)
Digitoxigen	1	
Elaeodendroside A	0.3	0.13
Elaeodendroside D	0.3	
Elaeodendroside E	0.3	0.23
Elaeodendroside I	0.3	0.029
31	0.09-0.06	
29	0.09-0.06	
30	0.09-0.06	

(Source: Kupchan *et al.*, 1977; Shimada *et al.*, 1985)

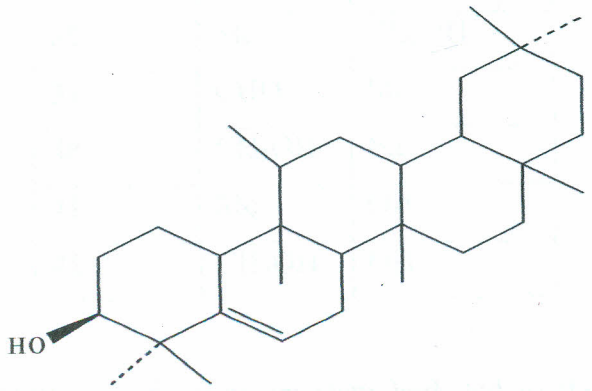
2.6.3 Triterpenoids

The triterpenoids balaenonol (**32**), balaenol (**33**) and isobalaendiol (**34**) were obtained from the hot hexane extract of the outer root bark of *E. balae* (Fernando *et al*; 1989).



Triterpene	R^1	R^2	R^3	R^4
32	Me	H	OH	O
33	Me	H	OH	H_2
34	H	Me	OH	β-OH, α-H

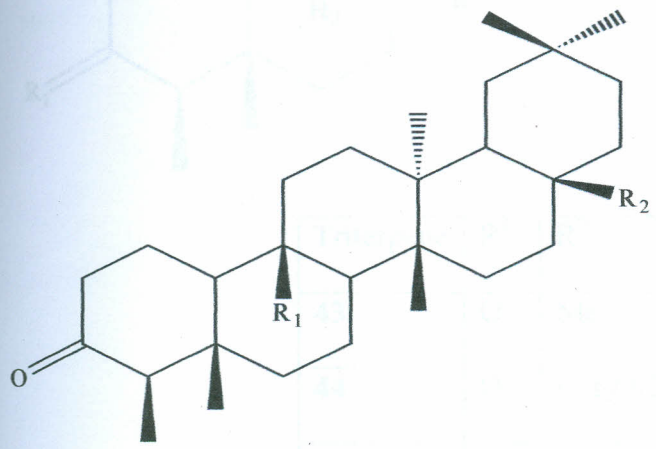
The friedooleananes have been found to occur in the stem bark of the two species of *Elaeodendron; E. glaucum* and *E. balae* (Anjaneyulu and Narayana, 1980). The petroleum ether extract of the root bark of *E .balae* produced friedooleanene (**35**) (Weeratunga *et al*., 1982).



35

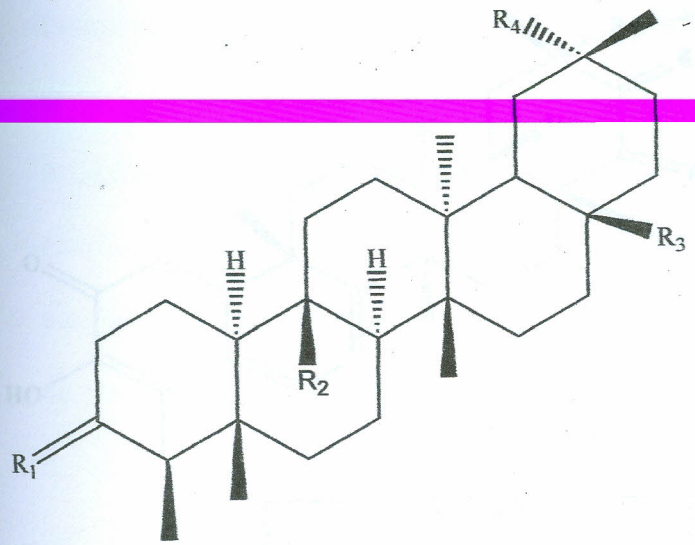
The biogenetic formation of the friedooleanenes has been well recognized as a biological transformation of a methyl group to –COOH and the loss of HCOOH results in the formation of the double bond (Anjaneyulu and Narayana, 1980). The compounds 3-oxofriedooleanane

(friedelin) (**36**), canophyllal (**37**), friedelan-3-one-25-al (**39**), 28-hydroxy-friedo-oleanan-3-one (canophyllol) (**38**) and friedelan-3-one-25-ol (**40**) were isolated from the bark of *E. glaucum* (Anjaneyulu and Narayana, 1980). The structures of elaeodendrol (**41**) and elaeodendrodiol (**42**) were established as 17β hydroxy-28-norfriedelan-3-one and 17β, 25-dihydroxy-28-norfriedelan-3-one respectively.



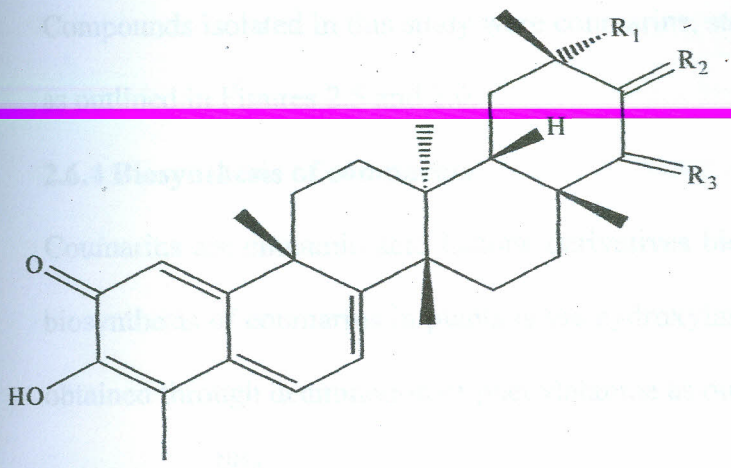
Triterpene	R^1	R^2
36	Me	Me
37	Me	CHO
38	Me	CH_2OH
39	CHO	Me
40	CH_2OH	Me
41	Me	OH
42	CH_2OH	OH

Investigation of benzene extract of *E. glaucum* stem bark led to the isolation of 29-hydroxy-friedooleanan-3-one (**43**) and the highly polar trioxygenated friedooleananes shown to be 25, 28-dihydroxy-friedooleanan-3-one (**44**) and 25-hydroxy-3-oxo-friedooleanan-28-al (**45**) (Weeratunga *et al.*, 1982).



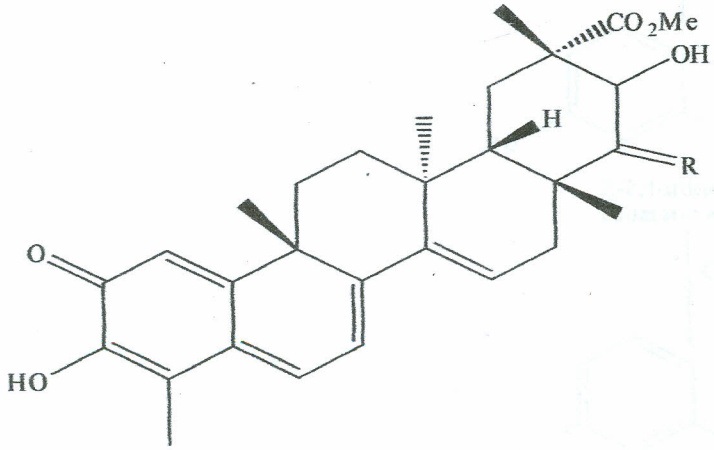
Triterpene	R^1	R^2	R^3	R^4
43	O	Me	Me	CH_2OH
44	O	CH_2OH	CH_2OH	Me
45	O	CH_2OH	CHOH	Me

Pristimerin (**46**), tingenon (**47**), 22-hydroxytingenon (**48**) and 20-hydroxytingenon (**49**) isolated from the hot hexane extract of the outer root bark of *E. balae* were cytotoxic compounds. Tingenon (**47**) was also isolated from *E. Croceum* (Fernando *et al*., 1989; Drewes and Mashimbye, 1993).



	R^1	R^2	R^3
46	CO_2Me	H_2	H_2
47	H	O	H_2
48	H	O	α-OH
49	OH	O	H_2

Netzahualcoyone (**50**) and Netzahualcoyonol (**51**) were also obtained from root bark *E. balae* (Fernando *et al*; 1989).



50 R=O

51 R=H, H

Compounds isolated in this study were coumarins, sterols and triterpenoids. Their biosyntheses are as outlined in Figures 2.5 and 2.6.

2.6.4 Biosynthesis of coumarins

Coumarins are cinnamic acid lactone derivatives biosynthesised through shikimate pathway. The biosynthesis of coumarins in plants is via hydroxylation and cyclization of cinnamic acid which is obtained through deamination of phenylalanine as outlined in Figure 2.5.

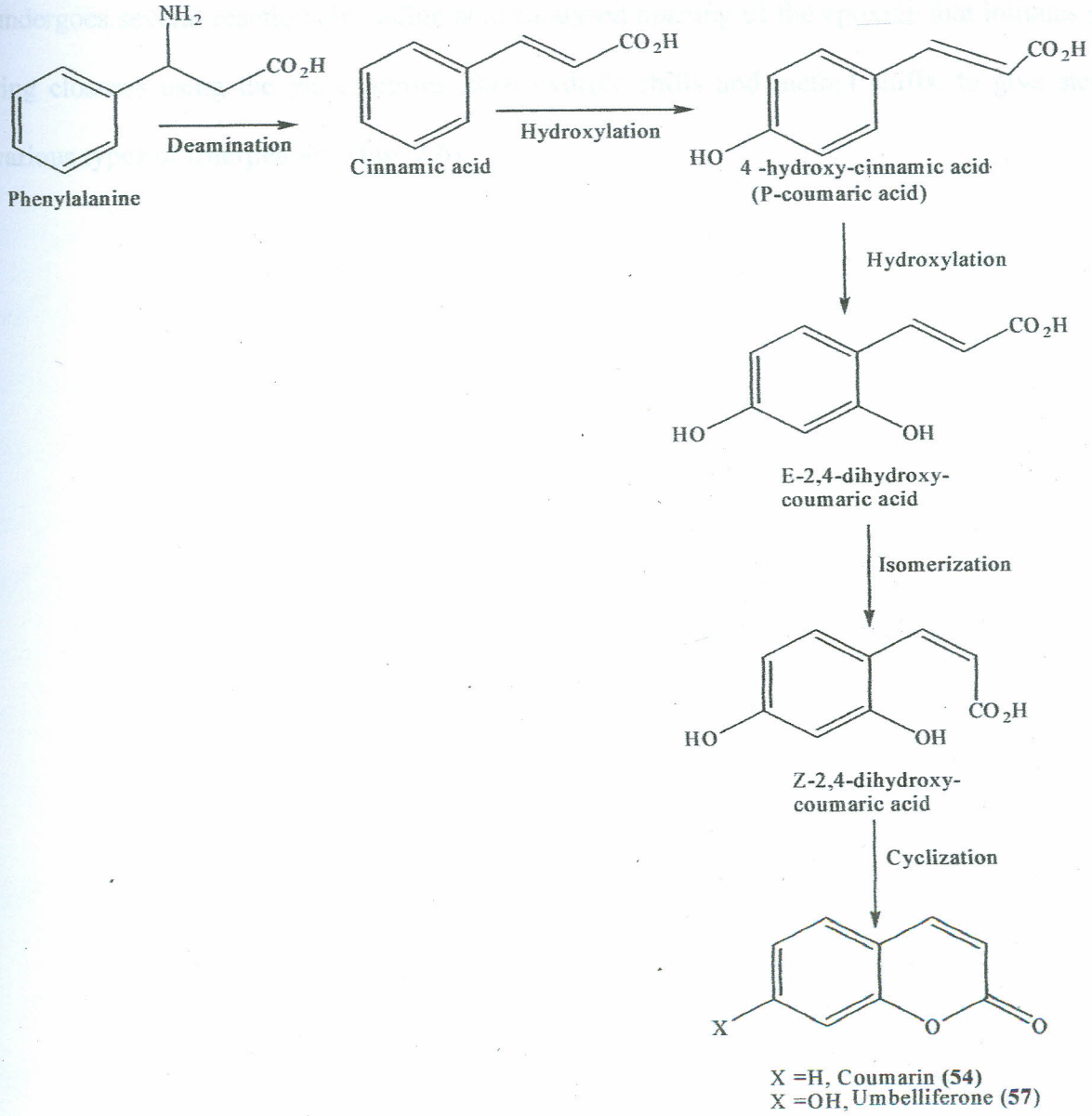


Fig. 2.5: Biosynthesis of coumarins (Source: Ryles *et al.*, 1988)

2.6.5 Biosynthesis of triterpenoids

Mevalonic acid (MVA) formed from acetyl coenzyme A (Acetyl co A) is the starting material for the squelene and hence triterpenoids. Phosphorylation of MVA leads to the formation of mevalonate -5-pyrophosphate which is converted to isopentenyl pyrophosphate (IPP). Coupling of IPP with dimethyl allyl pyrophosphate (DMAP) gives rise to geranyl pyrophosphate (GPP) which eventually forms squelene. Enzymatic oxidation of squelene forms epoxy squelene which undergoes several reactions including acid catalysed opening of the epoxide that initiates series of ring closures using the pie electrons, then hydride shifts and methyl shifts, to give sterols and various types of triterpenoids (Fig. 2.6).

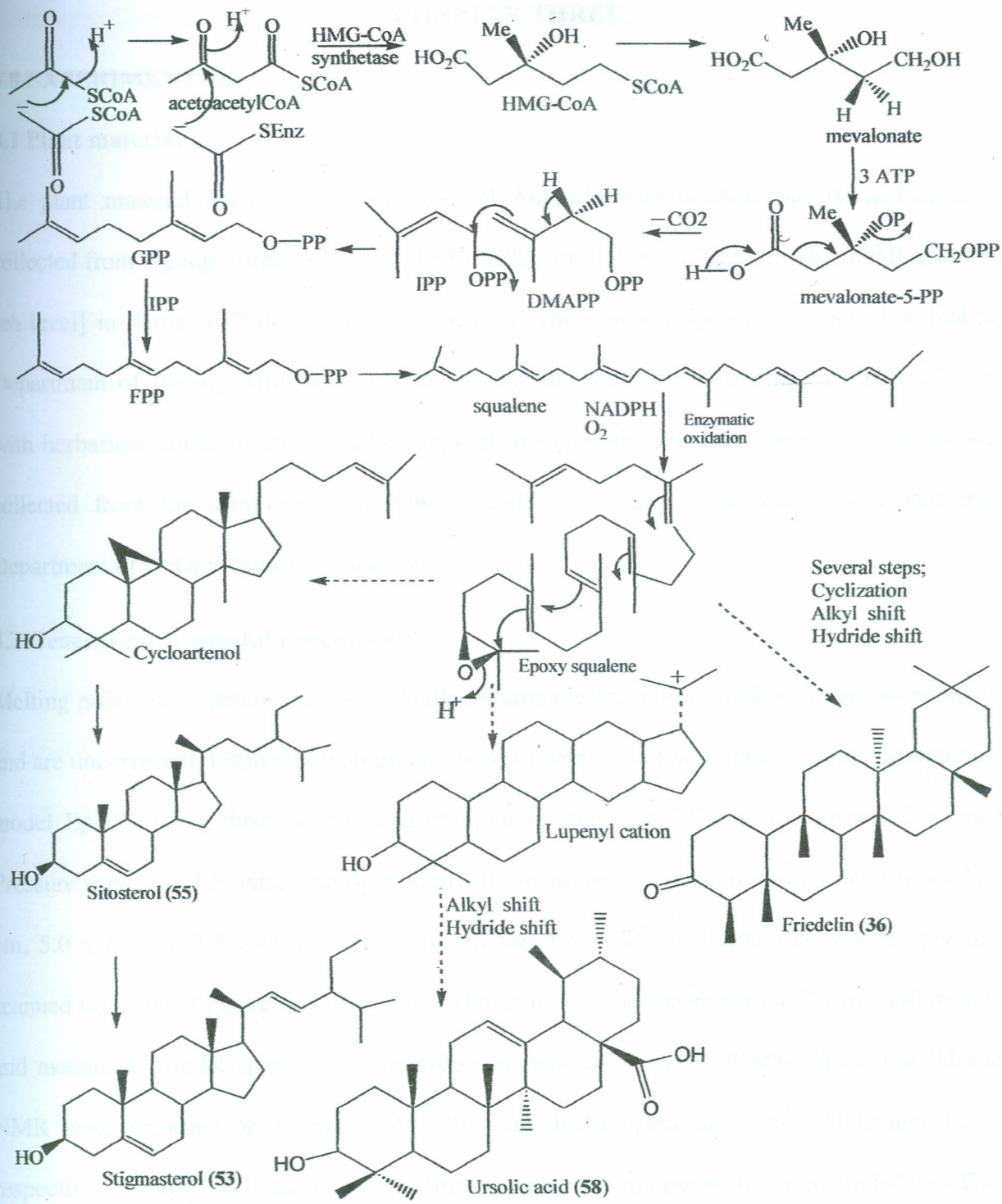


Fig. 2.6: Biosynthesis of sterols and triterpenoids (Source: Manitto, 1981).

CHAPTER THREE

3.0 EXPERIMENTAL

3.1 Plant material

The plant material (twigs and stem bark) of *Elaeodendron buchanannii* (*Celastraceae*) was collected from Ngong Forest, Kenya [1°18'13.62"S; 36° 43' 07.11" E; Altitude 5808ft above mean sea level] in April 2007 during the rainy season. The voucher specimens were identified at the Department of Botany, University of Nairobi by Mr. Mathenge (Taxonomist) after comparison with herbarium authentic samples. For phytochemical re-investigation the plant material was re-collected from the same plants in January 2008 and again identified by Mr. Mathenge of Department of Botany, Nairobi University.

3.2 General experimental procedures

Melting points were determined using Gallen-Kamp electrothermol melting point apparatus model and are uncorrected. Flash chromatography was performed on Eyela flash column chromatography model Ep-100 using three 60 cm long columns of diameter 7.5 cm, 3.5 cm and 2.5 cm each. Pressure used was 1.5 bars. Gravity column chromatography was done using columns of 7.5 x 60 cm, 5.0 x 60 cm, 3.5 x 60 cm, 2.0 x 40 cm and 1.5 x 40 cm. Ultraviolet visible spectra were scanned on Hewlett Puckered Diode Array Detector fitted spectrometer 8452A in dichloromethane and methanol. The IR spectra were recorded on Perkins-Elmer FTIR 600 series. The ^{1}H and ^{13}C NMR were recorded on Brucker AM-500 Ultra-shield operating at 500 MHz and 125 MHz respectively using $CDCl_3$ and chemical shift are expressed in ppm with tetramethylsilane (TMS) as internal standard. EIMS spectra were recorded on a 70eV MAT 311A Varian MAT Bremen instrument. The mass spectral data obtained by electron spray ionization mass spectrometer. I acknowledge the Institute of Organic Chemistry and Biochemistry, George August University,

Goettingen, Germany for providing the MS, IR and NMR spectral data. The reagents and solvents were of analytical grades and were supplied by Cobian, Kenya Limited.

3.3 Thin Layer Chromatography (TLC)

3.3.1 Chromatography

Analytical Thin Layer Chromatography (TLC) was performed on pre coated silica gel plates (Merck 60 FG $_{254}$) for the determination of the isolated compounds and for comparison with authentic samples. Silica gel 70-230 mesh ASTM and silica gel type 60 FG (0.02-0.7 mm) mesh (Merck) were used for gravity column chromatography and medium pressure chromatography (Flash chromatography), respectively.

3.3.2 Thin Layer Chromatography (TLC) solvent systems

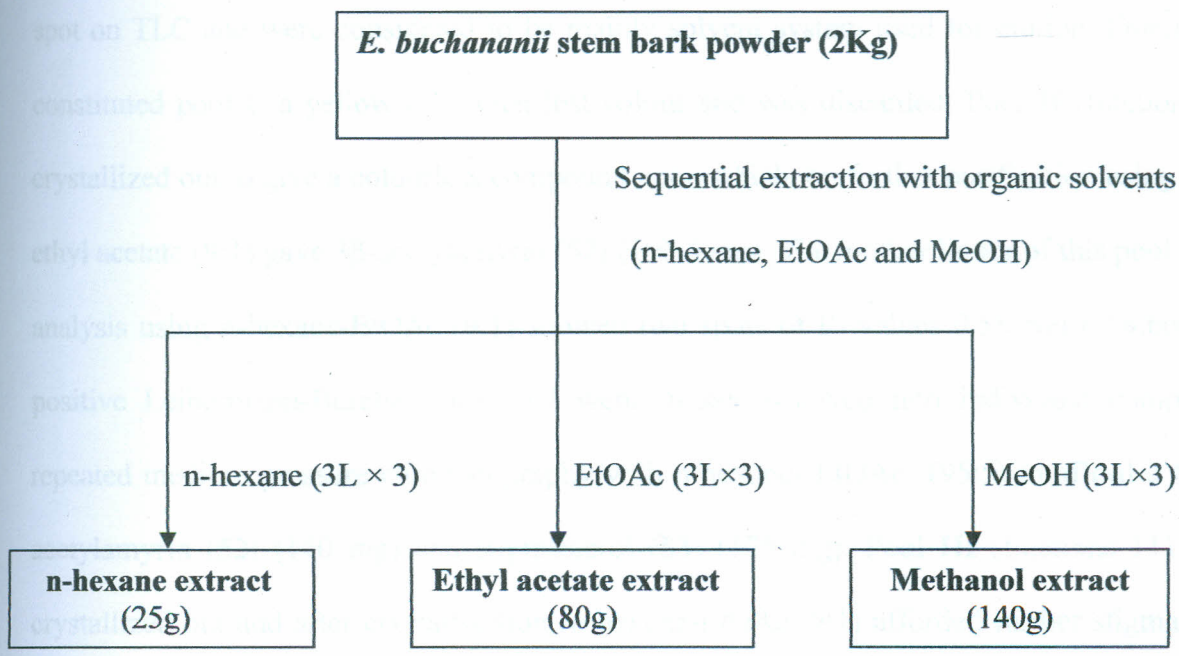
The following solvent systems were employed for TLC monitoring of pure substances, collected fractions as well as checking on composition of the crude extracts during the research work. The chromatograms were visualized using UV light (254 nm) and/or exposure to iodine vapour. The solvent systems employed included: n – hexane - EtOAc (9:1, 4:1, 3:2 and 1:1); CH_2Cl_2 - MeOH (9:5, 9:1, and 4:1) and n –BuOH – EtOAc - H_2O (4: 5: 1).

3.3.3 Chromatographic developing reagents

Three chromatographic developing reagents were used. In Leibermann – Burchard test, 5mls acetic anhydride was mixed with H_2SO_4 (5ml) and EtOH (50ml) under cooling condition. The sports were enhanced by blowing to dry. Vanillin reagent was prepared by adding 1% vanillin in conc. H_2SO_4. After spraying with the reagent, the chromatograms were kept at 110°C in a hot air oven until the colour of spots developed. Cerium sulphate was made by adding 100mg cerium sulphate in 5% H_2SO_4 solution. On spraying with the reagent the chromatograms were kept at 110°C in a hot air oven until the colours were developed.

3.4 Extraction of plant material

Two kilograms of the shade-dried ground powdered stem bark was transferred into two five litre conical flasks and exhaustively extracted sequentially using n- hexane, ethyl acetate and methanol (three litres each) with occasional shaking using an orbital shaker set at 150 revolutions per minute at room temperature (Scheme 3.1). The extracts were concentrated using a rotary evaporator to give yellow- brown, dark-brown and red materials in the yields of 25g, 80g and 140g, respectively.



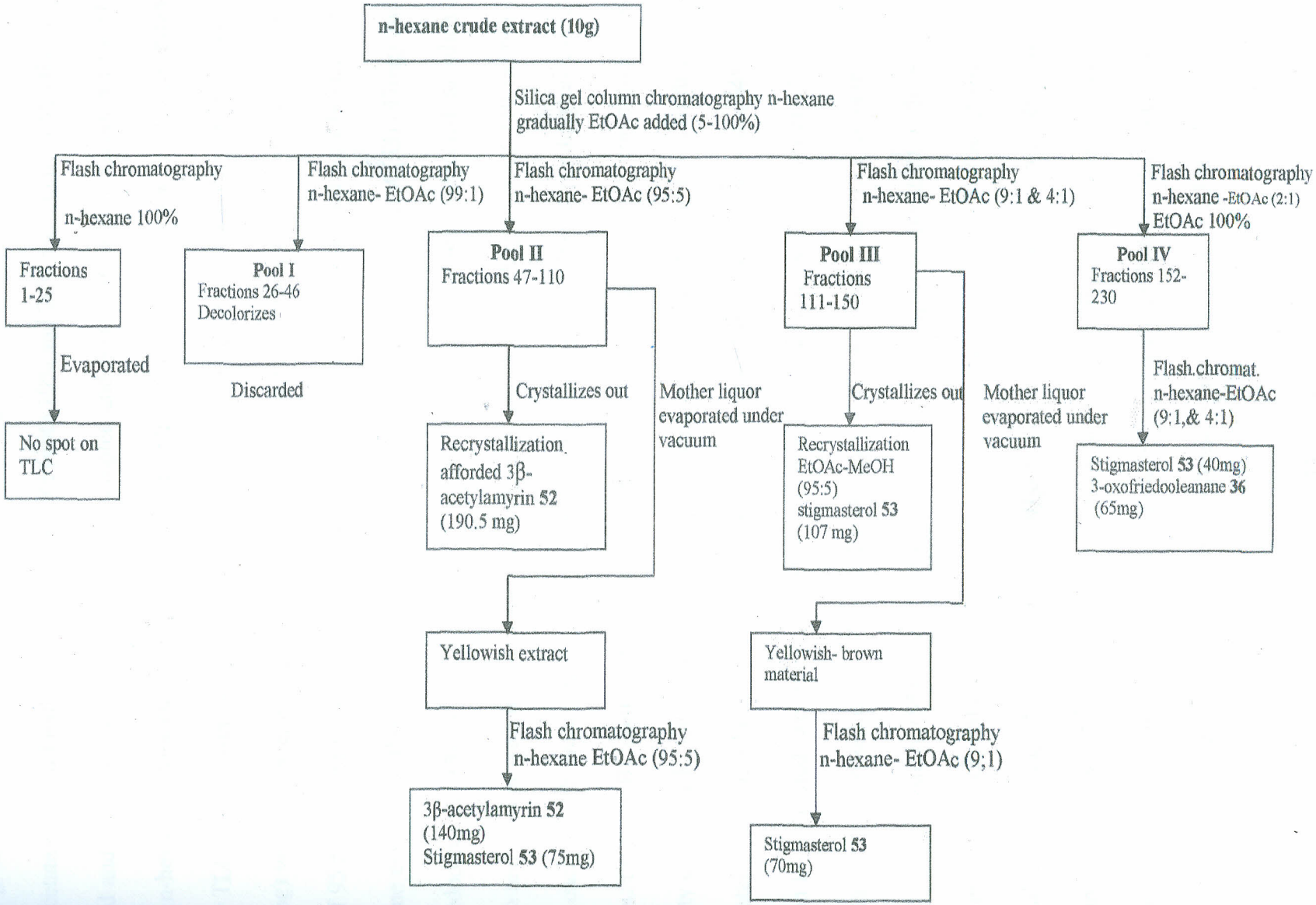
Scheme 3.1: Extraction of *E. buchananii* stem bark powder

3.5 Isolation of compounds from *Elaeodendron buchananii* stem bark

3.5.1 Isolation of components of n-hexane extract

Approximately 3.5g of the n-hexane crude extract was mixed with silica gel in a minimum amount of dichloromethane (CH_2Cl_2) in a round bottom flask. The solvent was removed in a *vacuo* and a free flowing yellowish-brown substance obtained. This was done to effect easy loading of the column and to reduce disturbances of silica gel as this allows the solvent to flow freely through the

column during elution. The free flowing material was chromatographed over silica gel packed column (medium pressure of 1.5 bars; column dimensions 2.5 by 60 cm: silica gel quantity, 100g) using n-hexane with increase of ethyl acetate up to 100%. A total of 230 fractions each of 20 mls were collected and the homogeneity of the fractions measured by TLC using solvent systems: n-hexane EtOAc, 9:1 and 4:1. Those showing similar TLC profiles were combined resulting into four pools (1-IV) (Scheme 3. 2). Fractions 1-25 after spoting and development did not show any spot on TLC and were considered to be mainly solvent system used for elution. Fractions 26-46 constituted pool **I**, a yellow oil which lost colour and was discarded. Pool **II** (fractions 47-110) crystallized out to give a colourless compound upon which, on further purification using n-hexane-ethyl acetate (9:1) gave 3β-acetylamyrin (**52**) (190.5 mg). The mother liquor of this pool upon TLC analysis using n-hexane-EtOAc (9:1) showed two spots of R_f values 0.58 and 0.54,both showed positive Leibermann-Burchard test and were further resolved into individual components by repeated medium pressure chromatography with n-hexane- EtOAc (95:5) to afford a further 3β-acetylamyrin (**52**) (140 mg) and stigmasterol (**53**) (175 mg). Pool **III** (fractions 111-180) also crystallized out and after crystallization (n-hexane-EtOAc, 9:1) afforded further stigmasterol (**53**) (107 mg). Similarly, the mother liquor of this pool (2.0 mg) afforded further stigmasterol (**53**) (70 mg) after crystallization (n-hexane-EtOAc, 9:1). Pool **IV**, (fractions151-230) were resolved into individual components by repeated medium pressure chromatography with n-hexane- EtOAc (9:1). TLC analysis using solvent system n-hexane-EtOAc (9:1) showed two spots of R_f values 0.54 and 0.76 and were further resolved into individual components stigmasterol (**53**) (40.0 mg) and 3-Oxofriedooleanane (**36**) (65 mg). Isolation of n-hexane extract components is summarised in Scheme 3.2.



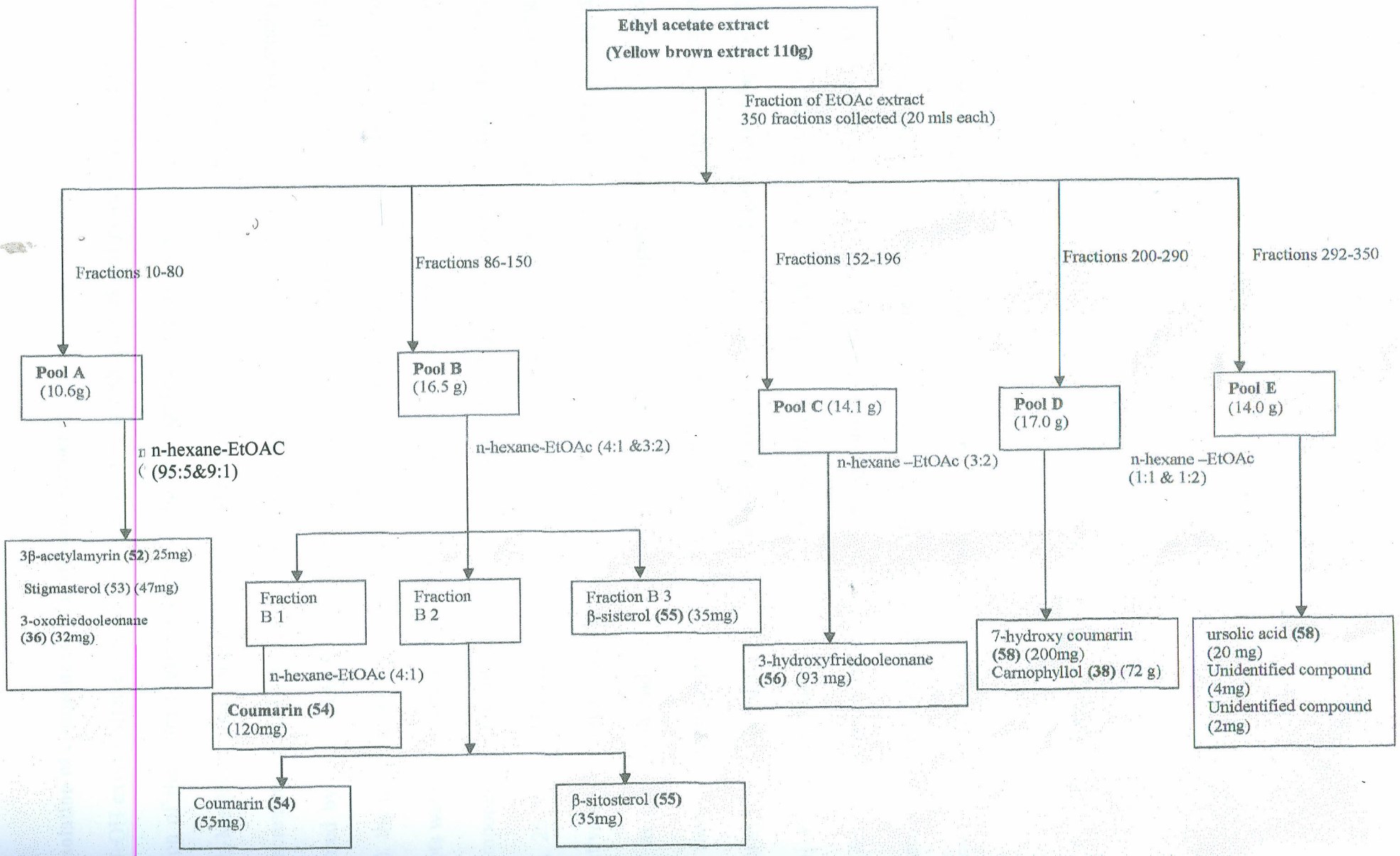
Scheme 3.2: Isolation of compounds from n-hexane extract of *Elaeodendron buchananii* stem bark

3.5.2 Isolation of components of ethyl acetate extract

The ethyl acetate extract approximately 110 g was subjected to pass over silica gel column (silica gel 250 g, column size 3.5 x 60 cm) with n-hexane-ethyl acetate (10% increment of ethyl acetate), ethyl acetate neat and finally with CH_2Cl_2-MeOH (with 10% and 20% increment of MeOH). Three hundred and fifty fractions each 20mls were sampled and their composition monitored by TLC, eluent: n-hexane-ethyl acetate (4:1, 3:2 and1:1) and CH_2Cl_2-MeOH (9:1 and 4:1). Those exhibiting similar TLC profiles were combined resulting into five major pools (A-E). Pool A (fractions 10-80,10.6g) was further applied to silica gel column and elution with n-hexane-ethyl acetate in the ratio of 95:5 followed by same solvent system 90:10 afforded compounds previously identified in n-hexane extract and this included 3β-acetylamyrin (**52**) (25mg), stigmasterol (**53**) (47mg) and 3-oxofriedooleonane (**36**) (32mg). Pool B (fractions 86-150, 16.5g) was subjected to silica gel column and elution with n-hexane-ethyl acetate (4:1) followed by (3:2) afforded 150 fractions of 20mls each, which were combined into three major fractions (B1-B3) depending on the TLC profiles. Fraction B1 contained one major compound with R_f value of 0.58 contaminated with impurity of that of R_f value 0.39 (solvent system, n-hexane- EtOAc, 4:1) and was further purified by crystallization in n-hexane-EtOAc -MeOH (10:40:50) mixture to give coumarin (**54**) (120mg). Fraction B2 exhibited two sports of R_f values 0.39 and 0.58 (solvent system n-hexane- EtOAc 4:1) and was further purified using n-hexane-EtOAc-MeOH (10:40:50) mixture to give coumarin (**54**) (55mg) and β-sitosterol (**55**) (35mg) respectively. Fraction B3 upon evaporation of the solvent on rotary evaporator crystallized out to give colourless crystals, which upon further purification by recrystallization using CH_2Cl_2-MeOH (10:90) afforded β-Sitosterol (**55**) (150mg). Fractions 152-196 constituted pool C (14.1g) which contained a single spot of R_f value of 0.66 (solvent system, n-hexane-EtOAc, 1:1) as was evident by exposure of UV light at 254nm. This pool was further purified by flash chromatography using solvent system n-hexane-EtOAc 3:2 to give 3-

hydroxyfriedooleanane (**56**) (93mg). Fractions 200-290 (pool D, 17g) on evaporation using rotary evaporator formed glassy colourless plates which were further purified by crystallization to give colourless crystals constituting 7-hydroxy coumarin (umbelliferone) (**57**) (200mg). The mother liquor from pool D exhibited two spots R_f value of 0.3 and 0.48 (solvent system: n-hexane-EtOAc, 1:2) both on spraying with anisaldehyde-H_2SO_4 and aniline-H_2SO_4 spraying reagent followed by heating at 110°C turned greenish and purple signifying the presence of triterpene and coumarin derivatives. The components of the mother liquor were resolved into individual components by repeated chromatography using n-hexane– EtOAc (1:1) followed by the same solvent system in the ratio 1:2 to give further 7-Hydroxy coumarin (**54**) (50mg) and canophyllol (28-Hydroxyfriedooleanan-3-one) (**38**) (72mg), respectively .

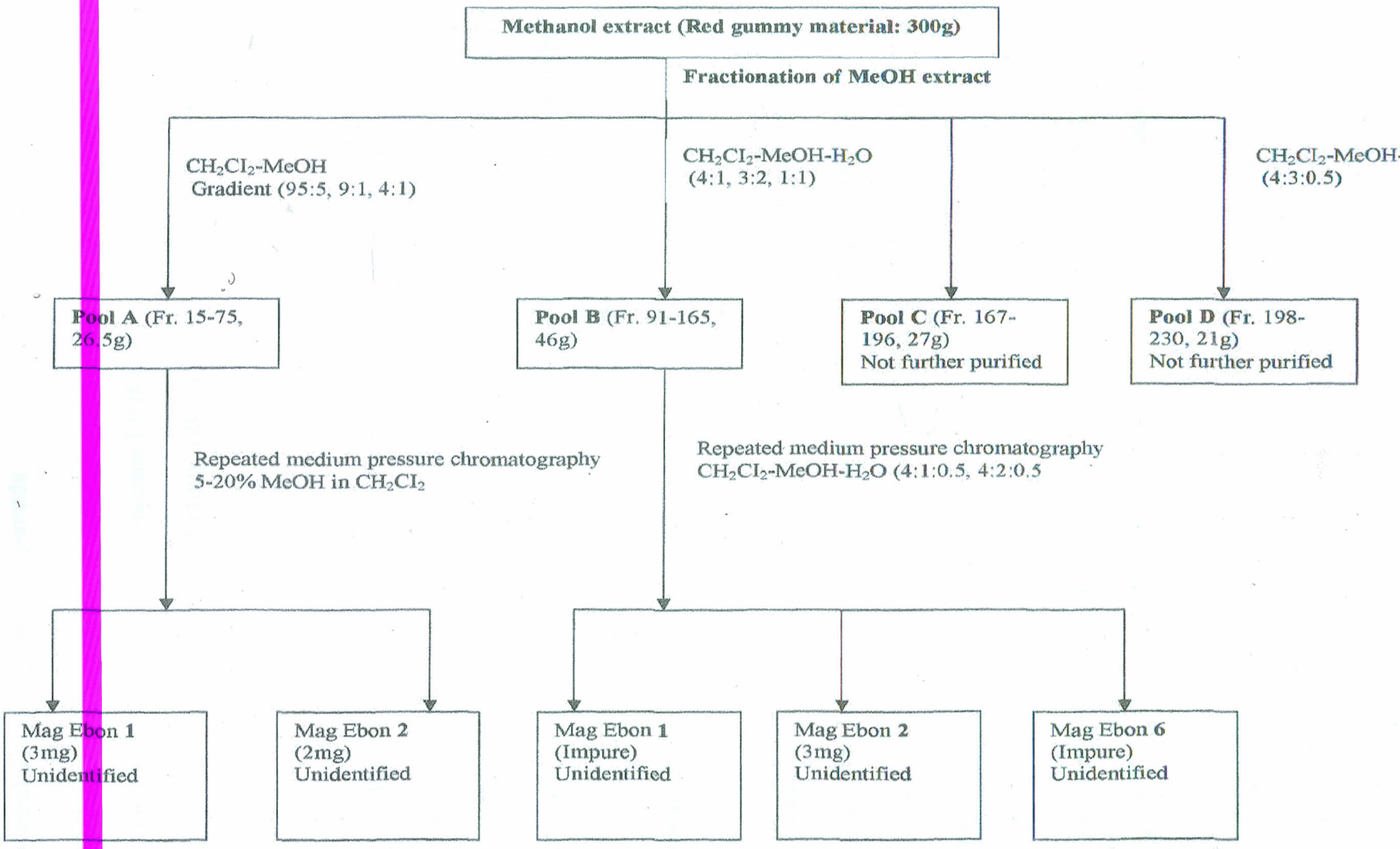
Fractions 292-350 which were mainly eluted by CH_2Cl_2-MeOH mixture from the column constituted pool E, which exhibited three spots of R_f values 0.63, 0.56 and 0.50, respectively using solvent system n-hexane–MeOH (9:1). Upon evaporation of the solvent, this fraction gave 14 mg of reddish-gummy material. Repeated silica gel chromatography using CH_2Cl_2-MeOH (95:5) followed by the same solvent system (9:1) afforded ursolic acid (**58**) (80mg) and two unidentified compounds (Scheme 3.3).



Scheme 3.3: Isolation of compounds from ethyl acetate extract of *Elaeodendron buchananii* stem bark

3.5.3 Isolation of components of methanol extract

The MeOH extract after evaporation of the solvent afforded a dark red gummy material of 450g. Aportion of the extract, approximately 300g was applied to pass over silica gel column using CH_2Cl_2-MeOH (5% increment of MeOH), MeOH neat and finally with MeOH-H_20 (with 5% and 10% increment of H_2O), a total of 200 fractions each of 50 mls were sampled and homogeneity monitored by TLC using CH_2Cl_2 -MeOH (4:1, 2:1 and 1:1) followed by CH_2Cl_2-MeOH-H_2O (6:3:1), the fractions were grouped into four pools (A-D) depending on TLC profile. In this case attempts were only made to purify pools A and B. The remaining pools showed high polarities and the components could not be resolved by column chromatographic technique. Pool A (fractions 15-90, 26.5g) on TLC analysis showed four spots of R_f values 0.41, 0.32, 0.30 and 0.28, respectively visualized after spraying with anisaldehyde-H_2SO_4 mixture followed by heating at 100°C as one bluish and three purple spots. Repeated chromatographic separation of this pool using flash chromatography with solvent system CH_2Cl_2-MeOH (95:5) followed by the same solvent system in the ratio 4:1 gave unidentified compounds [Mag Ebon **1** (3mg) and Mag ebon **2**, (2mg)]. Pool B (46g) similarly was chromatographed over silica gel column with CH_2Cl_2-MeOH-H_2O (4:1:0.5) followed by the same solvent system in the ratio (4:2:0.5) to give fractions which were resolved into Mag Ebon **1** and Mag Ebon **2** which were impure. The other unidentified compound was Eb **6** (1.5mg) which was also impure. The isolation is summarised in Scheme 3.4.



Scheme 3.4: Isolation of compounds from methanol extract of *Elaeodendron buchananii* stem bark

3.6 Physical and Spectral data of isolated compounds

3 α -Acetylamyrin (52)

Colourless crystals, M.p 153-155^0 C; R_f = 0.58 (n-hexane-EtOAc, 4:1). The EIMS *m/z* (%) 468.4 [M^+, $C_{32}H_{52}O_2$] (18), 409.4 (52), 249.1 (4), 218.2 (100), 189.1 (13), 135.0 (6). ^{1}H and ^{13}C NMR data: see Table 4.6.

Stigmasterol (53)

Colourless crystals, M.p 171.5-173.5 °C; R_f = 0.5 (n-hexane-EtOAc, 4:1). IR (KBr), λ_{max} 3500–3350 and 1654 cm^{-1} . The EIMS *m/z* (%): 412.2 [M^+, $C_{29}H_{48}O$] (88), 397.1 (15), 379.2 (16) 314.1(28), 300.0 (40), 271.0 (95), 281.0 (11), 229.0 (17), 213.0 (32), 187.0 (13), 158.9 (46), 144.9 (50), 133.0 (42), 109.0 (78), 94.9 (76), 68.9 (44), 54.9 (71). ^{1}H and ^{13}C NMR data: see Table 4.4.

3-Oxofriedooleanane (Friedelin) (36)

Colourless needles M.p 265-266°C; R_f = 0.68 (n-hexane-ethyl acetate, 4:1).

IR (KBr) λ_{max} 1712 and 2930 cm^{-1} . The EIMS *m/z* (%): 426.4 [M^+, $C_{30}H_{50}O$] (92), 411.4 (18), 344.3 (2), 342.4 (7), 307.3 (35), 302.3 (42), 273.3 $[C_{20}H_{33}]^+$ (52), 246.3 (32), 218.2 (53), 205.5 $[C_{15}H_{25}]^+$ (44), 179.2 (38), 137.2 (29), 125.2 (59), 123.2 $[C_9H_{15}]^+$ (62), 109.1 (73), 95.1 (83), 69.1 (92), 55.1 (58), 41.0 (32). ^{1}H and ^{13}C NMR data: see Table 4.7.

Coumarin (54)

Colourless needles M.p 298-299^{0}C; R_f = 0.39 (n-hexane-ethyl acetate, 4:1). The EIMS *m/z* (%): 146.1 [M^+, $C_9H_6O_2$] (100), 118.1 (70), 89.0 (16), 63 (10). ^{1}H and ^{13}C NMR data: see Table 4.2.

β-Sitosterol (55)

Colourless crystals M.p 135 – 136^{0}C; R_f = 0.56 (n-hexane-EtOAc, 3:2). EIMS *m/z* (%): 414.4 [M^+, $C_{29}H_{50}O$] (77), 397.4 (100), 381.4 (26), 329 (30), 303.3 (31), 271(17), 255.2 (21), 213.2 (18), 161.3 (14), 107.1(13), 82.1 (10). ^{1}H and ^{13}C NMR data: see Table 4.5.

3α -Hyroxyfriedooleanane (Hydroxyfriedelin) (56)

Colourless needles M.p 278-280°C. R_f = 0.66 (n-hexane-EtOAc 1:2).The IR (KBr) λ_{max} 3477, 2932-2869, 1450 and 1337cm^{-1}. EIMS *m/z* (%): 428.4 [M^+, $C_{30}H_{52}O$] (34), 413.4 (16), 395.4 (4), 275.3 (31), 248.3 (13), 220.2 (30), 206.2 (24), 177.2 (21), 165.1 (69), 137.1 (27), 123.1 (44), 109.1 (66), 69.1 (90), 55.1 (45), 41.0 (23). ^{1}H and ^{13}C NMR data: see Table 4.8.

Umbelliferone (57)

Colourless crystals M.p 230- 232°C; R_f = 0.3 (n-hexane-EtOAc 1:2). EIMS *m/z* (%): 162.0 [M^+, $C_9H_6O_3$] (92), 134.0 (100), 106 (10), 105 (20), 91 (22), 78 (28), 67 (8), 63 (12), 51(20). ^{1}H and ^{13}C NMR data: see Table 4.3.

28-Hydroxyfriedooleanan-3-one (Canophyllol) (38)

Colourless needles, M.p 280-282°C; R_f = 0.48 (n-hexane- EtOAc, 1:2)

^{1}H, ^{13}C NMR and HMBC data: see Table 4.9.

Ursolic acid (58)

Colourless needles M. p 282-284 °C. R_f = 0.5 (CH_2Cl_2-MeOH 9:1). EIMS *m/z* (%): 456.4 [M^+, $C_{30}H_{48}0_3$] (4), 439.4 (8), 411.4 (4), 249.2 (13), 248.2 (75), 203.2 (18), 147.1 (100), 118.1 (77), 89.0 (24), 63.0 (10). ^{1}H and ^{13}C NMR data: see Table 4.10.

3.7 Biological activities

3.7.1 Brine shrimp lethality assay

Lethality assay was conducted according to Meyer *et al* (1982). Eggs of *Brine shrimp*, 100 mg were sprinkled into large compartments of the two unequal compartment plastic chambers containing artificial sea water prepared from commercial sea salt, 38g/litre. The plastic chambers which were separated by a divider were well aerated with the aid of a pump. A bright light was left on to illuminate the smaller compartment while the larger compartment was darkened. The set up (Fig 3.1) was kept in a water bath at 28-30°C (Midletton *et al.*, 2005) for 24 hours.

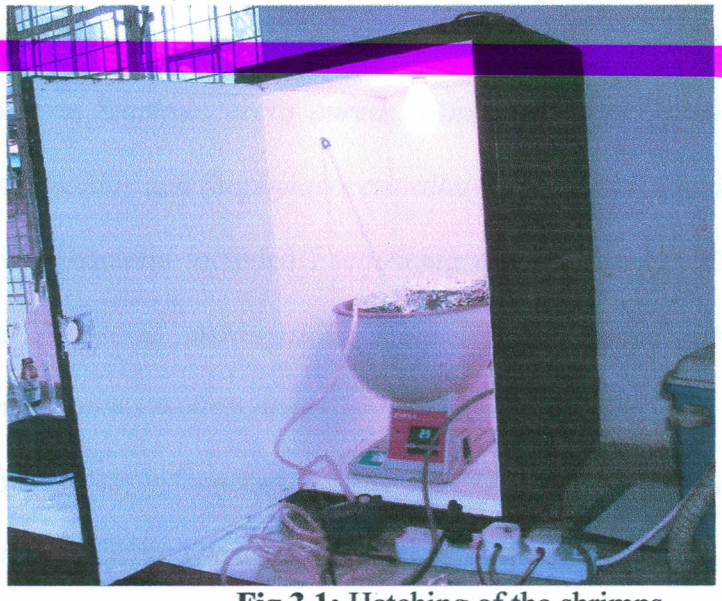


Fig 3.1: Hatching of the shrimps

The larvae were allowed to mature to *nauplii* by further incubating at 28^0C for 24 hours. After 48 hours the phototropic *nauplii* were collected by pipette from the lighted side, having been separated by the divider from their shells.

One gram of each of the extracts (n-hexane, ethyl acetate and methanol) was dissolved in 5mls of DMSO and then serially diluted with seawater to make concentrations of 200, 100, 50, 25, 12.5, 6.25, 3.13, 1.56 and 0.78µg/ml. Two days old *nauplii* were counted in groups of 10 and placed in 20mls of test solutions contained in 50mls beakers and maintained at 27°C for 24 hrs. Control experiments were set up using DMSO without the plant extracts. The tests were carried out in triplicate. The mortality of *nauplii* was determined after 24 hrs by recording the number of survivors. The percentage death was calculated and then converted to probit, which was plotted against concentration to determine the LD_{50} of the extracts (Finney, 1952).

3.7.2 Antibacterial and antifungal assays

3.7.2.1 Test organisms

Three gram-positive; *Staphylococcus aureus, (American Type Culture Collection* ATCC 25923), *Diplococcus pneumoniae* and *Staphylococcus albus* were used for antimicrobial activity screening. The gram-negative bacteria included *Eschirichia coli* (ATCC 25922), *Vibrio cholerae, Shigella dysenterae and Neisseria meningitidis*. Both fungi *Candida albicans* (ATCC 90028) and *Cryptococcus neoformans* were screened.

The test organisms *Staphylococcus aureus* ATCC 25923, *Eschirichia coli* ATCC 25922 and *Candida albicans* ATCC 90028 were obtained from the stock kept at the Microbiology Section of the New Nyanza Provincial General Hospital, Kisumu, Kenya. The rest of the test organisms were clinical isolates from the same Hospital. For positive controls; gentamicin, tetracycline and clotrimazole were used. Both antibacterial and antifungal assays were done at the Hospital Microbiology Laboratory.

3.7.2.2 Preparation of the agar culture media

Four types of agar media were used to isolate pure colonies of the micro organisms used. They were Sabouraud dextrose agar (SDA), Mueller Hinton Agar (MHA), Enriched Medium (EM) and Cystine Lactose Electrolyte Deficiency agar (C.L.E.D). All the agar media were prepared (Murray *et al*., 1999). About 30g of each of the medium powder was suspended in a litre of distilled water, and then heated to dissolve completely. Sterilization was done by autoclaving them at 121^0C for 15 minutes. All the sterile agar media were poured into sterile 90mm Petri dishes to a depth of 4mm. The surfaces of the agar were dried to remove excess moisture before use.

3.7.2.3 Disc diffusion assay

The Baur-Kirby procedure was used and is based on the diffusion through an agar gel of antimicrobial substances which are impregnated on paper discs (Murray *et al*., 1999). The dried

plant-extracts were dissolved in DMSO to give final concentrations of 0.5, 1.0 and 1.5mg/ml. Sterile paper discs (6 mm of diameter) prepared from Whatman No 1 filter paper were impregnated with 0.1 ml of the stock solution of plant extracts to give the final concentrations of 0.5, 1.0 and 1.5 mg per disk. These paper discs were kept in an incubator at 37^0C for 24 hours to evaporate the solvent. Overnight cultures of bacterial and fungal species were used for inocula preparation. The inocula were prepared by making microorganisms to grow in a sterile saline and turbidity was adjusted to yield 0.5 McFarland standard (approximately 10^8 colony-forming units per millilitres). Petri dishes containing Mueller-Hinton agar and Sabouraud dextrose agar were seeded with 100 µl of the prepared bacterial and fungal inocula, respectively. The discs were arranged and firmly pressed on the agar surface of each seeded plate and then incubated at 37^0C for 24 hours for bacteria and at 25^0C for 24 hours for fungi. Similarly, paper discs containing standard concentrations of antibiotics (Tetracyline, 30 µg per disk; gentamicin, 10µg per disk) and antifungal (clotrimazole, 30 µg per disk) were used as positive control. A disk soaked with the solvent (5% DMSO) was used as negative control. Antibacterial activity was determined by measuring the zone of growth inhibition (mm) around the disk. The result recorded for each bioassay was the average of three tests and the obtained results were statistically analysed.

3.7.2.4 Preparation of crude extracts and pure isolates for MIC determination

Minimum Inhibitory Concentration (MIC) is defined as the lowest concentration of the test sample in which there is no growth of microorganisms. The MIC of all extracts and isolates was determined by broth microdilution method (Murray *et al.*, 1999). Stock solutions of the crude extracts and pure isolates were prepared in DMSO at 2.0 mg/ml. 40µl of the DMSO solution was added to 1960 µl medium to obtain a final volume of 2ml. 1ml of this mixture was added to the next tube with 1ml medium, Mueller Hinton broth for bacteria and Sabouraud dextrose broth for fungi. This 2-fold serial dilution resulted in final concentrations of the dilutions 0.98, 1.96, 3.91,

7.82, 15.63, 31.25, 62.5, 125, 250, 500 and 1000µg/ml. The tubes were transferred from the incubator to the laminar flow hood from which an aliquot of 5µl of standardized suspension of microorganisms (0.5 McFarland standard turbidity containing 10^8 colony forming units (cfu)/ml) were added to 100µl of each extract or isolate dilution in the tubes. The same procedure was used to determine MIC values for gentamycin and clotrimazole. Each test included 2 growth controls consisting of the medium with the solvent (5% DMSO) and medium with bacterial or fungal suspension. The tubes were incubated for 24 hours at 37^0C and 25^0C for fungi and bacteria respectively. The assays were done in triplicate and analysed statistically using MSTAT-C statistical package (Michigan State University, M.I).

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 Preliminary biological tests results

The extracts (n-hexane, ethyl acetate and methanol) of the stem bark of *E. buchananii* were tested for activity using the brine shrimp (*Artemia salina*). The Brine shrimp lethality test results shown on Table 4.1 displayed the toxicity of the stem bark of *E. buchananii* against the shrimps. Dimethyl sulphoxide (DMSO) was used as a control, and no mortality was recorded.

Table 4.1: Mortality of *Artemia salina* by the crude extracts of *E. buchananii* stem bark

Concentration (µg /ml)	Mortality					
	n- hexane		Ethyl acetate		Methanol	
	No.	Probit	No.	Probit	No.	Probit
0.78	0.67±0.58	3.52	4.67±0.58	4.92	1.08±0.58	3.77
1.56	1.33±0.58	3.87	6.33±0.58	5.33	1.37±0.58	3.96
3.13	2.00±1.0	4.16	7.33±0.58	5.61	3.03±1.0	4.39
6.25	4.00±1.0	4.75	7.67±0.58	5.74	4.09±1.0	4.82
12.50	5.00±1.0	5.00	8.67±0.58	6.13	6.30±0.58	5.31
25.00	6.33±0.58	5.33	9.67±1.0	6.88	6.67±0.58	5.52
50.00	7.00±1.0	5.52	10.00±1.0	5.52	8.67±0.58	6.48
100.00	10.00 ±1.0	8.09	10.00±1.0	8.09	10.00±1.0	8.09
LC_{50} (µg /ml)	28.228		2.784		19.524	

Values are given as mean of replicate ± SD (standard deviation).

This toxicity to the shrimps was a manifestation of the activities of a broad range of active compounds in the plant extracts [INEPO (1994) http:www.inepo.com, 1994]. All the three extracts from the stem bark of *E. buchananii* were active. The ethyl acetate was the most toxic (LC_{50} 2.784 µg /ml) while n-hexane displayed comparatively low activity (LC_{50} 28.228µg /ml). Based on the

brine shrimp lethality results, the three extracts were separated by gravity and flash column chromatography on silica gel in order to isolate the active compounds responsible for the toxicity against the shrimps.

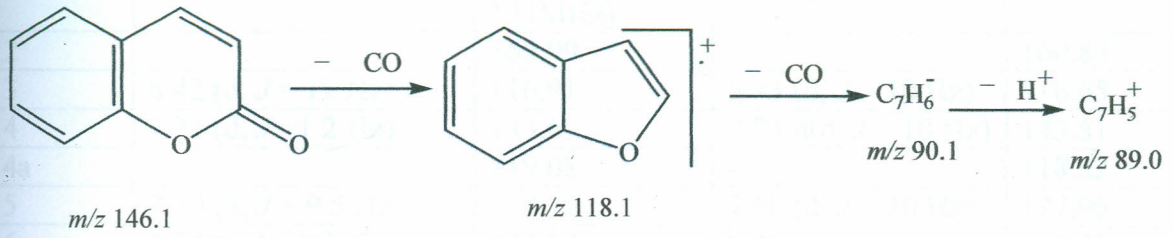
4.2 Structural elucidation of compounds

Fractionation of the extracts by gravity and flash column chromatography led to the isolation of twelve compounds of which nine were structurally identified. The identities of the isolated compounds were established by the use of a combination of spectroscopic analyses, literature data and authentic samples. The compounds isolated can be grouped as coumarins, sterols and triterpenoids as discussed herein.

4.2.1 Structural elucidation of coumarins from *E. buchanannii* stem bark

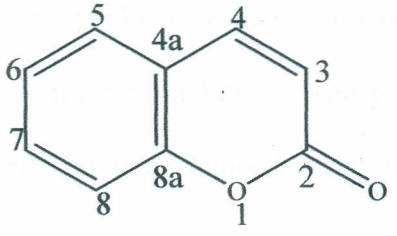
4.2.1.1 Coumarin (54)

Compound **54** was isolated as colourless needles from ethyl acetate extract, with M.p 298-299^{0}C and R_f of 0.39 (n-hexane- EtOAc, 9:1). The EIMS (70Ev) spectrum of compound **54** gave a molecular ion peak $[M]^+$ at *m/z* 146.1 corresponding to a molecular formula $C_9H_6O_2$, which further disintegrated into a benzofuran ion (*m/z* 118.1) $[M\text{-}CO]^+$, due to loss of carbon monoxide (CO), from the pyrone ring (Widodo *et al.*, 2009). The benzofuran ion is decomposed further by consecutive loss of CO and a hydrogen atom to give $[M\text{-}2CO\text{-}H]^+$ (*m/z* 89.0) (Murray *et al.*, 1982). The fragmentation pattern in the mass spectrum of compound **54** is illustrated in Scheme 4.1 which is characteristic of an unsubstituted coumarin derivative (Widodo *et al.*, 2009).



Scheme 4.1: MS fragmentation pattern of compound **54**

The ^{1}H-NMR ($CDCl_3$) spectrum of compound **54** displayed 4 doublet signals in the downfield region at δ 6.42 (J = 12.0Hz, H-3), 7.32 (J = 10.5Hz, H-8), 7.49 (J = 9.5 Hz, H-5) and 7.71 (J = 12 Hz, H-4). There were also two triplet signals centered at 7.28 (J = 9.5, 5 Hz) and 7.53 (J = 10.5, 9 Hz) attributed to H-6 and H-7, respectively. The ^{13}C NMR of compound **54** showed 9 signals consisting of three quaternary carbons at δ 119.08 (C-4a), 154.29 (C-8a) and 160.99 (C-2). The remaining signals were methine carbons which appeared at δ 116.91 (C-3), 117.10 (C-8), 124.67 (C-6), 127.12 (C-5), 132.06 (C-7) and 143.69 (C-4). The three quaternary and six methines carbons were further confirmed by DEPT experiments. The ^{1}H NMR and ^{13}C NMR spectral data of compound **54** were found to be similar to that of a coumarin previously isolated from *Ageratum conyzoides* leaves (Widodo *et al.*, 2009). Thus, based on the spectroscopic data (Appendix D) and comparative analysis with the literature data, compound **54** was concluded to be coumarin.



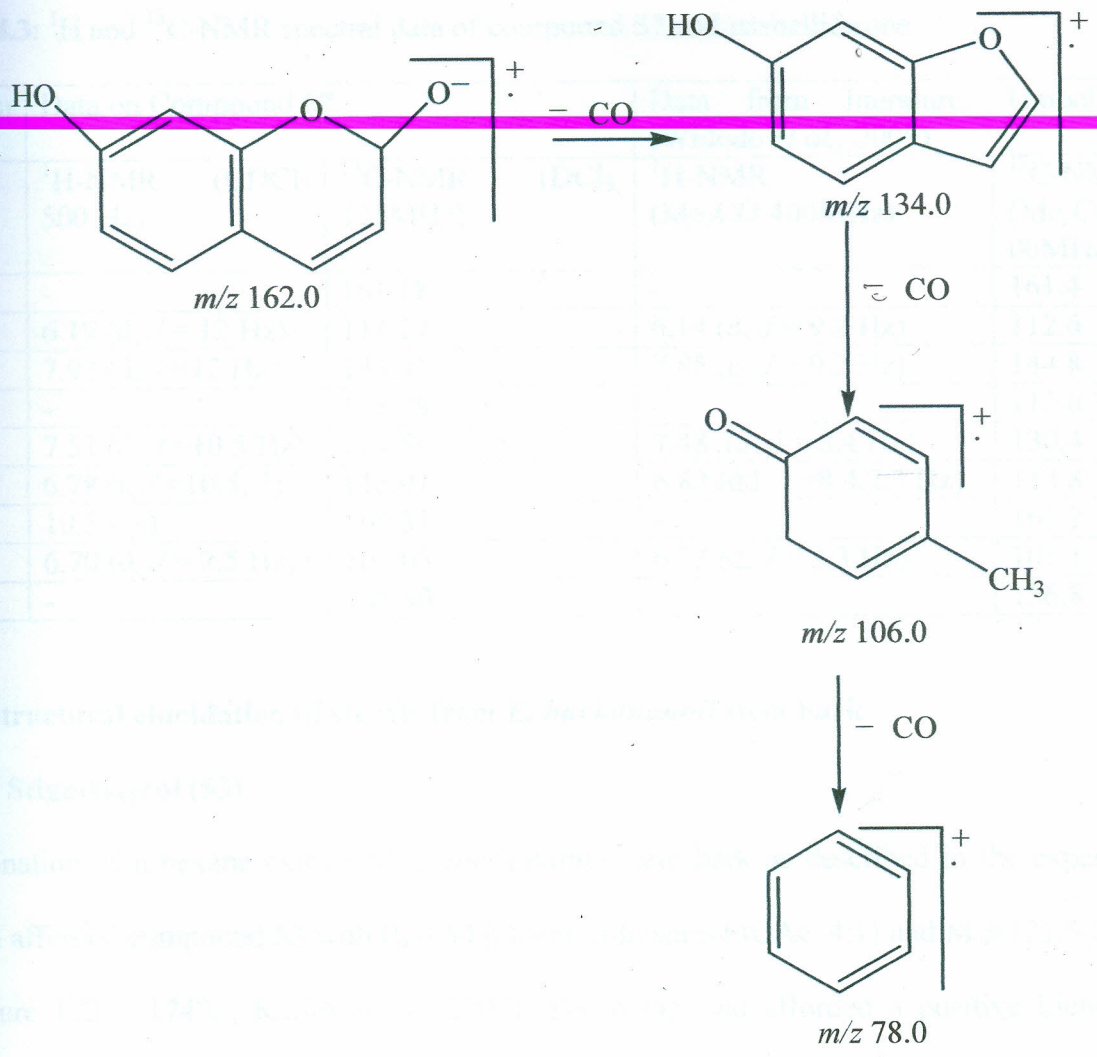
54

Table 4.2: ^{1}H and ^{13}C-NMR spectral data of compound **54** and coumarin

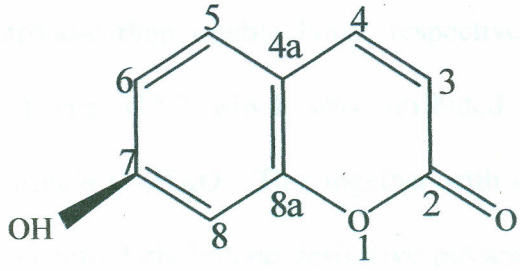
Position	Data on Compound **54**		Data from literature; coumarin Widodo *et al.*, 2009)	
	^{1}H-NMR ($CDCl_3$ 500 Hz)	^{13}C-NMR (DCl_3, 125MHz)	^{1}H-NMR (500 Hz)	^{13}C-NMR (125MHz)
2	-	160.99	-	160.84
3	6.42 (d, J = 12 Hz)	116.91	6.43 (d, J = 10 Hz)	116.65
4	7.71 (d, J =1 2 Hz)	143.69	7.73 d(d, J = 10 Hz)	143.51
4a	-	119.08	-	118.82
5	7.49 (d, J = 9.5 Hz)	128.12	7.50 (d, J = 10 Hz)	127.90
6	7.28 (t, J = 9.5, 5 Hz)	124.67	7.29 (m)	124.45
7	7.53 (t, J = 10.5, 9 Hz)	132.06	7.55 (m)	131.85
8	7.32 (d, J = 10.5 Hz)	117.10	7.33 (d, J = 10 Hz)	116.83
8a	-	154.29	-	154.01

4.2.1.2 Umbelliferone (57)

Compound **57** was obtained as colourless crystals with M.p 230-232^{0}C from ethyl acetate extract, R_f 0.3 (n-hexane-EtOAc, 1:2). Its ^{13}C NMR spectrum exhibited 9 carbon atoms resolved into five methines and four quaternary carbons by 135 DEPT experiments. The ^{1}H NMR spectrum shows four doublet methine protons at δ 6.19 (H-3), 6.70 (H-8), 7.51 (H-5) and 7.92 (H-4) together with a methine triplet at δ 6.78 (H-6) and a singlet at δ 10.54, respectively which after comparison with those of compound **54** suggested the presence of a substituted coumarin derivative (Loomis *et al.*, 2004). A substituted coumarin derivative was supported by EIMS spectrum which exhibited a molecular ion peak $[M]^+$ at *m/z* 162.0 corresponding to a molecular formula $C_9H_6O_3$. The other fragments at *m/z* 134.0 $[M-CO]^+$ and 106.0 $[M-2CO]^+$ (Scheme 4.2) were typical of hydroxylated coumarin derivative (Widodo *et al.,* 2009). Data from the MS indicated a secondary metabolite with structure similar to that of compound **54** with major structural difference being the OH group confirmed by mass difference of 16 amu. On the basis of spectroscopic data (^{1}H, ^{13}C NMR, Table 4.3 and MS, Appendix G) with published data (Widodo *et al,* 2009), compound **57** was concluded to be umbelliferone (7-hydroxycoumarin).



Scheme 4.2: MS fragmentation pattern of compound **57**



57

Table 4.3: ^{1}H and ^{13}C-NMR spectral data of compound **57** and umbelliferone

Position	Data on Compound **57**		Data from literature; Umbelliferone (Widodo *et al.*, 2009)	
	^{1}H-NMR ($CDCl_3$ 500 Hz)	^{13}C-NMR (DCl_3, 125MHz)	^{1}H-NMR (Me_2CO,400MHz)	^{13}C-NMR (Me_2CO 00MHz)
2	-	161.18	-	161.4
3	6.19 (d, J = 12 Hz)	111.17	6.14 (d, J = 9.2 Hz)	112.6
4	7.92 (d, J =12 Hz)	144,41	7.85 (d, J = 9.2 Hz)	144.8
4a	-	111.29	-	112.6
5	7.51 (d, J =10.5 Hz)	129.59	7.48 (d, J = 8.4 Hz)	130.4
6	6.78 (t, J =10.5, 3)	113.01	6.82 (dd, J =8.4,2.3 Hz)	113.8
7	10.54 (s)	160.31	-	162.2
8	6.70 (d, J = 2.5 Hz)	102.05	6.73 (d, J = 2.3 Hz)	103.1
8a	-	155.40	-	156.8

4.2.2 Structural elucidation of sterols from *E. buchanannii* stem bark

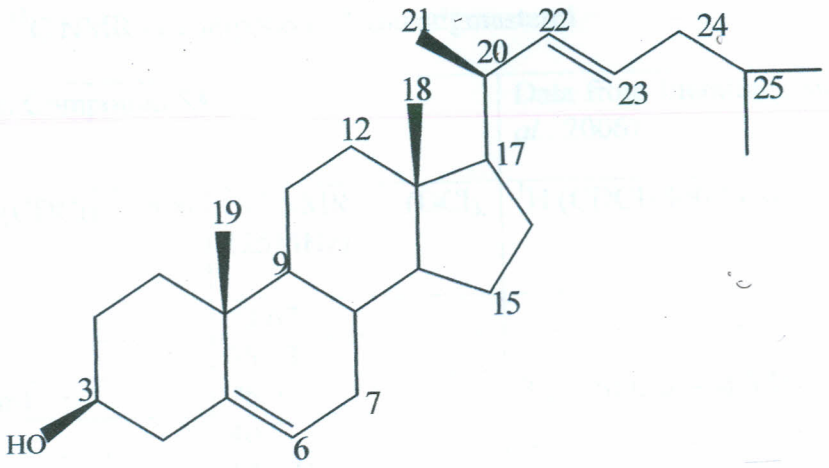
4.2.2.1 Stigmasterol (53)

Fractionation of n-hexane extract of *E. buchanannii* stem bark as described in the experimental section afforded compound **53** with R_f 0.54 (eluent: n-hexane-EtOAc, 4:1) and M.p 171.5-173.5 °C (literature 172 – 174°C; Karim *et al.*, 2007). The compound afforded a positive Liebermann-Burchad test but failed the ceric sulphate test for triterpenes suggesting that it could be a sterol derivative. Its IR spectrum afforded significant peaks at λ_{max} 3500 and 1654 cm^{-1} suggesting the presence of hydroxyl and carbon-carbon double bond, respectively. The EIMS (70eV) mass spectrum exhibited a peak at *m/z* 412.2 which was attributed to the molecular ion, $[M^+]$ corresponding to molecular formula $C_{29}H_{48}O$. This together with other significant peaks at *m/z* 379.2, and 271.0 (base peak) signified cholestane derivative possessing a hydroxyl group at C-3 (Holland *et al.*, 1976). The ^{13}C NMR spectrum showed a total of 29 carbon atoms which were resolved into six methyls, nine methylenes, eleven methines and three quaternary carbons by DEPT experiments.

Out of the eleven methine carbons, two sets of olefinic carbon signals appearing at δ 140.74 and 121.73 due to trisubstituted double bond functionality (Holland *et al.,* 1976) and an isolated double bond at δ 137.21 and 109.52 were observed.

Furthermore, the presence of methyl resonances at δ 21.08, 21.06, 19.40, 18.67, 18.65, and 11.84 together with another methine carbon signal at δ 76.80 further support the presence of a cholestane skeleton with hydroxyl group at C-3 (Li *et al.,* 2006).

The ^{1}H NMR spectrum determined in deuterated chloroform further corroborated the ^{13}C NMR results. Careful examination of the spectrum revealed the presence of a peak at δ 3.52 which appeared as a multiplet attributable to an oxymethine proton possibly at C-3 (Holland *et al,* 1976). In addition, the multiplet peaks at δ 5.28 and 4.68 represented olefinic protons H-5 and H-22 / H-23, respectively and are characteristic features of sterol (Guyot *et al.,* 1982). In fact, the latter peak is identical with the chemical shift of H-22 / H-23 of stigmasterol (Li *et al.,* 2006). Further support for the structure of compound (**53**) was evidenced by the ^{1}H NMR data in which two tertiary methyl groups appeared as singlets at δ 0.90 and 1.02 while the signals for three secondary methyls resonated as doublet at δ 0.81 and singlets at δ 0.91, 1.02 and comparison of spectroscopic data of compound **53** with those of stigmasterol shows some resemblance. Similarly, comparison of spectroscopic data (Appendix B) of compound **53** with those of sitosterol **55**, indicated that the latter had a molecular ion peak at *m/z* 414, two amu higher while the ^{1}H NMR lacked the peak at δ 4.68 signifying the absence of an isolated double bond. Therefore from the above results, compound **53** was confirmed as stigmasterol.



53

Table 4.4: ^{1}H and ^{13}C NMR of compound **53** and stigmasterol

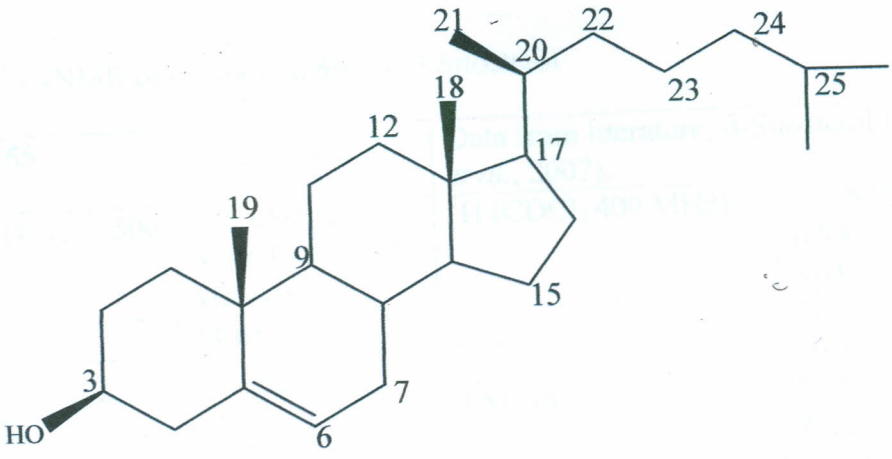
Position	Data on Compound **53**		Data from literature; Stigmasterol (Li *et al.*, 2006).	
	^{1}H ($CDCl_3$ 500 MHz)	^{13}C-NMR (DCl_3, 125MHz)	^{1}H ($CDCl_3$ 400 MHz)	^{13}C-NMR ($CDCl_3$ 400 MHz)
1		33.67		32.9
2		35.53		34.7
3	3.52 (m)	76.67	3.25 (tdd, J = 4.5 Hz)	79.0
4		40.2		42.0
5		140.74		142.6
6	5.28 (m)	121.73	5.14 (m)	124.4
7		28.72		31.6
8		28.17		28.7
9		42.29		42.0
10		36.51		39.6
11		20.80		19.4
12		31.91		31.9
13		42.25		40.8
14		49.53		47.7
15		21.06		21.4
16		20.80		20.8
17		51.99		50.1
18	1.02 (s)	18.67	1.08 (s)	18.3
19	0.91 (s)	18.65	1.16 (s)	18.2
20		36.49		33.4
21	0.81 (d, J = 6.5JHz)	20.23		17.4
22	4.68 (m)	109.52	4.62 (m)	107.1
23		137.21		139.5
24		50.12		47.7
25		31.67		30.6
26	1.02 (s)	19.40	1.01 (3s)	20.2
27	0.91(s)	21.08	0.98 (s)	19.3
28	0.90 (s)	25.20		24.2
29		11.84	0.96 (3s)	11.8

4.2.2.2 β-Sitosterol (55)

The Compound was obtained as colourless crystals from n-hexane-ethyl acetate mixture with M.p 135 – 136^{0}C. It gave an R_f value of 0.58 (eluent: n-hexane-EtOAc, 3:2) and afforded a positive test with Liebermann-Burchard reagent but failed the ceric suphate test for triterpenes suggesting that it

may be a sterol. Its ^{13}C NMR spectrum displayed 29 distinct carbon resonances attributable to six methyls, eleven methylenes, nine methines and three non-protonated carbon atoms from DEPT experiment corresponding to 49 protons of the molecule. The EIMS analysis indicated the presence of a molecular ion at *m/z* 414.4 corresponding to a molecular formula of $C_{29}H_{50}O$. Thus from the *m/z* data,the additional proton was part of the hydroxyl functionality as evidenced by ^{1}H NMR oxymethine peak at δ 3.53. The ^{1}H NMR spectrum corroborated the ^{13}C NMR data findings by displaying two tertiary methyl groups as singlet at δ 0.68 and 0.80, another three secondary methyl signals at δ 0.82 (d, J = 10.25 Hz), 0.84 (d, J = 6.9 Hz) and 0.92 (d, J = 8.2 Hz) and a primary methyl group at δ 0.86 (t, J = 13.7, 6.8 Hz).

Another characteristic of the ^{1}H NMR spectrum was an olefinic proton which resonated as a multiplet at δ 5.32 indicated the presence of a trisubstituted olefinic bond as evidenced by ^{13}C NMR peaks at δ 121.70 and 140.75 and typical of endocyclic double bond between C-5 and C-6 of cholestane moiety (Guyot *et al.*, 1982). Further support for cholestane skeleton with hydroxyl group and endocyclic double bond between C-5 and C-6 came from other prominent peaks in the mass spectrum besides the molecular ion $[M]^+$ 414.4, which appeared at *m/z* 397.4 $[M^+ + H - H_2O]^+$, 381.4 $[M^+ - CH_3 - H_2O]^+$, and 255.2 [M-side chain – H_2O $]^+$ which are consistent with already reported data for β-sitosterol (Karim *et al.*, 2007). The identification of compound **55** as β-sistosterol was further accomplished by comparing it with authentic sample on TLC and comparison of the spectroscopic data (Appendix E) with corresponding data reported in the literature (Karim *et al.*, 2007; Table 4.5).



55

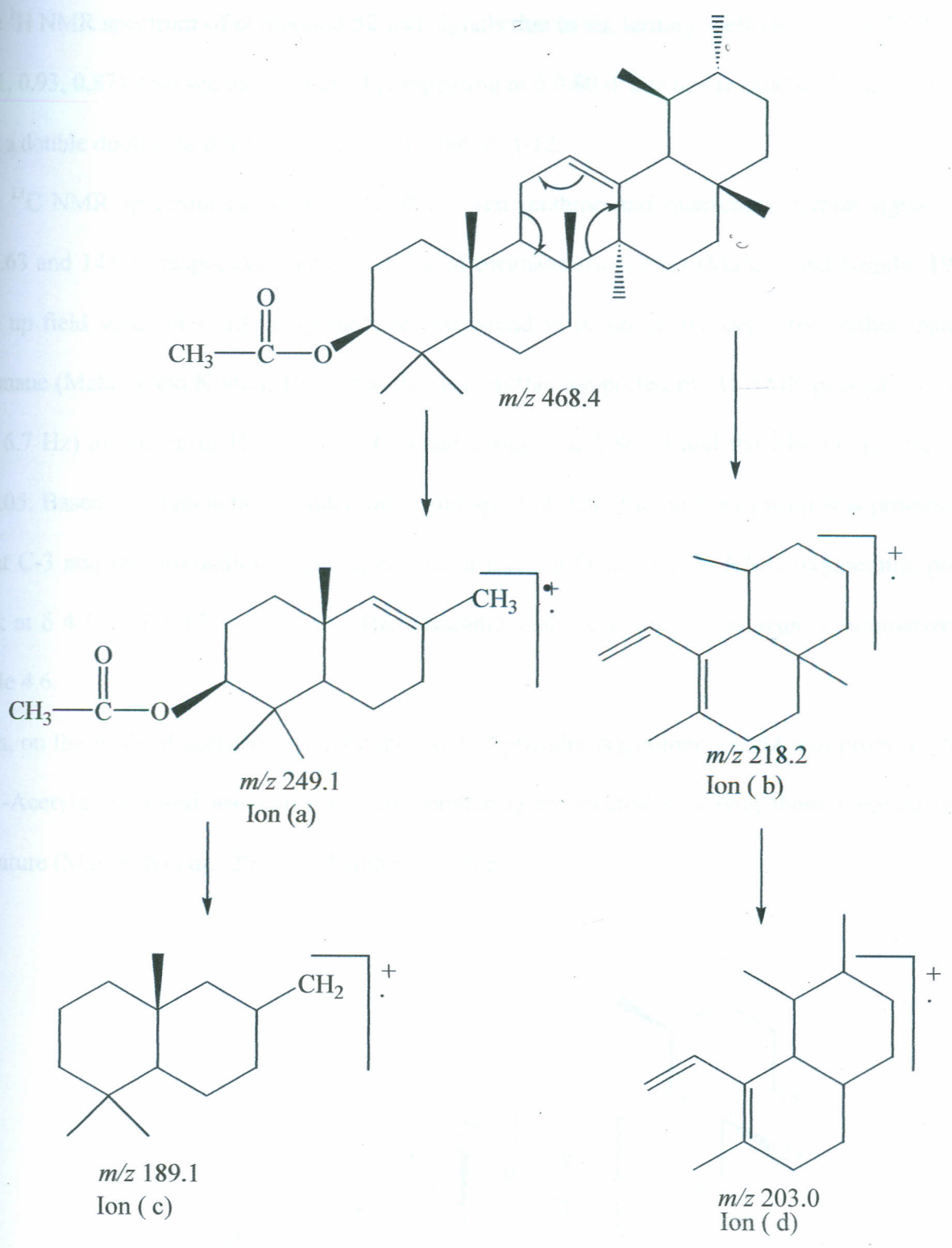
Table: 4.5: ^{1}H and ^{13}C-NMR of compound **55** and β Sitosterol

Data on compound **55**			Data from literature; β-Sitosterol (Karim *et al.*, 2007).	
Position	^{1}H ($CDCl_3$ 500 MHz)	^{13}C-NMR ($CDCl_3$, 125 MHz)	^{1}H ($CDCl_3$ 400 MHz)	^{13}C-NMR ($CDCl_3$ 400 MHz)
1		31.65		32.4
2		36.13		36.1
3	3.53 (m)	71.79	3.51 (m)	71.8
4		42.95		42.3
5		140.75		140.7
6	5.32 (m)	121.70	5.35 (m)	121.7
7		31.65		31.6
8		28.23		28.2
9		42.88		42.3
10		39.77		39.7
11		21.52		20.9
12		31.90		31.8
13		33.94		40.4
14		45.84		45.8
15		23.07		21.2
16		21.07		21.0
17		50.13		50.1
18	0.68 (s)	19.80	0.65 (s)	20.5
19	0.80 (s)	19.38	0.80 (s)	20.2
20		26.09		29.6
21	0.92 (d, J = 8.2 Hz)	18.77	0.90 (d, J = 7.4Hz)	18.7
22		33.96		33.7
23		29.16		29.1
24		42.30		42.3
25		32.19		30.5
26	0.84 (d, J = 6.9 Hz)	19.80	0.84 (d, J = 7.4Hz)	19.8
27	0.82 (d, J = 10.25)	19.38	0.81 (d J,= 7.4Hz)	19.3
28		24.29		24.2
29	0.86 (t, J = 13.7, 6.8 Hz)	11.84	0.87 (t, J = 13.3, 7.2Hz)	11.8

4.2.3 Structural elucidation of triterpenoids from *E .buchanannii* stem bark

4.2.3.1: 3α -Acetylamyrin (52)

Compound **52** was obtained as colourless crystals from n-hexane-EtOAc, (9:1) with M.p of 153-155^{0}C and R_f value of 0.58 (eluent: n-hexane-EtOAc, 4:1). It showed a bluish colour with acidified anisaldehyde and also positive tests with Liebermann-Burchard test as well as the ceric sulphate indicating that it is a triterpene derivative. The EIMS spectrum afforded a molecular ion at *m/z* 468.4 (M^+, $C_{32}H_{52}O_2$). Other characteristic ions at *m/z* 249.1 (ion a) and 218.2 (ion b) (Scheme 4.3) are due to retro-Diels-Alder cleavage commonly found in the spectra of olean-12-ene or urs-12-ene possessing acetyl group in rings A/B part of the molecule (Marizeth *et al.*, 2002). The ready loss of acetic acid from ion (a) and a methyl group at C-17 gave fragments at *m/z* 189.1 and 203.0, respectively, thus restricting the location of double bond at C-12 in ring C.



Scheme 4.3: MS fragmentation pattern of compound **52**

The ^{1}H NMR spectrum of compound **52** had signals due to six tertiary methyls (δ 1.33, 1.32, 1.12, 1.01, 0.93, 0.87), two secondary methyls (appearing at δ 0.80 d, J = 6.3Hz) and 0.83 (d, J = 4.1Hz) and a double doublet at δ 5.17 (J = 13.7, 5.1) due to H-12.

The ^{13}C NMR spectrum of compounds **52** showed methine and quartenary carbon signals at δ 121.63 and 145.22 respectively characteristic of oleanane triterpenes (Mahato and Kundu, 1994). The up field value of C-13, suggested the compound to be an ursane derivative rather than the oleonane (Mahato and Kundu, 1994) and this was further supported by ^{1}H NMR peak at δ 1.34 (d, J = 6.7 Hz) attributed to H = 18. The C-3 had a signal at δ 80.94 and the MeCO appeared at δ 170.05. Based on biogenetic considerations and spectral data, the acetoxy group was proposed to be at C-3 and the orientation being equatorial, a fact confirmed by ^{1}H NMR oxymethine proton peak at δ 4.5 (t, J = 11.35, 5.2 Hz). The assignment of the ^{1}H and ^{13}C-signals is summarized in Table 4.6.

Thus, on the basis of accrued spectroscopic data (Appendix A), compound **52** was proposed to be 3 α -Acetylamyrin and was confirmed by comparing its spectral data with those reported in the literature (Marizeth *et al.,* 2002) and authentic sample.

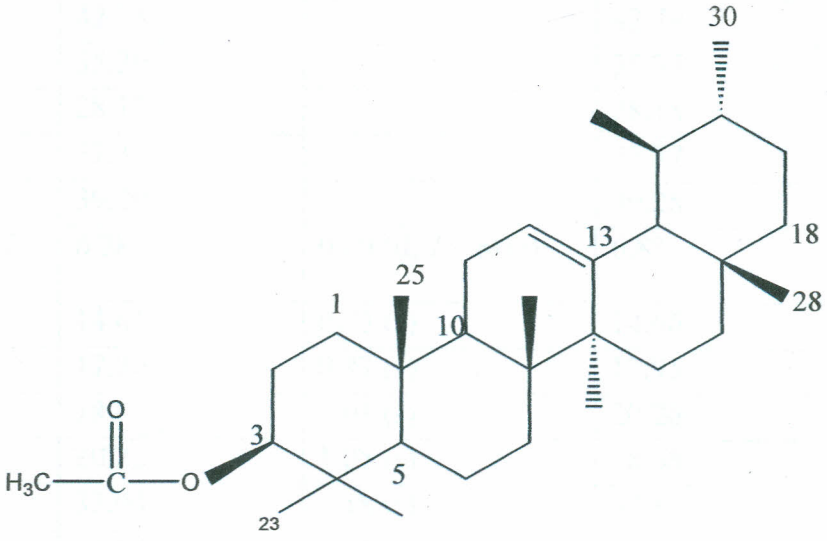


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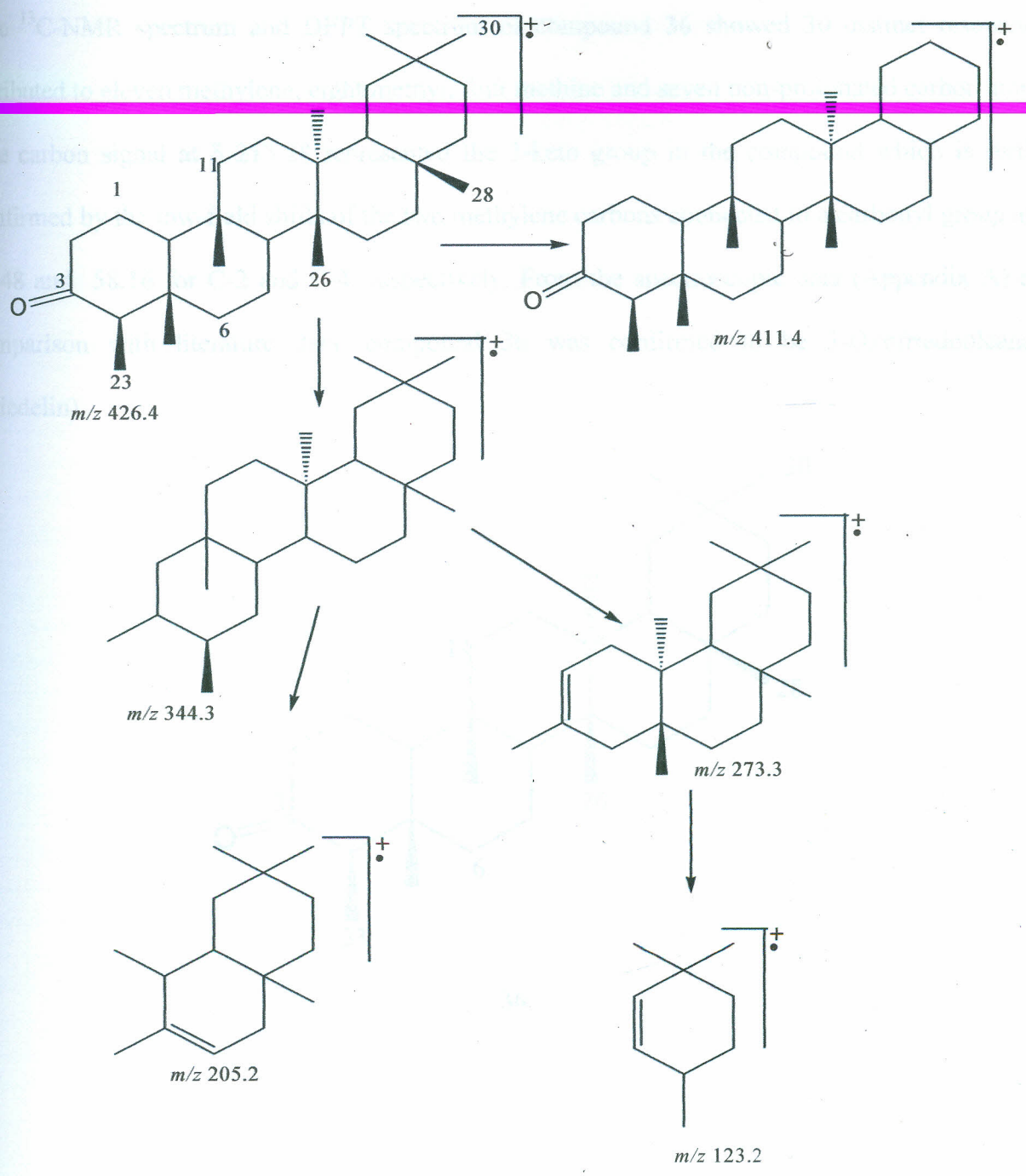
Table **4.7**: ^{1}H and ^{13}C-NMR of 3-Oxofriedooleanane and compound **36**

Data on compound **36**			Data from literature; 3-Oxofriedooleanane (Monkodkaew *et al.*, 2009).	
Position	^{1}H ($CDCl_3$ 500 MHz)	^{13}C-MR ($CDCl_3$, 125MHz)	^{1}H (400 MHz in $CDCl_3$)	^{13}C-NMR ($CDCl_3$, 400 MHz)
1		22.40		22.30
2	2.39 (m)	41.48	2.42 (dd, J = .1,1.9)	41.54
3		213.28		213.32
4	2.23 (m)	58.16	2.25 (m)	58.23
5		42.14		42.16
6		41.23		41.29
7		18.19		18.24
8		53.05		53.10
9		37.38		37.45
10		59.41		59.47
11		35.57		39.63
12		30.46		30.51
13		38.24		39.70
14		39.64		38.30
15		32.71		32.42
16		35.95		36.01
17		29.94		30.03
18		42.73		42.79
19		35.29		35.35
20		28.12		28.18
21		32.36		32.77
22		39.20		39.26
23	0.88 (d, J = 7.5 Hz)	6.28	0.89 (d, J = 8Hz)	6.83
24	0.71 (s)	14.61	0.73 (s)	14.66
25	0.85 (s)	17.90	0.87 (s)	17.95
26	0.98 (s)	18.63	1.01 (s)	20.26
27	1.03 (s)	20.22	1.05 (s)	18.68
28	1.16 (s)	32.04	1.18 (s)	32.10
29	0.94 (s)	31.74	0.95 (s)	35.03
30	0.96 (s)	34.99	1.01 (s)	31.79

4.2.3.2: 3-Oxofriedooleanane (Friedelin) (36)

Compound **36** was isolated as colourless needles from n-hexane-ethyl acetate mixture, M.p 265-266°C and R_f value of 0.68 (hexane-ethyl acetate, 4:1). The IR spectrum showed significant absorption bands at λ_{max} 1712 and 2930 cm^{-1} which indicated the presence of a 6-membered ring ketone and C-H stretching of sp3 hybridization respectively (Monkodkaew *et al.*, 2009). The EIMS of compound **36** gave a parent ion $[M]^+$ peak at *m/z* 426.4 corresponding to the molecular formula of $C_{30}H_{48}O$.

The mass spectrum of the compound showed fragmentation pattern typical of saturated pentacyclic triterpenes, a fact supported by characteristic daughter ions at *m/z* 411.4 $[C_{29}H_{47}O]^+$, 344.3 $[C_{25}H_{44}]^+$, 273.3 $[C_{20}H_{33}]^+$, 205.2 $[C_{15}H_{25}]^+$ and 123.2 $[C_9H_{15}]^+$ attributed to the fragments derived from cleavage of the C13-18 and C16-17 bonds (Siddiqui *et al.*, 1995) as illustrated in Scheme 4.4. The fragment ion at *m/z* 109.1 for $[C_7H_9O]^+$ further confirmed the presence of the carbonyl group in the molecule.



Scheme 4.4: MS fragmentation pattern of compound **36**

The ^{1}H NMR spectrum of compound **36** showed seven tertiary singlet methyl signals at δ 1.16, 1.03, 0.98, 0.96, 0.94, 0.85, 0.71 and one secondary methyl signal at δ 0.88 (d, J = 7.5 Hz, H-23). The ^{1}H NMR spectrum also showed multiplets at δ 2.39 and 2.23 for α-proton ketone at position 2 and 4 respectively (Monkodkaew *et al.*, 2009).

The ^{13}C-NMR spectrum and DEPT spectrum of compound **36** showed 30 distinct resonances attributed to eleven methylene, eight methyl, four methine and seven non-protonated carbon atoms. The carbon signal at δ 213.28 represented the 3-keto group in the compound which is further confirmed by the low-field shifts of the two methylene carbons connected to a carbonyl group at δ 41.48 and 58.16 for C-2 and C-4, respectively. From the spectroscopic data (Appendix A) and comparison with literature data, compound **36** was confirmed to be 3-Oxofriedooleanane (Friedelin).

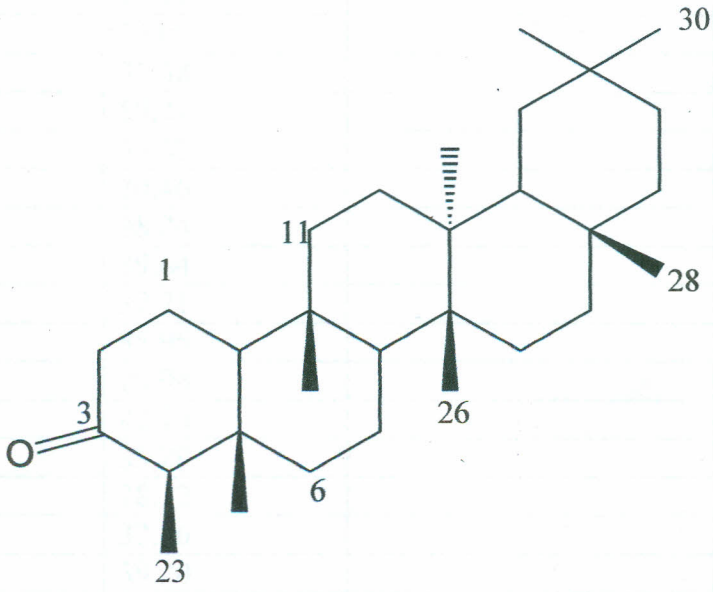


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Table 4.7: ^{1}H and ^{13}C-NMR of 3-Oxofriedooleanane and compound **36**

Data on compound **36**			Data from literature; 3-Oxofriedooleanane (Monkodkaew *et al.*, 2009).	
Position	^{1}H ($CDCl_3$ 500 MHz)	^{13}C-MR ($CDCl_3$, 125MHz)	^{1}H (400 MHz in $CDCl_3$)	^{13}C-NMR ($CDCl_3$, 400 MHz)
1		22.40		22.30
2	2.39 (m)	41.48	2.42 (dd, J = .1,1.9)	41.54
3		213.28		213.32
4	2.23 (m)	58.16	2.25 (m)	58.23
5		42.14		42.16
6		41.23		41.29
7		18.19		18.24
8		53.05		53.10
9		37.38		37.45
10		59.41		59.47
11		35.57		39.63
12		30.46		30.51
13		38.24		39.70
14		39.64		38.30
15		32.71		32.42
16		35.95		36.01
17		29.94		30.03
18		42.73		42.79
19		35.29		35.35
20		28.12		28.18
21		32.36		32.77
22		39.20		39.26
23	0.88 (d, J = 7.5 Hz)	6.28	0.89 (d, J = 8Hz)	6.83
24	0.71 (s)	14.61	0.73 (s)	14.66
25	0.85 (s)	17.90	0.87 (s)	17.95
26	0.98 (s)	18.63	1.01 (s)	20.26
27	1.03 (s)	20.22	1.05 (s)	18.68
28	1.16 (s)	32.04	1.18 (s)	32.10
29	0.94 (s)	31.74	0.95 (s)	35.03
30	0.96 (s)	34.99	1.01 (s)	31.79

4.2.3.3: 3α -Hyroxyfriedooleanane (Hydroxyfriedelin) (56)

Compound **56** was obtained as colourless needles from hexane-ethyl acetate (4:1) with an R_f value of 0.66 in hexane-ethyl acetate (1:1), M.p 278-280^{0}C. The IR (KBr) spectrum of the compound exhibited absorption bands at λ_{max} 3477 and 2932-2869cm^{-1} which were due to O-H stretching and

C-H stretching of sp3 hybridization, respectively, suggesting that the compound had friedelane skeleton (Monkodkaew *et al.*, 2009). The other peaks at 1450 and 1337cm^{-1} were attributed to CH_2 and CH_3 bending respectively (Silverstain *et al.,* 1981). The EIMS spectrum displayed a molecular ion peak at *m/z* 428.4, corresponding to the molecular formula $C_{30}H_{52}O$. The peaks at *m/z* 413.4 and 395.4 were due to the loss of a methyl group from the molecular ion and the loss of water from the daughter ion at *m/z* 413.4 respectively. The ^{1}H NMR spectrum of the compound displayed seven tertiary methyl signals (δ 0.85, 0.95, 0.96, 0.97, 0.98, 1.01, 1.17), and a secondary methyl signal at δ 0.94 d (*J* = 7.2 Hz, H-23). Compound **56** is related to compound **36**, the differences being the absence of ketone signal (δ 213.28, C-3 in **36**) and the presence of OH group (δ 3.72 d (*J* = 2.4Hz, H-3) and δ 72.79, C-3) in **56**. The small coupling constant allowed the assignment of alpha and axial orientation of the OH group (El-Lahlou *et al.*, 1999). The ^{13}C-NMR of compound **56** afforded 30 signals; eight methyl, eleven methlene, five methine and six quartenary carbon atoms. The ^{1}H-^{1}H COSY and HMQC allowed complete assignment of compound **56** as summarized in Table 4.8. Based on the spectroscopic data (Appendix F) as well as comparison with the reported data in the literature, compound **56** was concluded to be 3α-Hydroxyfriedooleanane.

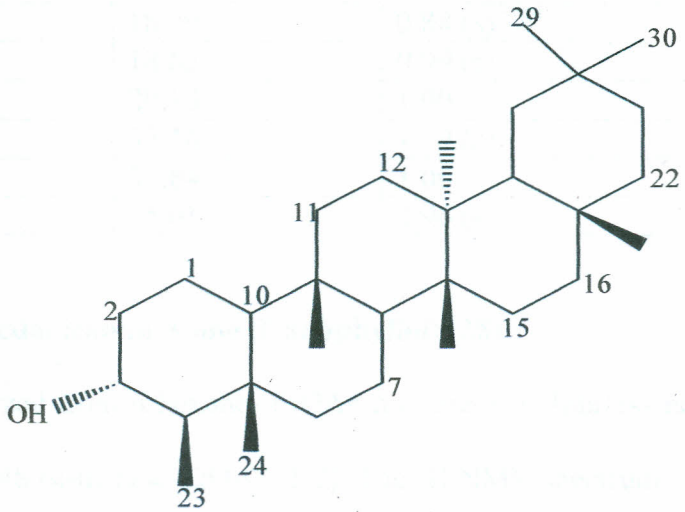

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Table 4.8: ^{1}H and ^{13}C-NMR of compound **56** and 3 α -Hyroxyfriedooleanane

Data on compound **56**			Data from literature; 3α - Hydroxyfriedooleanane (Monkodkaew *et al.*, 2009).	
Position	^{1}H–NMR ($CDCl_3$ 500 MHz)	^{13}C-NMR ($CDCl_3$, 125MHz)	^{1}H -NMR ($CDCl_3$ 300 MHz)	^{13}C-NMR ($CDCl_3$, 300 MHz)
1		16.43		17.57
2		35.46		35.03
3	3.72 (d, J = 4.5 Hz)	72.79	3.73 d (J = 4.5 Hz)	72.77
4		49.39		49.17
5		37.99		37.10
6		41.92		41.72
7		17.65		15.79
8		53.34		53.20
9		37.27		37.12
10		61.58		61.34
11		35.71		35.34
12		32.72		30.64
13		38.51		39.67
14		39.81		38.37
15		32.48		32.33
16		36.25		36.08
17		30.13		30.03
18		43.05		42.81
19		35.39		35.18
20		28.22		28.18
21		33.00		32.81
22		39.36		39.28
23	0.94 (d, J=7.2)	11.59	0.94 (s)	11.63
24	0.96 (s)	15.90	0.98 (s)	16.40
25	0.85 (s)	18.29	0.88 (s)	18.25
26	0.95 (s)	18.62	0.99 (s)	20.13
27	1.01	20.13	1.00	18.66
28	1.17 (s)	32.16	1.19 (s)	32.09
29	0.98	31.84	1.00	31.80
30	0.97 (s)	35.01	0.96 (s)	35.00

4.2.3.4 28-Hydroxyfriedooleanan-3-one (Canophyllol) (38)

Compound **38** crystallized from n-hexane- EtOAc mixture as colourless needles, M.p 280-282°C. It had an R_f value of 0.48 (n-hexane-EtOAc, 1:2). The ^{1}H NMR spectrum of compound **38** showed the signals due to six tertiary methyl groups (δ 0.72, 0.86, 1.03, 1.05, 1.02, 1.03) and one

secondary methyl at δ 0.88 (d, J = 7.1), corroborated by seven carbon signals at δ 6.71, 14.62, 17.84, 18.43, 18.88, 20.74 and 30.56 (Table 4.9) in the ^{13}C-NMR spectrum, which were consistent with the characteristics of triterpene skeleton (Sun *et al.*, 2009). The ^{1}H-NMR spectrum of compound **38** presented a double of doublet at δ 3.28 (J = 12.9, 7.7 Hz) typical of hydrogen bonded to an oxygenated carbon, suggesting the presence of hydroxyl group in the structure. It also presented a signal at δ 2.34, characteristic of hydrogen bonded to a carbon adjacent to a carbonyl group (Duarte *et al.*, 2009). The ^{13}C-NMR spectrum of compound **38** showed thirty distinct signals sorted for seven methyls, nine methylenes, seven methines and six quartenary carbon atoms corresponding to a molecular formula $C_{30}H_{50}O_2$. The ^{13}C-NMR of **38** presented a signal at δ 213.3, attributed to a carbonyl, confirming the presence of a ketone group, and also a signal at δ 74.74, which was attributed to a carbon bonded to a hydroxyl (Mahato and Kundu 1994). The HMBC correlations (Table 4.9) for the signals H-2 (δ 2.37), H-23 (δ 0.95) and H-4 (δ 2.12) with carbonyl carbon at δ 213.3 and for the signals H-28 (δ 3.28, 3.24) with C-16 (δ 29.74) and C-22 (δ 33.1) were consistent with a 28-hydroxyl-3-one type friedolane triterpene (Sun *et al.*, 2009; Silvia, 2006). A comparison of its ^{1}H and ^{13}C-NMR data (Appendix H) with those of the literature (Duarte *et al.*, 2009; Sun *et al.*, 2009; Silvia, 2006) confirmed the compound to be 28-Hydroxyfriedooleanan-3-one.

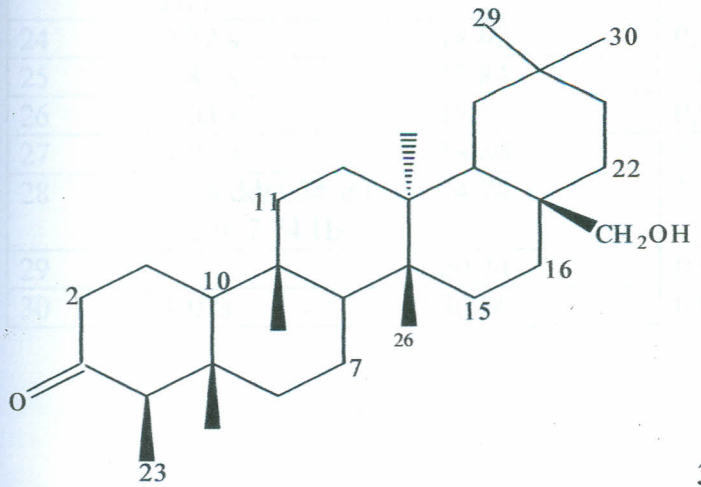

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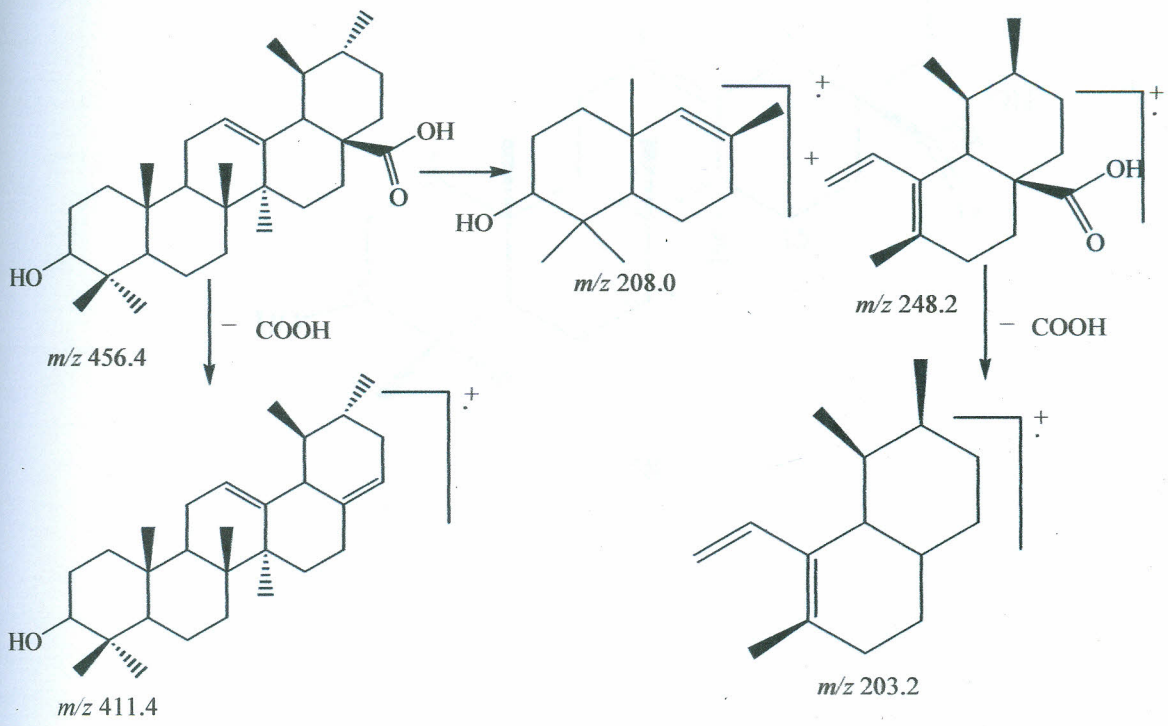
Table 4.9: ^{1}H and ^{13}C-NMR of compound **38** and 28-Hydroxyfriedooleanan-3-one

Data on compound **38**			Data from literature; 28-Hydroxyfriedooleanan 3-one (Duarte *et al.*, 2009)	
Position	^{1}H-NMR ($CDCl_3$ 500 MHz) & HMBC	^{13}C-NMR ($CDCl_3$, 125MHz)	^{1}H-NMR ($CDCl_3$ 400 MHz) & HMBC	^{13}C-NMR ($CDCl_3$, 400MHz)
1	1.5 m/1.73 m	22.26	1.62 m/1.86 m	22.77
2	2.37 m/2.38 m	41.50	2.34 m/2.44 m	41.90
3	-	213.23	-	212.12
4	1.97/2.12	58.21	2.20 m	58.28
5	-	42.13		-
6	1.10 m/1.54	41.26	1.54 m	41.54
7	1.23 m	30.50	1.64 m	32.67
8	1.39 m	53.38	1.51 m	50.82
9	-	37.41	-	37.97
10	1.40 m	59.44	1.56 m	59.77
11	1.30 m	35.62	1.32 m	35.64
12	1.80 m	29.74	1.29 m/1.40 m	30.42
13	-	39.93	-	39.95
14	-	38.21	-	40.25
15	1.41 m	32.71	1.66 m/2.05 m	40.25
16	1.52 m/2.44 m	29.74	1.62 m	36.95
17	-	35.85	-	37.75
18	1.89 m	41.84	1.63 m	46.66
19	1.60 m/2.62 m	35.62	1.35	34.28
20	-	27.77	-	28.62
21	1.77 m/2.82 m	35.63	1.62 m	34.97
22	2.44 m/1.79 m	33.09	1.97/2.05 m	27.76
23	0.88 d (J = 7.1 Hz)	6.71	0.87d (J=6.7Hz)	7.55
24	0.72 s	14.62	0.72s	15.08
25	0.86 s	17.84	0.86 s	19.43
26	1.03 s	18.43	0.98 s	17.70
27	1.05 s	18.88	1.12 s	18.95
28	3.28 dd/3.24 dd (J = 2.9, 7.74 Hz)	74.74	3.31 dd (J = 11.2, 6.5 Hz)	74.63
29		20.74	0.99s	20.02
30	1.02s	30.56	0.95 s	30.13

4.2.3.5: Ursolic acid (58)

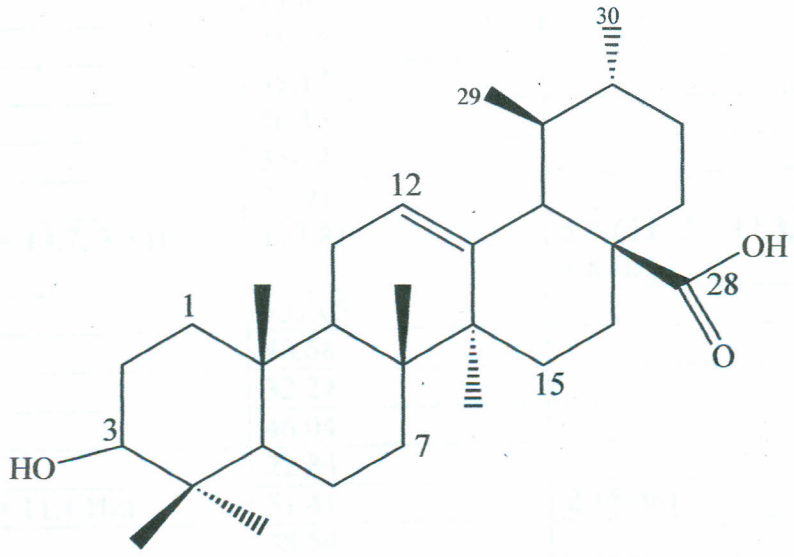
Compound **58** was isolated as colourless crystals using CH_2Cl_2-MeOH mixture. It had M.p of 282-284 °C and R_f of 0.5 (CH_2Cl_2-MeOH, 9:1). The EIMS spectrum of compound **58** showed a molecular ion peak at *m/z* 456.4, corresponding to a molecular formula $C_{30}H_{48}O_3$. Besides the molecular ion peak, EIMS exhibited other diagnostic peaks at *m/z* 411.4 representing the loss of COOH group from the molecular ion [M^+]. A prominent peak at *m/z* 248.2 represented Retro-Diels-Alder fragmentation characteristic of ursane type triterpene with COOH at C-17 (Burnouf-Radosevich *et al.*, 1985). The base peak at *m/z* 203.2 was attributed to the loss of COOH from the fragment at *m/z* 248.2 (Scheme 4.5).



Scheme 4.5: MS fragmentation pattern of compound **58**

The ^{1}H NMR spectrum of compound **58** displayed five tertiary methyls at δ 0.68, 0.87, 0.88, 0.90 and 1.05; two secondary methyls at δ 0.82 (d, J = 5.9 Hz) and δ 0.92 (d, J = 6.8 Hz) which were indicative of ursane skeleton. The presence of a peak at δ 3.40 which appeared as double of doublet (J = 5.2, 9.5 Hz) was attributable to an oxymethine proton. The olefinic proton appeared at

δ 5.14 (dd, J = 13.7; 3.5 Hz). The ^{13}C-NMR spectrum and DEPT experiments of compound **58** revealed the presence of thirty carbon signals indicating seven methyls, nine methylenes, seven methines and six quartenary carbon atoms. The carbon signals at δ 123.81 and 137.05 represented the olefinic carbons. The signals at δ 76.44 (C-3) and 177.99 (C-28) indicated the presence of OH and COOH groups, respectively. The comparison of spectral data (Appendix I) with those in the literature identified compound **58** as ursolic acid earlier reported from *Ocimum tenuiflorum* (Silva *et al.*, 2008).



58

Table 4.10: ^{1}H and ^{13}C-NMR of compound **58** and Ursolic acid

Data on compound **58**			Data from literature: Ursolic acid (Silva *et al.*, 2008).	
Position	^{1}H-NMR ($CDCl_3$ 500 MHz)	^{13}C-NMR ($CDCl_3$, 125MHz)	^{1}H-NMR ($CDCl_3$ 400 MHz)	^{13}C-NMR ($CDCl_3$, 400MHz)
1		38.19		38.22
2		26.64		26.79
3	3.40 (dd, J = 9.5, 5.2 Hz)	76.44	3.3 (m)	76.82
4		38.33		38.35
5		53.91		54.77
6		17.92		17.97
7		31.72		30.77
8		39.17		39.09
9		46.13		47.00
10		35.58		36.51
11		25.91		23.80
12	5.14 (dd, J = 13.7, 3.5 Hz)	123.81	5.3 (dd, J = 12.3, 3.8 Hz)	124.54
13		137.05		138.18
14		40.68		41.62
15		32.24		32.69
16		46.04		46.81
17		22.84		22.82
18	2.12 (d, J = 11.1 Hz)	51.41	2.15 (m)	52.37
19		38.54		38.42
20		38.75		38.48
21		27.09		27.52
22		35.41		36.29
23	0.90 (s)	29.36	0.75 (s)	28.23
24	0.88 (s)	16.87	0.89 (s)	16.90
25	0.87 (s)	15.93	0.87 (s)	16.03
26	0.68 (s)	15.16	0.68 (s)	15.18
27	1.05 (s);	23.31	1.04 (s)	23.24
28		177.99		178.23
29	0.82 (d, J = 5.9 Hz)	17.31	0.81(d, J = 5.4 Hz)	16.97
30	0.92 (d, J = 6.8 Hz),	21.89	0.90 (d, J = 5.8 Hz),	21.10

4.3 Biological activities

The n-hexane, ethyl acetate and methanol extracts as well as the pure isolates from *E. buchananii* stem bark were tested for their potency against bacteria and fungi and the results are shown in Tables 4.11, 4.12, 4.13, 4.14, 4.15 and 4.16.

The n-hexane extract had low potency on tested micro organisms (Table 4.11). The n-hexane antibacterial activities on both *Staphylococcus aureus* and *Neisseria meningitidis* (at 1.50 mg/ml) were comparable to commercial tetracycline used as positive control with zone diameters of 12.20 mm and 14.17mm respectively for the extract and 12.13 mm and 13.17 mm, respectively for the tetracycline. The minimum inhibitory concentration (MIC), defined as the lowest concentration of the test sample in which there is no growth of microorganisms (Table 4.16) correlated with zones of inhibition in which both *S. aureus* and *N. meningitidis* had the best MIC (125 µg/ml). However the n-hexane extract did not exhibit antifungal activity. Dimethyl sulphoxide (DMSO) was used as the negative control, and there was no inhibition recorded.

Table 4.11: Antibacterial and antifungal activities of the n-hexane stem bark extract of *E. buchananii*

Extract/standard	Microorganisms								
	S. aureus	*D .pneumoniae*	*S. albus*	*E. coli*	*V. cholerae*	*S. dysenterae*	*N. meningitidis*	*C. neoformans*	*C. albicans*
Tetracycline	12.13±0.15	14.2±0.2	14.83±0.05	14.17±0.15	10.10±0.10	9.2±0.20	13.17±0.15	NT	NT
Gentamycin	21.13±0.11	17.17±0.15	18.17±0.15	17.17±0.15	15.17±0.15	14.07±0.15	16.83±0.15	NT	NT
Clotrimazole	NT	NT	NT	NT	NT	NT	NT	15.10±0.10	22.17±0.1
0.5mg/ml sample	6.10±0.10	4.03±0.058	3.13±0.15	7.17±0.15	0.00±0.00	0.00±0.00	10.23±0.21	0.00±0.00	0.00±0.00
1.0mg/ml sample	9.07±0.058	7.10±0.15	6.10±0.10	8.23±0.21	2.13±0.20	3.11±0.13	11.13±0.58	0.00±0.00	0.00±0.00
1.50mg/ml sample	12.20±0.20	10.13±0.15	11.30±0.15	9.27±0.25	5.20±0.20	5.17±0.15	14.17±0.15	3.17±0.15	5.10±0.15
CV%	0.76	1.13	0.70	1.02	1.58	1.99±±0.20	0.96	2.30	1.49
LSD $P \leq 0.05$	0.1138	0.2087	0.1308	0.1992	0.1796	0.2203	0.2189	0.1469	0.1418

Values are inhibition zones diameter (mm) given as mean of replicate±SD (standard deviation). NT= Not tested. Tetracycline and clotrimazole 30µg/ disc, gentamycin 10 30µg/ disc

The ethyl acetate extract displayed the best antibacterial activity (Table 4.12) amongst the three extracts. It had better antibacterial activities on all the tested bacteria than the tetracycline used as the positive control with zone diameters ranging from 12.20 mm to 24.13 mm, for the crude extract (at 1.5 mg/ml) and 9.20 mm to 14.73 mm for tetracycline The ethyl acetate extract also showed better antibacterial activities against *S. aureus, Staphylococcus albus* and *N. meningitidis* than gentamycin (a commercial positive control used, which was stronger than tetracycline) with zones of inhibition caused by the extract as 22.20 mm, 19.23 mm and 24.13 mm respectively and that of gentamycin as 21.10 mm, 18.17 mm and 16.43 mm, respectively. The extract showed a much lower antifungal activity compared to antibacterial activity with the maximum zones of inhibition of 12.13 mm against the fungus, *Candida albicans* and 24.13 mm against bacterium, *N. meningitidis*. Just like the n-hexane extract, the MIC of ethyl acetate showed strong correlation with the zones of inhibition. It had a MIC of 15.62 µg /ml against *N. meningitidis* compared with 31.25 µg /ml of gentamycin against the same bacterium. DMSO was used as the negative control, and there was no inhibition recorded.

Table 4.12: Antibacterial and antifungal activities of ethyl acetate stem bark extract of *E. buchananii*

Extract/standard	Microorganisms								
	S. aureus	*D. pneumoniae*	*S. albus*	*E. coli*	*V. cholerae*	*S. dysenterae*	*N. meningitidis*	*C. neoformans*	*C. albicans*
0.5mg/ml Sample	14.40±0.15	10.20±0.20	12.20±0.10	12.20±0.10	9.20±0.10	7.10±0.10	16.00±0.10	5.10±0.10	8.10±0.12
1.0mg/ml Sample	17.20±0.10	14.23±0.12	15.07±0.12	13.20±0.10	11.20±0.10	9.23±0.06	20.17±0.06	7.13±0.06	10.23±0.06
1.5mg/ml Sample	22.20±0.10	16.10±0.00	19.23±0.06	15.20±0.10	12.20±0.10	13.13±0.06	24.13±0.06	13.50±0.10	15.13±0.10
Tetracyline	12.00±0.10	14.03±0.06	14.73±0.06	14.80±0.06	9.97±0.00	9.20±0.10	13.13±0.06	NT	NT
Gentamycin	21.10±0.10	17.20±0.10	18.17±0.06	17.33±0.06	15.17±0.15	14.23±0.06	16.43±0.06	NT	NT
Clotrimazole	NT	NT	NT	NT	NT	NT	NT	15.1±0.10	22.17±0.15
CV %	0.67	0.77	0.60	2.18	0.77	0.88	1.00	0.91	1.01
LSD P ≤0.05	0.19	0.19	0.16	0.55	0.15	0.16	0.31	0.12	0.18

Values are inhibition zones diameter (mm) given as mean of replicate ±SD (standard deviation). NT= Not tested. Tetracycline and clotrimazole 30µg/ disc, gentamycin 10 30µg/ disc

The methanol extract exhibited mild antibacterial activities but very strong antifungal activities on both the fungi tested (at 1.5mg/ml) better than the commercial clotrimazole used as positive control (Table 4.13). The zones of inhibitions due to the extract on *Cryptococcus neoformans and C. albicans* were 16.10 mm and 25.13 mm, respectively while those due to clotrimazole on the same fungi were 15.10mm and 22.17mm, respectively. There was a strong relationship between these zones of inhibition and the MIC values as reflected in Table 4.16. The MIC value for methanol extract against *C. albicans* (31.25 µg/ml) was the same as that of clotrimazole on the same fungus.

Table 4.13: Antibacterial and antifungal activities of the methanol stem bark extract

Extract/standard	Microorganisms								
	S. aureus	*D. pneumoniae*	*S. albus*	*E. coli*	*V. cholerae*	*S. dysenterae*	*N. meningitidis*	*C. neoformans*	*C. albicans*
0.5mg/ml Sample	4.21±0.10	7.15±0.06	9.11±0.10	10.167±0.12	5.10±0.10	3.13±0.10	11.20±0.10	13.13±0.06	20.13±0.06
1.0mg/ml Sample	8.15±0.15	10.17±0.06	10.2±0.10	11.2±0.10	7.19±0.15	5.2±0.10	13.13±0.15	14.20±0.10	23.17±0.12
1.5mg/ml Sample	14.23±0.10	12.2±0.10	13.2±0.10	13.06±0.06	9.12±0.10	10.2±0.10	16.20±0.10	16.10±0.10	25.13±0.15
Tetracyline	12.00±0.10	14.07±0.10	14.73±0.06	14.13±0.06	9.97±0.15	9.2±0.10	13.13±0.06	NT	NT
Gentamycin	21.13±0.06	17.17±0.06	17.97±0.06	17.33±0.10	15.10±0.10	14.07±0.12	18.53±0.06	NT	NT
Clotrimazole	NT	NT	NT	NT	NT	NT	NT	15.10±0.06	22.17±0.15
CV %	1.07	0.70	0.61	0.0347	1.30	1.39	0.63	0.61	0.72
LSD $P \leq 0.05$	0.22	0.21	0.19	0.25	0.29	0.06	0.22	0.17	0.32

NT = Not tested.

Values are inhibition zones diameter (mm) given as mean of replicate±SD (standard deviation). NT= Not tested. Tetracycline and clotrimazole 30µg/ disc, gentamycin 10 30µg/ disc

The n-hexane, ethyl acetate and methanol extracts of the stem bark of E. *buchananii* displayed concentration dependant antifungal and antimicrobial activities (Tables 4.11, 4.12 and 4.13). A comparison of the activities of the three extracts showed that, as the concentration of the extract was increased from 0.5 mg/ml to 1.5 mg/ml, the diameter of zones of inhibition also increased for the three extracts. The ethyl acetate extract inhibited both gram positive and gram negative bacteria significantly compared to methanol and n-hexane extracts (Table 4.14) indicating that most of the active components of the stem bark were extracted by ethyl acetate. The n-hexane showed the least activity against most of the organisms tested, which may be due to poor solubility of active compounds in it. The methanol extract was more potent against the fungi, similar to the results reported earlier by Hamza *et al* (2006) that the methanol extract of the stem bark of *E. buchananii* exhibited strong activity against *C. albicans.* The overall results of these extracts are comparable to those of the reference drugs; tetracycline, gentamyacin and clotrimazole (Table 4.14) thus indicating promising baseline information for the potential uses of the methanol and ethyl acetate extracts of *E. buchananii* against pathogens.

Table 4.14: Comparison of the antibacterial and antifungal activities of the crude extracts of *E.buchananii* stem bark

Extract/standard		Microorganisms								
		S. aureus	*D. pneumoniae*	*S. albus*	*E. coli*	*V. cholerae*	*S. dysenterae*	*N. meningitidis*	*C. neoformans*	*C. albicans*
Sample (1.5mg/ml)	n-hexane	12.13±0.20	10.13±0.15	11.3±0.15	9.27±0.25	5.2±0.15	5.17±0.10	14.17±0.15	3.17±0.10	5.1±0.10
	Ethyl acetate	22.2±0.10	16.1±0.00	19.23±0.06	15.2±0.10	12.2±0.10	13.13±0.06	24.13±0.06	13.5±0.10	15.13±0.10
	methanol	14.23±0.10	12.2±0.10	13.2±0.10	13.07±0.06	9.12±0.10	10.2±0.10	16.2±0.10	16.1±0.10	25.13±0.05
Tetracycline (30µg /disc)		12.13±0.15	14.2±0.20	14.83±0.05	14.17±0.15	10.1±0.10	9.2±0.20	13.17±0.15	0±0.00	0±0.00
Gentamyacin (10 µg /disc)		21.13±0.11	17.17±0.15	18.17±0.15	17.17±0.15	15.17±0.15	14.07±0.10	16.83±0.06	NT	NT
Clotrimazole (30µg /disc)		NT	NT	NT	NT	NT	NT	NT	15.10±0.06	22.17±0.15

NT = Not tested.

Values are inhibition zone diameter (mm) given as mean of replicate ± SD (standard deviation). NT=Not tested. Tetracycline and clotrimazole 30µg/ disc, gentamycin 10µg/ disc

All the nine isolated compounds were screened for both antibacterial and antifungal activities (stigmasterol (**53**) was inactive). Coumarin (**54**), umbelifferone (**57**) and hydroxyfriedelin showed antifungal activities against both *C. neoformans and C. albicans* (Table 4.15). Canophyllol **(38)** 3α -acetylamyrin (**52**) and friedelin (**36**) exhibited antibacterial activities against most of the bacteria tested. Ursolic acid (**58**) demonstrated both antifungal and antibacterial activities. These findings compare with the previous data reported by Mahmud *et al* (1998) that canophyllol **(38)** demonstrated good activity compared with ambicillin and amoxicillin against *S. aureus* and that friedelin exhibited good antifungal activity against *Trichophyton schoenleinii,* a cutaneous fungus ocuring in hair, skin and nails unlike our findings that friedelin (**36**) had antibacterrial activity and no antifungal activity.

Table 4.15 Antibacterial and antifungal activities of the Isolates from *E. buchananii* stem bark extract

	Microorganisms								
Compound	*S. aureus*	*D. pneumoniae*	*S. albus*	*E. coli*	*V. cholerae*	*S. dysenterae*	*N. meningitidis*	*C. neoformans*	*C. albicans*
Canophyllol (**38**)	11.20±0.10	10.2±0.10	12.17±0.15	11.20±0.10	8.17±0.15	5.20±0.10	15.10±0.10	3.13±0.06	3.20±0.10
3β-acetyl amyrin (**52**)	10.10±0.10	8.20±0.10	11.20±0.10	3.20±0.10	3.10±0.10	7.20±0.10	10.27±0.06	0.00±0.00	0.00±0.00
Coumarin(**54**)	4.20±0.10	5.20±0.10	5.19±0.12	0.00±0.00	0.00±0.00	3.20±0.10	9.20±0.10	11.20±0.10	10.3±0.10
Umbelliferone(**57**)	9.20±0.10	5.10±0.10	7.23±0.10	4.20±0.10	3.20±0.10	3.23±0.12	5.20±0.10	10.80±0.10	13.60±0.06
Ursolic acid (**58**)	13.20±0.10	10.23±0.15	12.27±0.15	8.20±0.10	5.20±0.10	3.17±0.15	7.20±0.10	9.20±0.10	12.20±0.10
Hydroxy friedelin (**56**)	5.20±0.10	0.00±0.10	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	9.20±0.10	10.20±0.10	9.20±0.10
Friedelin (**36**)	14.23±0.058	13.20±0.10	10.20±0.10	12.20±0.10	10.30±0.06	9.13±0.15	14.17±0.06	3.20±0.10	7.20±0.10
β Sitosterol (**55**)	5.20±0.10	5.15±0.10	3.20±0.10	0.00±0.00	0.00±0.00	0.00±0.00	6.20±0.10	10.20±0.10	7.20±0.10
Tetracycline	11.93±0.152	14.17±0.06	14.79±0.06	14.06±0.06	10.07±0.06	9.13±0.06	13.07±0.12	NT	NT
Gentamycin	21.07±0.12	17.23±0.12	17.63±0.12	17.13±0.23	15.07±0.12	14.10±0.10	16.47±0.12	NT	NT
Clotrimazole	NT	NT	NT	NT	NT	NT	NT	15.20±0.10	21.90±0.46
CV%	1.06	1.14	1.17	1.356	1.53	1.90	0.72	0.99	0.74
LSD P≤ 0.05	0.186	0.167	0.182	3.203	0.139	0.171	1.27	0.125	0.114

Values are inhibition zone diameters (mm), NT = Not tested, Isolates tested at conc. 5 mg/ml, tetracycline30µg /disc, clotrimazole 30µg /disc, gentamycin 10 µg /disc.

Table 4.16 Minimum Inhibitory Concentration for Crude extracts and isolates

Crude extract/compound		Micro organisms								
		S. aureus	*D. pneumoniae*	*S. albus*	*E. coli*	*V. cholerae*	*S. dysenterae*	*N. meningitidis*	*C. neoformans*	*C. albicans*
Crude extract	n-Hexane	125±0.00	250±0.00	333.3±0.04	333.3±0.04	500±0.00	414.67±0.00	125±0.00	1000±0.00	1000±0.00
	Ethyl acetate	31.25±0.00	62.5±0.00	31.25±0.00	62.5±0.00	125±0.00	104.2±0.00	15.62±0.00	250±0.00	125±0.00
	Methanol	104.08±0.08	104.08±0.00	125±0.00	125±0.00	208±0.00	250±0.00	52.17±0.00	62.5±0.00	31.25±0.00
Isolate	Canophyllol (38)	125±0.00	125±0.00	125±0.00	125±0.00	250±0.00	250±0.00	62.5±0.00	NA	NA
	β-acetyl amyrin (52)	250±0.00	250±0.00	250±0.00	NA	NA	500±0.00	250±0.00	NA	NA
	Coumarin (54)	1000	1000±0.00	1000±0.00	NA	NA	NA	1000±0.00	125±0.00	125±0.00
	Umbelliferone (57)	250±0.00	1000±0.00	250±0.00	NA	NA	NA	1000±0.00	125±0.00	125±0.00
	Ursolic acid (58)	125	250±0.00	125 ±0.00	250±0.00	1000±0.00	NA	500±0.00	250±0.00	125±0.00
	Hydroxy friedelin (56)	1000±0.00	NA	NA	NA	NA	NA	250±0.00	125±0.00	125±0.00
	Friedelin (36)	62.5±0.00	125±0.00	62.5±0.00	125±0.00	250±0.00	250	125±0.00	NA	500±0.00
	β sitosterol (55)	1000±0.00	1000±0.00	NA	NA	NA	NA	500±0.00	125±0.00	500±0.00
Standard	Gentamycin	31.25±0.00	62.5±0.00	62.5±0.00	62.5±0.00	125±0.00	250±0.00	31.25±0.00	NT	NT
	Clotrimazole	NT	NT	NT	NT	NT	NT	NT	62.5±0.00	31.25±0.00

MIC values are in µg /ml, NT = Not tested, NA = Not active.

CHAPTER FIVE

5.0 SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

The stem bark of *E. buchananii* was sequentially extracted using n-hexane, ethyl acetate and methanol. The preliminary activities of the extracts were investigated using Brine shrimps, *Artemia salina.* The compounds were isolated using gravity column and flash chromatography. Both the crude extracts and isolates were tested for *in vitro* antibacterial and antifungal activities using two fungal and seven bacterial strains. The findings drawn in this study are summarized below:

5.1 Summary

1. The three extracts were active against *Artemia salina.* The ethyl acetate extract exhibited the highest toxicity (LC_{50} 2.784 µg /ml) while n-hexane showed mild toxicity (LC_{50} 28.228 µg /ml) to the shrimps.
2. Two coumarins, two sterols and five triterpenoids were isolated and characterized.
    i. The n-hexane extract yielded 3 α -Acetylamyrin (**52**), stigmasterol (**53**) and 3-Oxofriedooleonane (friedelin) (**36**).
    ii. Ethyl acetate extract gave nine compounds; coumarin (**54**), β-Sitosterol (**55**), 3 α -Hydroxyfriedooleonane (Hydoxyfriedelin) (**56**), umbelliferone (**57**), 28-Hydroxyfriedooleanan-3-one (Carnophyllol) (**38**), ursolic acid (**58**) and the three compounds isolated from n-hexane extract.
    iii. Compounds isolated from methanol extract were in negligible amounts hence not characterised.
3. The crude extracts showed varied antibacterial and antifungal activities.

4. The ethyl acetate extract showed strong antibacterial activities especially against *N. meningitidis* (zone of inhibition 24.2 mm and MIC of 15.6 µg /ml) and moderate antifungal activities.
5. Methanol extract displayed strong antifungal activities especially against *C. albicans* (zone of inhibition 25.13 mm and MIC of 31.5 µg /ml).
6. The n-hexane extract showed mild antibacterial and no antifungal activities.
7. The isolates also displayed antibacterial and antifungal activities. Coumarin (**55**) and umbelliferone (**57**) showed antifungal activities while friedelin (**36**) and carnophyllol (**38**) exhibited antibacterial activities. Ursolic acid (**58**) displayed both antibacterial and antifungal activities.
8. The results of this study have expanded our knowledge on the phytochemistry of *Elaeodendron buchananii* since the nine compounds have been reported on this plant for the first time.
9. The findings of this study have justified the ethnomedicinal uses of *Elaeodendron buchananii* in the management of both bacterial and fungal infections.

5.2 Conclusions

1. The n-hexane, ethyl acetate and methanol extracts were active. This indicated that the active compounds from *E. buchananii* stem bark were soluble in the extracts.
2. The crude extracts and some isolates displayed varied antibacterial and antifungal activities.
3. Nine compounds were characterised; two coumarins, two sterols and five triterpenoids.

5.3 Recommendations

1. The extracts of the stem bark of *E. buchananii* were active against the brine shrimps, indicating that it may have some medicinal value hence there is need to conserve the plant.
2. This study has justified the use of *E. buchananii* stem bark in the management of both fungal and bacterial related ailments hence the plant can still be used to manage these diseases.

3. Different classes of compounds were isolated and characterised from *E. buchananii* stem bark, indicating that the plant is a potential source of secondary metabolites hence the need to conserve it.

5.4 Suggestions for further study

1. Antibacterial and antifungal activities of the extracts and the active isolates should be tried *in vivo* to determine their potential application.

2. The methanol extract should be subjected to diverse chromatographic methods such as reverse phase column and preparative high performance liquid chromatography in order to isolate compounds in large quantities for both spectroscopic analysis and biological activities.

3. The SAR studies on active isolated compounds should be done to determine the pharmacophores and to evaluate if their activity can be enhanced.

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