ISOLATION AND CHARACTERIZATION OF ACTIVE ANTIHYPERGLYCEMIC COMPONENT OF *URTICA DIOICA* LEAVES

BY

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DECLARATION

DECLARATION BY THE CANDIDATE

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DEDICATION

I dedicate this project to my father, mother and the entire family, friends, the common man and junior researchers in the field of Organic Chemistry and Biochemistry.

ABSTRACT

Urtica dioica (*U.dioica*) commonly known as the stinging nettle is a herbaceous perennial plant belonging to the plant family *Urticaceae.* The aqueous extract of the plant have been shown to have antihyperglycemic activity though the bioactive compounds of the extract have not been previously isolated. This study isolated and characterized four compounds which include 1',4',7-trimethylcyclohex-2-ylnaphthalene given the trivial name urticol and three terpenoids from the leaves extract of the plant. It further established the general mechanism of action of the extract in treatment of diabetes. The air dried plant material (1.5 kg) was macerated in DCM/MeOH solvent system (1:1 v/v) for 72h to yield 40 g of the crude extract. The extract was then separated and purified using silica gel column chromatography under n-hexane/DCM/EtOAc/MeOH gradient followed by sephadex LH-20 column chromatography. Structure elucidation of the compounds was done by employing spectroscopic techniques such as IR, mass spectrometry, 1D NMR and 2D NMR experiments. Urticol and the terpenoids were assayed for their potential stimulation of hepatic glucose uptake using freshly isolated primary rat hepatocytes incubated in a humidified 5 % CO_2 incubator and the glucose concentrations for 15, 30, 45 and 60 minutes intervals determined spectrophotometrically using 3,5-dinitrosalicyclic acid colorimetric assay method. Urticol like pioglitazone significantly upregulated the uptake of glucose by 28.57 % compared to the untreated control in rat hepatocytes cultured in DMEM supplemented with 100 μM of urticol and 100 μM of pioglitazone ($p<0.05$). The other compounds were found to be non-bioactive at both 50 μM and 100 μM. Although urticol had comparable activity to pioglitazone, it increased the uptake of glucose by 11.45 % relative to pioglitazone. This showed that urticol is better than pioglitazone in stimulation of glucose uptake. Therefore, the findings show urticol as a potent antihyperglycemic agent. This work may provide a scientific proof of the folklore antidiabetic activity of *U.dioica*. Previous studies found out that the crude extract of *U.dioica* leaves inhibited α–glucosidase enzyme and activated PPAR-γ in the liver. α–glucosidase is an enzyme which is responsible for endogenous production of glucose in the liver while PPAR-γ is responsible for improving insulin sensitivity. Increased glucose uptake in the liver enhances hepatic insulin sensitivity which leads to a decrease in endogenous glucose production. Urticol could possibly inhibit α–glucosidase but activate PPAR-γ.

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LIST OF ABBREVIATIONS

ACC	Acetyl CoA Carboxylase
ADA	American diabetes association
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BB	Bicarbonate buffer
CAD	Coronary artery disease
ChREBP	Carbohydrate response element binding protein
CoA	Coenzyme A
CYP4-A	Cytochrome P450 subfamily A
DCM	Dichloromethane
DMEM	Dulbecco's Minimum Essential Medium
DNSA	3, 5-dinitrosalicyclic acid
EDTA	Ethylene diamine tetra acetic acid
EIMS	Electron impact mass spectrometry

EtOAc	Ethyl acetate
FAS	Fatty acid synthase
FBS	Fetal bovine serum
FTIR	Fourier Transform Infra Red
GC	Gas chromatography
GDM	Gestational Diabetes Mellitus
GLUT4	Glucose transporter isotype 4
HDL-C	High density lipoprotein cholesterol
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
HMBC	Heteronuclear multiple bond correlation
HSQC	Heteronuclear single quantum coherence
IDDM	Insulin Dependent Diabetes Mellitus
IDF	International diabetes federation
IR	Infrared
LD_{50}	Lethal dose for 50 % of the tested population in the tested time
LXR	Liver X Receptors
MEM	Minimum Essential Media

MeOH	Methanol
Mp	Melting point
MS	Mass spectrometry
NF-kB	Nuclear factor kappa-B
NIDDK	National Institute of Diabetes and Digestive and Kidney Diseases
NIDDM	Non-Insulin Dependent Diabetes Mellitus
NIH	National Institute of Health
NMR	Nuclear Magnetic Resonance
NO	Nitrous oxide
NR1C1	Nuclear receptor subfamily 1, group C, member 1
PAD	Peripheral artery disease
PBS	Phosphate buffered saline
PCD	Programmed cell death
PPAR	Peroxisome proliferator activated receptors
PPRE	Peroxisome proliferator response element
PPs	Peroxisome proliferators
RXR	Retinoid X Receptor

SCD	Sterol CoA Desaturase
SDS	Sodium dodecyl sulfate
SPSS	Statistical package for social sciences
SREBP	Sterol regulatory element binding protein
STZ	Streptozotocin
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
TG	Triglyceride
TLC	Thin layer chromatography
TZD	Thiazolidinedione
U.dioica	*Urtica dioica*
UV	Ultra violet
W.H.O	World Health Organization

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I thank the almighty God for His unending love, good health and wisdom he gave me during my Masters program. It has been by his grace that I've come this far. Thanks to my supervisors: Dr. Clare Muhanji and Dr. Geoffrey Maiyoh for their guidance, comments, support in designing and executing the experiments in this project, advice, criticisms in writing my thesis and the strong mentorship that they have offered me in research. They have been a force behind my progress in research. Also, this project could have taken me long if not for the funding from the National Council for Science and Technology (NCST/5/003/3rd CALL M.Sc/113), much thanks to them for appreciating this work as one of the innovations worth funding. I also thank Mr. Maritim P, Mr. Korir R, Mr. Muchiri J and Mr. Mutuku M.M, all from Kemri Nairobi for their assistance especially in the execution of the bioassay. I acknowledge University of Eldoret Chemistry and Biochemistry technical staffs for their cooperation and assistance when carrying out the experimental work. On the same note, thanks to Mr. Lagat and other Biotechnology technical staffs of University of Eldoret for the technical support they offered me while conducting bioassays. Thanks to Mr. Apollo who played a role in getting me the rats for the assays. I acknowledge my father (Mr. Chepkwony) for his financial and moral support. He played a role in nurturing my potential by providing me an opportunity, through paying fees in part, to pursue my dreams. Besides, I thank my entire family for the moral support they gave me while undertaking my Masters program. Last but not least, I thank all my colleagues and friends who directly or indirectly encouraged

and supported me morally. They have been a force in part behind my progress in both course work and research.

CHAPTER ONE

INTRODUCTION

1.1 Natural products in drug discovery

Herbal medicine sometimes referred to as herbalism or botanical medicine involves the use of plants extract for their therapeutic or medicinal value, and has been used by all cultures throughout history. Plants produce a variety of chemical substances that act upon the body. Herbalists use the leaves, flowers, stem, berries, and roots of plants to prevent, relieve and treat various ailments. In 2001, 122 compounds used in mainstream medicine which were derived from "ethnomedical" plant sources were identified; 80% of these compounds were used in the same or related manner as the traditional ethnomedical use (Farnsworth, 1998; Fabricant and Farnsworth, 2001). Many drugs listed as conventional medications were originally derived from plants. For example, Salicylic acid, a precursor of aspirin, was originally derived from “white willow bark” (Mahdi *et al.*, 2006).

In Africa, the use of plant extracts for medicines is a very common practice from ancient time, and it is considered as a much safer and less expensive therapeutic strategy for treatment of various diseases. Pravin (2006) reported that about 70% of the human population is dependent (wholly or partially) on plant-based medicines and the World Health Organization estimates that in some Asian and African countries, 80% of the population presently uses herbal medicine for some aspect of primary health care (WHO, 2009). The potential of medicinal plants research results in health care is no longer in doubt, having gained recognition in several parts of the

world. Collection of information and documentation of traditional knowledge in herbal medicine plays an important role in scientific research on drug development. A mounting body of research is hoisting the credibility of traditional knowledge in meeting the challenges of primary healthcare and management of natural resource globally (Ragupathy *et al.*, 2008).

The use of plant medicines for treatment of diabetes has been reported and there is plethora of literature that is available for antidiabetic plants (Modak *et al*., 2007; Carai *et al*., 2009; Mishra *et al*., 2010; Konkon *et al*., 2010). Many studies have identified a number of plants whose extracts have shown hypoglycemic effects in the management of diabetes mellitus. The antihyperglycemic effects of these plants extracts may delay the development of diabetic complications and correct the metabolic abnormalities (Bnouham *et al*., 2006). For instance, *Aloe vera* juice stimulates the release of insulin from the *beta*-cells in human (Bolkent *et al.*, 2005), *Acacia catechu* wood extract enhances the regeneration of pancreatic *beta* cells in rabbits (Singh *et al.*, 2009) and *Momordica charantia* fruit extract enhances insulin secretion by the islets of Langerhans. A significant proportion of the plants with antidiabetic activity have been observed to possess potent antioxidant activity, which may contribute to anti-diabetic property in streptozotocin/alloxan, induced animal model (Grover *et al*., 2002).

1.2 Botanical background of the genus *Urtica*

The genus name “Urtica” from the plant family *Urticaceae* is derived from the Latin word “urere”, which means to burn, because of its urticant hairs (Marrassini *et al.*, 2011). There are 80 species of the genus *Urtica* which include *Urtica urens*(*U.urens*), *Urtica pilulifera*, *Urtica circularis* (*U.circularis*), *Urtica dioica* (*U.dioica*) and *Urtica massaica*. *U.dioica* and *Urtica massaica* are native to Africa, for example Kenya, Rwanda, Uganda, Congo and Tanzania. Much of the historical evidence of use of the nettles in medicine, folk remedies and fiber production relate to one species – *U.dioica*, but a fair amount also refers to the use of *U.urens*. *U.dioica* is a herbaceous perennial plant native to Europe, Asia, North America and Africa (Golalipour *et al.*, 2009). In Kenya, the plant is native to Rift Valley, Central and Western parts of Kenya. *U.dioica* has many hollow stinging hairs called trichomes on its leaves and stems (Figure 1.1), which act like hypodermic needles that inject histamine and other chemicals that produce the stinging sensation when contacted by humans and other animals. *U.dioica* has creeping roots, sharp pointed leaves and green small, inconspicuous flowers. They have opposite, stalked and cordate leaves which are heart-shaped, serrated and tapered at the ends. The leaves are also covered on both sides with stinging hairs. The flowers of the plants are unisexual and dioecious. Besides, the flowers are also arranged in drooping panicles, growing in groups from the upper leaf axils. *U.dioica* plant can grow about anywhere but mostly in moist nitrogen-rich soils.

Figure 1.1: *Urtica dioica* leaves and the hairy stem of the plant. (Source: Author, 2012)

1.3 Ethnomedical applications of *U.dioica*

Urtica dioica is widely used in folk medicine for improving certain diseases such as diabetes (Kavalali *et al.*, 2003; Roman *et al*., 1992; Farzami *et al*., 2003), rheumatoid arthritis, hypertension and allergic rhinitis (Mittman, 1990; Sezik *et al.*, 1997; Riehemann *et al.*, 1999). The plant is also used against liver insufficiency and to treat stomachache in Turkish folk medicine (Ye¸silada *et al.*, 2001). *U.dioica* in folk medicine of most cultures has emerged the best drug candidate as antidiabetic (Gulcin *et al*., 2004). For example, in folk medicine of the Kalenjin community in Kenya, the leaves of the herb are used as antidiabetic. Traditional eating habits of the community included *U.dioica* leaves in their diet, and through this kind of self-

oriented health care, diabetes has not been common in the community until recently when the shift in eating habits to more refined sugary diet did the prevalence of diabetes increase. A further study conducted by Gulcin *et al.,* (2004) revealed vast medicinal value of the plant some of which include analgesic, anti-ulcer, antimicrobial and antioxidant activities of the nettle. The water extract of the *U.dioica* was observed to have a powerful antioxidant activity against various oxidative systems *in vitro*. The various antioxidant mechanisms of the extract may be attributed to strong hydrogen donating ability, metal chelating ability, and their effectiveness as scavengers of hydrogen peroxide, superoxide, and free radicals. In the study, phenolic compounds were suggested to be responsible for the antioxidant activity of the water extract of the nettle. Therefore, the water extract of the nettle can be used as an accessible source of natural antioxidants and as a possible food supplement or in pharmaceutical industry. Briefly, free radicals have been demonstrated to be a contributing factor in the tissue injury and modulation of pain in diabetics (Khalil *et al*., 1999; Van der Laan *et al*., 1997).

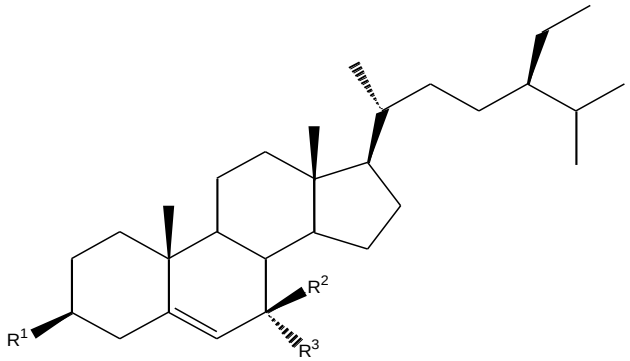
According to Kavalali *et al*., (2003) and Petleveski *et al.,* (2003) the nettle has a lot of medicinal value and anti-diabetic properties. Onal *et al.,* (2005) investigated α-glucosidase inhibition activity of *U.dioica* extract to identify a prophylactic effect for diabetes. They found out that the plant's extract and some common antidiabetic drugs had inhibitory effects against the enzyme source in the liver and the small intestine. Daher's *et al.,* (2006) reported that orally administered *U.dioica* extract improve the blood lipid profile. Hyperglycemia is responsible for the development of

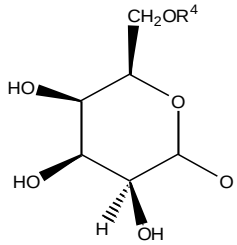
oxidative stress via glucose auto-oxidation and protein glycotion which is characterized by increased lipid peroxide production (malondialdehyde) and/or decreased antioxidative defence (Petlevski *et al*., 2003; Ozturk *et al.,* 1996; Singh *et al*., 2000; Rajasekaran *et al*., 2005; Rauscher *et al.,* 2000; Levy *et al*., 1999). In addition, Golalipour *et al.,* (2009) found out that *U.dioica* plays a major role in the prevention and therapy of the liver disease. Further work on antidiabetic activity of *U.dioica* by Farzami *et al.,* (2003) revealed that there is induction of insulin secretion by a component of *U.dioica* extract in perfuses islets of Langerhans and its *in vivo* effects in STZ induced diabetic rats. Bnouham *et al.,* (2003) observed that aqueous extract of *U.dioica* showed antihyperglycemic activity on STZ induced rats. Traditional preparations from plant sources are widely used in the world to treat this disease despite the availability of many synthetic drugs (Bennet and Brown, 2000; Ryan *et al.*, 2001).

1.4 Secondary metabolites isolated from the genus *Urtica*

Chaurasia and Witchtl (1987) reported the isolation of sterols and steryl glycosides: sitosterol (**1**), sitosterol-3-*O*-*β*-D-glucoside (**2**), (6'-*O*-palmitoyl)-sitosterol-3-*O*-*β*-D-glucoside (**3**), (24R)-ethyl-5*α*–cholestan-3*β*,6*α*-diol (**4**), 7β-hydroxysitosterol (**5**), 7*α*-hydroxysitosterol (**6**), 7*β*-hydroxysitosterol-3-*O*-*β*-D-glucoside (**7**) and 7α-hydroxysitosterol-3-*O*-*β*-D-glucoside (**8**) (Figure 1.2) from the roots and flowers of

U.dioica, however they didn't report any biological evaluation of these compounds.





2a: R^4 = H
2b: R^4 = $C_{16}H_{31}O$

	R^1	R^2	R^3
1	OH	H	H
2	2a	H	H
3	2b	H	H
5	OH	OH	H
6	OH	H	OH
7	2a	OH	H
8	2a	H	OH

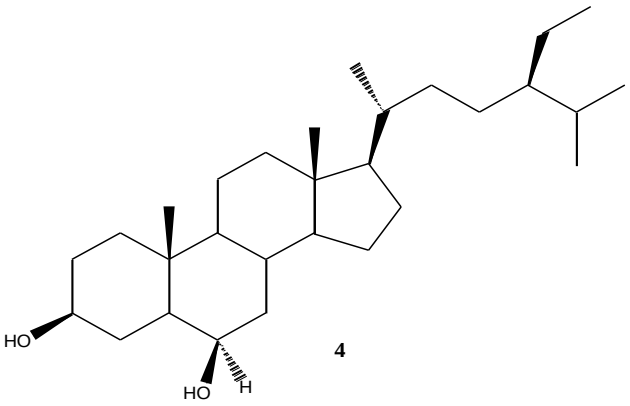


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Figure 1.2: Isolated sterols and steryl glycoside from the roots and flowers of *U.dioica.* (Chaurasia and Witchtl, 1987)

Marrasinni *et al.,* (2011) isolated a flavonoid glycoside characterized as vicenin-2 (**9**) from an ethanolic extract of the aerial parts of *Urtica circularis.* Vicenin-2 was found to possess significant anti-inflammatory activity. In cultured murine

macrophages, vicenin-2 modified lipopolysaccharide-induced total nitrite and tumour necrosis factor-α production, in addition to the lipopolysaccharide-induced translocation of the nuclear factor kappa B (NF-κB).

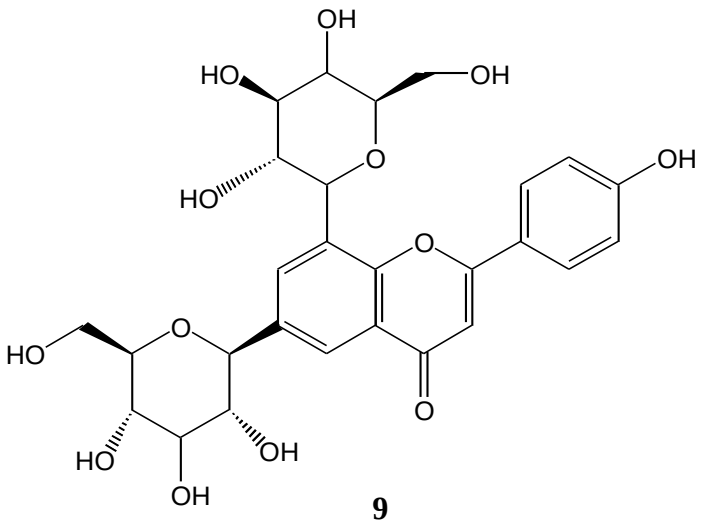

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Figure 1.3: Vicenin-2, isolated from *U.circularis.* (Marrasinni *et al.,* 2011)

1.5 Problem statement

The treatment of diabetes with synthetic drugs is costly, inaccessible and is associated with major complications, for example rosiglitazone can increase the risk of heart failure (Martin, 2006). Although there are some other advancement in therapeutic techniques recently developed which include islets transplantation, there are a lot of problems associated with this technique. These include severe shortage of islets available for transplant and the need for patients/islets recipients to take immunosuppressive drugs, often with undesired side effects, to stop the immune system from rejecting the transplanted islets (NIH, 2010 and NIDDK, 2010).

Therefore, plant extracts with antihyperglycemic properties are considered to be the alternative sources for finding new leads for antihyperglycemic agents (Das *et al.*, 2009). Das *et al.,* (2011) found out that the nettle has antihyperglycemic activity. However, its mechanism of action has not been known. Therefore, understanding the structure of the active antihyperglycemic components of *U.dioica* leaves extract may assist in gaining insight into the probable mechanism of action of the extract which in turn will lead to development of more potent drugs and better methods of diabetes therapy.

1.6 Main objective

To isolate and characterize bioactive compounds from the leaves of *U.dioica*

1.7 Specific objectives

1. To use freshly isolated primary rat hepatocytes to determine whether the isolated compounds may possess antihyperglycemic activity.
2. To establish the probable mechanism of action of the active compound(s) by comparison with those of known agents like pioglitazone.
3. To compare the compound's potency with that of known agent pioglitazone

CHAPTER TWO

LITERATURE REVIEW

2.1 Diabetes Mellitus

Diabetes mellitus is a disease characterized by high blood sugar (glucose) levels, that results from defects in insulin secretion or action, or both (Ruchi, 2011). Diabetes is a clinical syndrome characterized by hyperglycemia caused by a relative or absolute deficiency of insulin at the cellular level. It is the most common endocrine disorder affecting mankind all over the world, prevalence of which is increasing every day (Das *et al*., 2009, Tong and Cockrum, 2003). Diabetes has been divided into three different types: The first type is Type 1 diabetes mellitus or insulin dependent diabetes mellitus (IDDM) or juvenile onset diabetes. The second one is Type 2 diabetes mellitus or noninsulin dependent diabetes mellitus (NIDDM) or adult - onset diabetes is found in individuals who are insulin-resistant and who usually have relative insulin deficiency. Finally, Gestational diabetes mellitus (GDM), the third type, is defined as any degree of glucose intolerance with onset or first recognition during pregnancy. Recently, diabetologists have added a fourth category, tropical diabetes, suggested first in 1907, but made popular by Hugh Jones (J-type diabetes) during the mid-1950s following his study on 13 Jamaican patients, (Azevedo and Alla, 2008). There are many types of diabetes but Type 1 diabetes mellitus (T1DM) and Type 2 diabetes Mellitus (T2DM) account for around 98% of all cases (WHO, 2009).

2.1.1 Type 1 diabetes mellitus

T1DM is an autoimmune disease in which T-lymphocytes (white blood cells) attack insulin-producing pancreatic *beta* cells, and it accounts for 5-10 percent of all diabetes cases (Herold, 2002). However, the exact cause of T1DM is yet to be identified but risk factors may be genetic or environmental. There are three genetic causes of diabetes. The first one is genetic defect of the *beta* cells which causes monogenic forms of diabetes. Monogenic diabetes results from mutation or changes in a single gene. It is either inherited or the gene mutation develops spontaneously. Monogenic diabetes is diagnosed by genetic testing (NIDDK, 2010). The second cause is as a result of genetic defect in insulin action which leads to changes in insulin receptor. This may cause mild hyperglycemia or severe diabetes with acanthosis as one of its symptoms. The last one is the diseases of the pancreas or injuries of the pancreas from trauma or disease. These include: pancreatitis, infection and cancer of the pancreas, cystic fibrosis and homochromatosis (NIDDK). In addition, no method of preventing T1DM has been identified. The prevalence of T1DM world-wide is increasing but not nearly at the rate that T2DM is (NIH, 2010).

2.1.2 Type 2 diabetes mellitus

T2DM makes up 90% of all diabetes cases worldwide (WHO, 2009). T2DM refers to an impaired sensitivity to glucose in the cells of the body. At first, the pancreas increases insulin production to compensate for the decreased effectiveness (leading to hyperinsulinemia and hence insulin resistance) but over time loses its ability to produce enough insulin (ADA, 2006). The result is consistently high blood sugar.

T2DM results from an interaction between a genetic predisposition and behavioral and/or environmental risk factors, such as obesity. It is associated with old age, physical inactivity, and race/ethnicity (Tuomilehto, 2001). T2DM is usually diagnosed in overweight and obese adults, but diagnosis of adolescents is increasing around the world as well. Furthermore, overweight and obese are some of the lifestyle causes of hyperlipidemia that leads to insulin resistance which in turn leads to Type 2 diabetes mellitus (NIH, 2010).

2.2 Insulin resistance, hyperlipidemia and T2DM

Diabetes mellitus is associated with several structural and functional liver abnormalities that affect glycogen and lipid metabolism (Golalipour et al., 2009; Sanchez *et al*., 2000; Koyuturk *et al*., 2005; Bolkent *et al.*, 2004). Poor lipid metabolism leads to hyperlipidemia. Hyperlipidemia means high lipid levels and includes several conditions, but usually means high cholesterol or high triglyceride (TG) levels. Hyperlipidemia is caused in part by lifestyle changes like obesity, overweight, smoking, lack of exercise, and treatable medical conditions such as diabetes, kidney diseases, underactive thyroid glands and pregnancy. The other hyperlipidemia is inherited especially if you have a normal body weight. Hyperlipidemia leads to insulin resistance which in turn leads to T2DM (NIH, 2010).

Insulin resistance in fat cells results in hydrolysis of stored TG which elevates free fatty acid in the blood plasma. Moreover, insulin resistance in the muscles reduces glucose uptake whereas insulin resistance in the liver reduces glucose storage, with

both effects serving to elevate blood glucose. Insulin-mediated glucose disposal varies widely in apparently healthy human beings, and the more insulin resistance an individual is the more insulin they must secrete in order to prevent development of T2DM. However, the combination of insulin resistance and the compensatory hyperinsulinemia increases the likelihood that the individual will be hypertensive, and have dyslipidemia characterized by high blood TG and low high density lipoprotein cholesterol (HDL-C) concentration (NIH, 2010). The most common type of insulin resistance is associated with a disease state known as metabolic syndrome. Metabolic syndrome is a group of conditions which include high blood sugar, high blood pressure, high TG and low HDL-C among others that subject individuals at the risk of heart disease and diabetes. It is caused by insulin resistance and hence also known as insulin resistance syndrome (NIH, 2010).

Insulin resistance can progress to full T2DM when hyperglycemia develops after a meal due to pancreatic β–cells being unable to produce adequate insulin to maintain normal blood sugar levels (euglycemia). The inability of β–cells to produce more insulin in a condition of hyperglycemia is what characterizes the transition from insulin resistance to T2DM (NIDDK, 2010).

2.3 Hepatic steatosis, lipotoxicity and lipoaptosis

The TG content of hepatocytes is regulated by integrated activities of cellular molecules that facilitate hepatic TG uptake, fatty acid synthesis and esterification on one hand (“input”) and hepatic fatty acid oxidation on the other hand (“output”).

Steatosis occurs when “input” exceeds the capacity for “output”. The accumulation of TG in insulin-sensitive tissues such as the liver and pancreas has been associated with insulin resistance and T2DM (McGarry and Dobbins, 1998; Unger, 2002). The term used to describe this phenomenon is Lipotoxicity. Lipotoxicity refers to dysfunction of non-adipose tissues such as pancreatic β–cells, the myocardium, the liver and the skeletal muscle to bring about fat accumulation (Unger, 2002). It is thought that lipotoxicity or accumulation of TG in insulin-sensitive tissues impairs insulin action in the respective organs causing insulin resistance (McGarry and Dobbins, 1998; Unger, 2002).

When consumption of energy far exceeds the combustion of calories, there is increased deposition of long chained fatty acids, principally in the form of TG in adipose and other tissues leading to obesity. All the three members of peroxisome proliferators-activated receptors (PPAR), that is PPAR-α,–β/δ and-γ, subfamily of nuclear receptors have been shown to participate in energy metabolism in that PPAR-α and PPAR-β/δ function mostly as catabolic regulators of energy expenditure, while PPAR-γ regulates anabolic metabolism in that it plays a role in energy storage (Evans *et al*., 2004). The PPAR-α or NR1C1 is a nuclear hormone receptor activated by a structurally diverse array of synthetic chemicals known as peroxisome proliferators (PPs). PPs is a group of structurally diverse chemicals that lower serum lipids, and induce massive proliferation of peroxisomes in liver cells, with associated coordinated transcriptional activation of peroxisomal fatty acid β–oxidation system genes. PPAR-α serves as a xenobiotic and lipid sensor to

regulate energy combustion, hepatic steatosis, lipoprotein synthesis, inflammation and liver cancer (Reddy *et al*., 2010).

The liver is a central player in the whole body energy homeostasis by its ability to orchestrate fatty acid and glucose metabolism. Hepatic lipid metabolism principally involves three aspects: lipogenesis, oxidation of fatty acids and secretion of lipids. Lipogenesis consists of *de novo* fatty acid synthesis and subsequent conversion of these fatty acids into TG. In the liver, lipogenesis is regulated by transcription factors, sterol regulatory-element binding protein-1c (SREBP-1c), Carbohydrate response-binding protein (ChREBP) and PPAR-γ (Reddy *et al*., 2010). SREBP-1c regulates the expression of constellations of glycolytic and lipogenic genes, including stearoyl CoA desaturase (scd-1) and fatty acid synthase (FAS) (Horton *et al*., 2002, Rao and Reddy, 2004). Activation of lipogenic transcriptional factors, SREBP-1c, FAS and Acetyl CoA Carboxylase (ACC) appears to be a major contributing factor underlying the increase in lipogenesis (Zhou *et al*., 1998).

Emerging evidence suggests that PPAR-α also influences lipogenesis by transcription of scd-1 and other lipogenic genes by regulating the primary transcription factors SREBP-1c and Liver X receptor-α (LXR-α) (Horton *et al*., 2002). It is suggested that PPAR-α participates in the generation of an endogenous LXR-α ligand, since a synthetic, non-steroidal ligand of LXR-α has been found effective in the induction of lipogenic genes in mice deficient in PPAR-α (Horton *et al*., 2002, Reddy *et al*., 2010).

However, the role of PPAR-α in lipogenesis, albeit modest, is intriguing, as it appears paradoxical to the well known role of this transcription factor in the regulation of fatty acid oxidation (Reddy and Hashimoto, 2001). The involvement of PPAR-α in lipogenesis may suggest a fail-safe compensatory mechanism for the removal of important fatty acids, for example during starvation. The liver plays an important role in fatty acid oxidation and this catabolic energy burning is regulated by PPAR-α (Reddy *et al*., 2010). Oxidation of fatty acids occurs in three subcellular organelles with the bulk of β−oxidation confined to mitochondria and peroxisomes, while CYP4-A catalyzed ω−oxidation takes place in the endoplasmic reticulum (Reddy and Hashimoto, 2001; Rao and Reddy, 2004). Some of the key enzymes in these three fatty acid oxidation systems possess peroxisomes proliferator response element (PPRE) and are regulated by PPAR−α, though PPAR−β/δ has also been shown to regulate these genes.

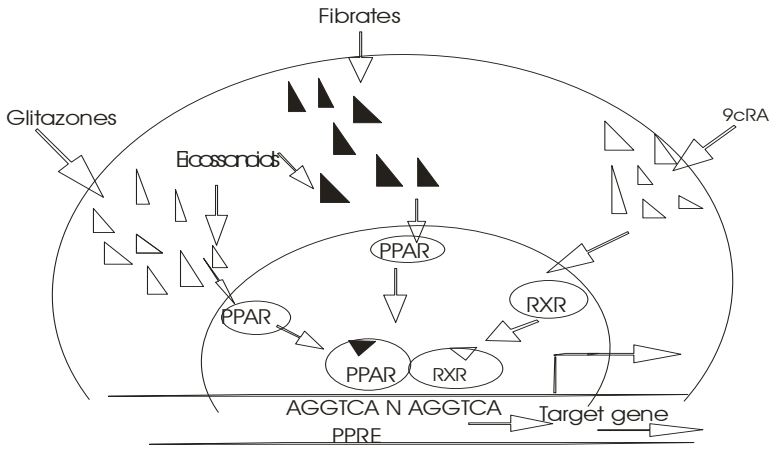

Figure 2.1: The PPAR signaling pathway and its natural and synthetic activators which include TZDs (Staels *et al.*, 1998).

After activation by its respective ligands, PPARs heterodimerize with the receptor for 9-cis-retinoic acid, RXR, and bind to specific response elements in the regulatory regions of target genes, termed PPREs, which are composed of 2 degenerate hexanucleotide repeats (arrows in Figure 2.1) arranged in tandem as direct repeats spaced by 1 nucleotide (Staels *et al.*, 1998).

Fatty acids are converted into acyl CoAs prior to oxidation (Reddy *et al*., 2010). Excess deposition of TG in non-adipocytes leads to impairment of cellular functions. The normal fat itself is probably relatively innocuous but it provides an intracellular source of fatty acids in excess of oxidative needs of the cell and increases ceramide formation, which triggers nitric oxide-mediated lipotoxicity and fatty acid induced apoptosis (lipoapoptosis) (Unger 2002). The most well known functions of ceramides as cellular signals include regulating the differentiation, proliferation, programmed cell death (PCD) and apoptosis (Type 1 PCD) of cells. Due to these functions, the ceramides are sometimes called “messengers of cell death”.

2.4 T2DM, hyperlipidemia, atherosclerosis and hypertension

Atherosclerosis is a form of hardening of the blood vessels, caused by fatty deposits such as cholesterol and local tissue reaction in the walls of the arteries. Blood supply beyond the affected parts of the artery is usually compromised by the narrowing and, sometimes, occlusion of the artery. The deposits, called plaques, may rupture with disastrous consequences (depositing more oxidized cholesterol into the artery wall, which leads to hardening and narrowing of the arteries reducing blood flow and

increasing blood pressure) (Sparrow and Olszewski, 1993). Heart disease and stroke, arising mainly from the effects of atherosclerosis, account for 65 percent of deaths among diabetics. Normal blood vessels have an inner lining, called endothelium, that keeps blood flowing smoothly by producing local nitrous oxide (NO). NO serves to relax the smooth muscles in the walls of the vessels and prevent cells from sticking to the walls. A disruption of this mechanism is thought to be at the heart of the increased formation of plaques in diabetes. High blood sugar, elevated fatty acids and triglycerides leads to stickier walls, encouraging the attachment of cells that produce local tissue reaction. The local tissue reaction further traps floating particles and different blood cells, heaping up and hardening the vessel walls.

Insulin stimulates the production of NO by the cells lining the blood vessels. In diabetics who are resistant to the actions of insulin (who suffer insulin resistance), this stimulatory effect is lost resulting in increased tendencies towards plaque formation. In the presence of raised blood sugar and resistance to insulin, the cells lining the blood vessels not only reduce production of NO but also increase the production of substances that constrict the blood vessel, further encouraging plaque formation. The smooth muscles of the blood vessels are also hyperactive in diabetes. Platelets and clotting factors are also affected by the high blood sugar, fatty acids and free radicals in diabetes. The blood cells are much stickier and the factors that inhibit clots do not work well under the peculiar circumstances of diabetes (Ikechik, 2009) leading to microvascular complications (Jiffri and Al-Dahr, 2010).

Atherosclerosis can affect any blood vessel with disastrous effects to the organs supplied by the vessel involved. When it involves the arteries supplying the heart, it leads to Coronary Artery Disease (CAD). This usually results in cases of angina or heart attacks. Involvement of other arteries leads to Peripheral Artery Disease (PAD). When it involves the arteries of the brain, the condition can result in strokes. Adequate treatment of diabetes with lifestyle modifications has a highly positive impact, restoring normal function and reducing plaque formation.

2.5 Complications of diabetes

Diabetes affects over 150 million people worldwide with this number expected to double by 2025 as shown in Figure 2.2 below (ADA). Diabetes and its complications are considered an epidemic in Africa, compelling African governments to start paying more attention to its impact as thousands of Africans run the risk of dying young. The potential severity of diabetes is such that some epidemiologists predict that its economic impact and death toll will surpass the ravages of HIV/AIDS in the near future (Azevedo and Alla, 2008). International Diabetes Federation (IDF) Diabetes Atlas (2009) estimated that 12.1 million people in sub-Saharan Africa will have diabetes in 2010 (3.8%), with the number projected to double by 2030. The complications of undetected and untreated diabetes are serious and cause huge human suffering and disability, and have huge socioeconomic costs resulting from premature morbidity and mortality.

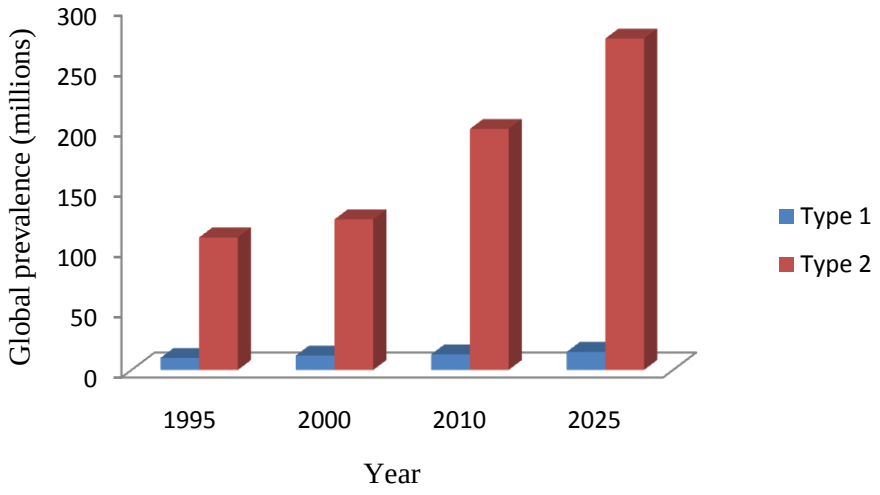

Figure 2.2: Prevalence of T1DM and T2DM in the world (ADA)

Diabetes is one of the leading causes of blindness, renal failure and lower limb amputation. It also triggers cardiovascular disease which is the leading cause of deaths in diabetes patients. Acute complications do occur such as diabetes ketoacidosis, and diabetes non-ketotic coma (hyperosmolar) (Tuei *et al.*, 2010). In Kenya, the prevalence of diabetes has shot up to 7.2% which is among the highest in the world and 80% of cardiovascular disease deaths occur in third world countries.

Both T1DM and T2DM have been consistently shown to increase the risk of developing microvascular and macrovascular complications such as stroke, nephropathy (kidney failure), myocardial infarction (heart attack), retinopathy (loss of vision), and neuropathy (nerve damage which often leads to numbness) (Tasci *et al.*, 2011). The reason for these harmful and restrictive complications is that long-term hyperglycemia (high blood sugar) leads to damage of the vascular endothelium (a thin layer of cells that line blood vessels) as a result of oxidative stress.

Encouragingly, all of these complications are reduced or avoided completely when patients are able to maintain a blood sugar level in the healthy range through a combination of diet, exercise, medications (often including insulin), and blood sugar management. In a person without diabetes, the blood sugar level is normally regulated by the body to remain within a narrow range of 4.4 – 6.1 mmol/L (82 to 110 mg/dl) (ADA, 2006).

2.6 Predisposing Factors

The National Institute of diabetes and digestive and Kidney (NIDDK, 2010) diseases estimates that diabetes affects 23.6 million people in the United States. More than 90% of diabetics have T2DM among adults, adolescents and children who are overweight or obese. Some of the other non-genetic predisposing factors include: advancing age, excessive alcohol consumption, physical inactivity, stress, unhealthy diet e.g. consumption of refined carbohydrate and high-fat diets as new lifestyles, imported dietary practices, and chronic use of steroids (ADA, 2011).

2.7 Pharmacotherapy of T2DM and hyperglycemia

Synthetic drugs used in the treatment of diabetes include sulfonylureas, Metformin, Thiazolidinediones and insulin.

2.7.1 Sulfonylureas

Sulfonylureas are a class of antidiabetic drugs used in the management of type 2 diabetes mellitus. According to WHO (2007), glibenclamide (**10**) is a second

generation of sulfonylureas and is one of the only two oral antidiabetics in the WHO model list of essential medicines (the other being metformin). Other sulfonylurea drugs (Figure 2.3) include gliclazide (**11**), glimepiride (**12**), tolazamide (**13**) and chlorpropamide (**14**).

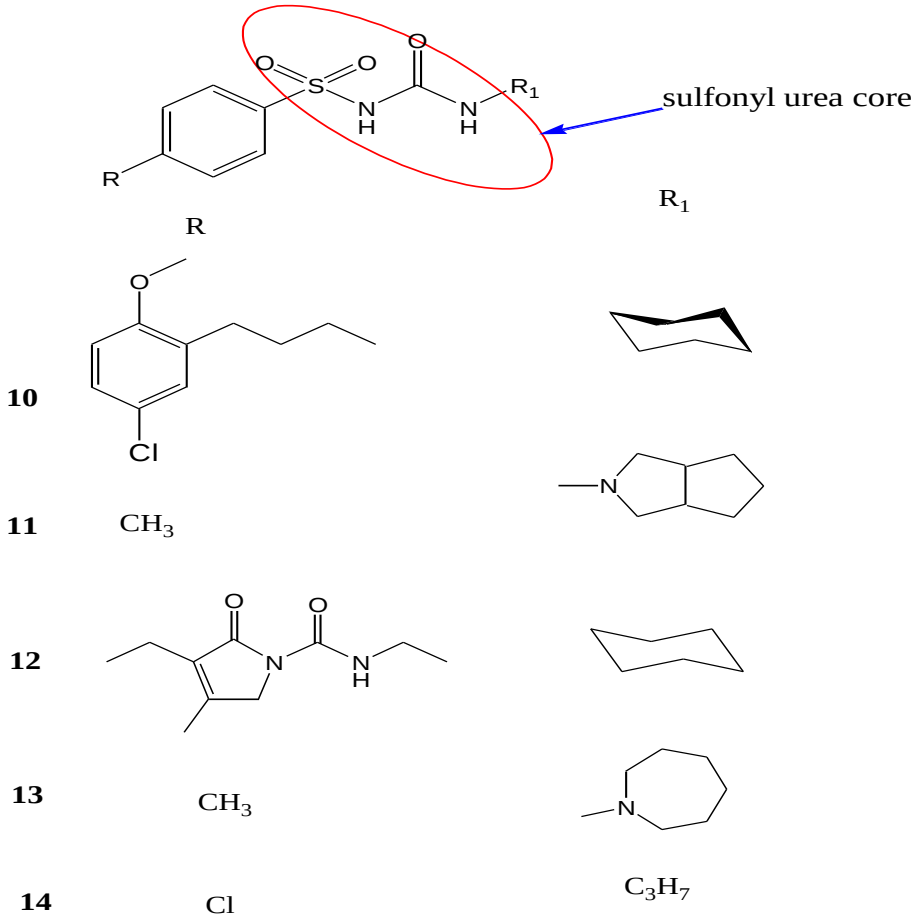

Figure 2.3: Sulfonylurea drugs for treating diabetes. (Kunte *et al.*, 2007)

Kunte *et al.*, (2007) found out that sulfonylureas improve the health outcome in people with T2DM and acute ischemic stroke. The drug works by inhibiting ATP-sensitive potassium channels in pancreatic *beta*-cells (Serrano-Martin *et al*., 2006). This inhibition causes cell membrane depolarization resulting in voltage-dependent

calcium channels to open. This increases intracellular calcium in the *beta* cells which stimulates insulin release. Sulfonylureas, as opposed to metformin, the thiazolidinediones, and other newer treatment agents may induce hypoglycemia as a result of excess insulin production and release.

2.7.2 Metformin

Metformin (**15**) is an oral antidiabetic drug in the biguanide class. It is the first-line drug of choice for the treatment of type 2 diabetes, in particular, in overweight and obese people and those with normal kidney function.

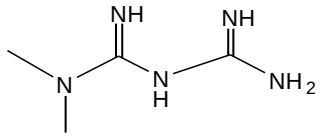

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Figure 2.4: Metformin drug for diabetes. (Jones *et al*., 2003)

Metformin is contraindicated in people with any condition that could increase the risk of lactic acidosis, including kidney disorders, lung and liver disease (Jones *et al*., 2003). It improves hyperglycemia primarily by suppressing glucose production by the liver (hepatic gluconeogenesis) (Kirpichnikov *et al*., 2002). The "average" person with type 2 diabetes has three times the normal rate of gluconeogenesis and metformin treatment reduces this by over one third (Hundal *et al.,* 2000). Metformin activates AMP-activated protein kinase (AMPK), an enzyme that plays an important role in insulin signaling, whole body energy balance, and the metabolism of glucose

and fats. Activation of AMPK is required for metformin's inhibitory effect on the production of glucose by liver cells. In addition to suppressing hepatic glucose production, metformin increases insulin sensitivity, enhances peripheral glucose uptake (by phosphorylating GLUT-4 enhancer factor), increases fatty acid oxidation and decreases absorption of glucose from the gastrointestinal tract.

2.7.3 Thiazolidinediones

Another class of antidiabetic drugs is thiazolidinediones (TZDs). Examples include troglitazone (**16**), pioglitazone (**17**) and rosiglitazone (**18**). TZDs are PPAR-γ agonists prescribed as insulin sensitizing drugs for clinical management of T2DM (Elte and Blickle, 2007). The activation of PPAR-γ by TZDs leads to a redistribution of fat from visceral to subcutaneous adipose tissue, increased trapping of fatty acids in adipose, and a modified secretion of hormones from adipose tissue, all factors known to improve insulin sensitivity. However, administration of TZDs has been associated with severe side effects such as oedema, weight gain, heart enlargement and hepatotoxicity. The occurrence of undesirable side-effects has been linked to the use of TZDs behaving like full PPAR-γ agonists. Partial PPAR-γ agonists are ligands that upon binding to PPAR-γ induce a conformation of the ligand-binding domain which differ from that induced by full agonists, and thereby also recruit a different set of co-factors than these. It is generally recognized that the selective recruitment of co-factors in response to administration of a partial agonist do not induce the same

magnitude of side effects as observed for the full agonist TZDs (Mikami *et al.*, 2012; Berger and Moller, 2002).

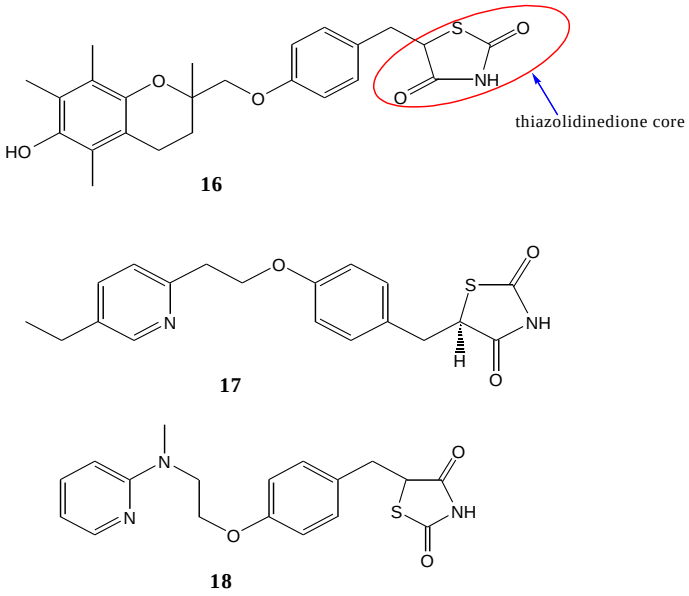


Figure 2.5: Thiazolidinediones class of drugs for treating diabetes. (Mikami *et al.*, 2012; Berger and Moller, 2002)

Thiazolidinediones has other side effects like, upper respiratory infections and retention of fluid in the body which may lead to heart failure and muscle pain (NIDDK). Another complication can occur when a diabetic swallows pills or drugs of different medications hand in hand and the risk is closing of the door of the pancreas from functioning. The reflection seen is high blood sugar due to insufficient insulin production to burn calories (Varadharajan, 2011).

CHAPTER THREE

EXPERIMENTAL

3.1 General experimental procedures

Column chromatography was run on Sigma Aldrich silica gel 60, 230-430 mesh. TLCs were run on Merck silica gel 60 F_{254} coated on aluminium foil. TLC profiles were developed using hexane/DCM solvent system (100:0, 90:10, 80:20, 60:40, 50:50, 40:60 and 20:80). Visualization of spots on TLC was done under UV lamp (254 nm and 365 nm) and by spraying with *p*-anisaldehyde/sulfuric acid/acetic acid in methanol reagents followed by heating of the plate in an electric oven at 110 ^{o}C and/or spraying with 5% concentrated sulfuric acid in methanol followed by heating the plate at 110 ^{o}C. All solvents were of analytical grade. Spectroscopic analyses were done using IR (Shimadzu IR-408) and FTIR (Shimadzu FTIR-8400) spectrometers as potassium bromide disks. NMR was done in University of Cape Town, South Africa, using Varian 400 MHz. Mass spectrometry was done in Jomo Kenyatta University of Science and Teachnology using Funnigan GC 8000 series interfaced with a Voyager EI-MS detector. Melting point was done in using Stuart melting point apparatus. Hepatocytes viability was done by trypan blue test using hemocytometer and then counted using inverted microscope (Olympus BX 41). Statistical analysis was done using SPSS V16, Tukey posthoc one-way analysis of variance (ANOVA) and T-test, and graphs were generated using Microsoft Excel software.

The outline for experimental procedures followed that led to isolation of urticol and the three terpenoids in this study is as shown in Figure 3.1 below.

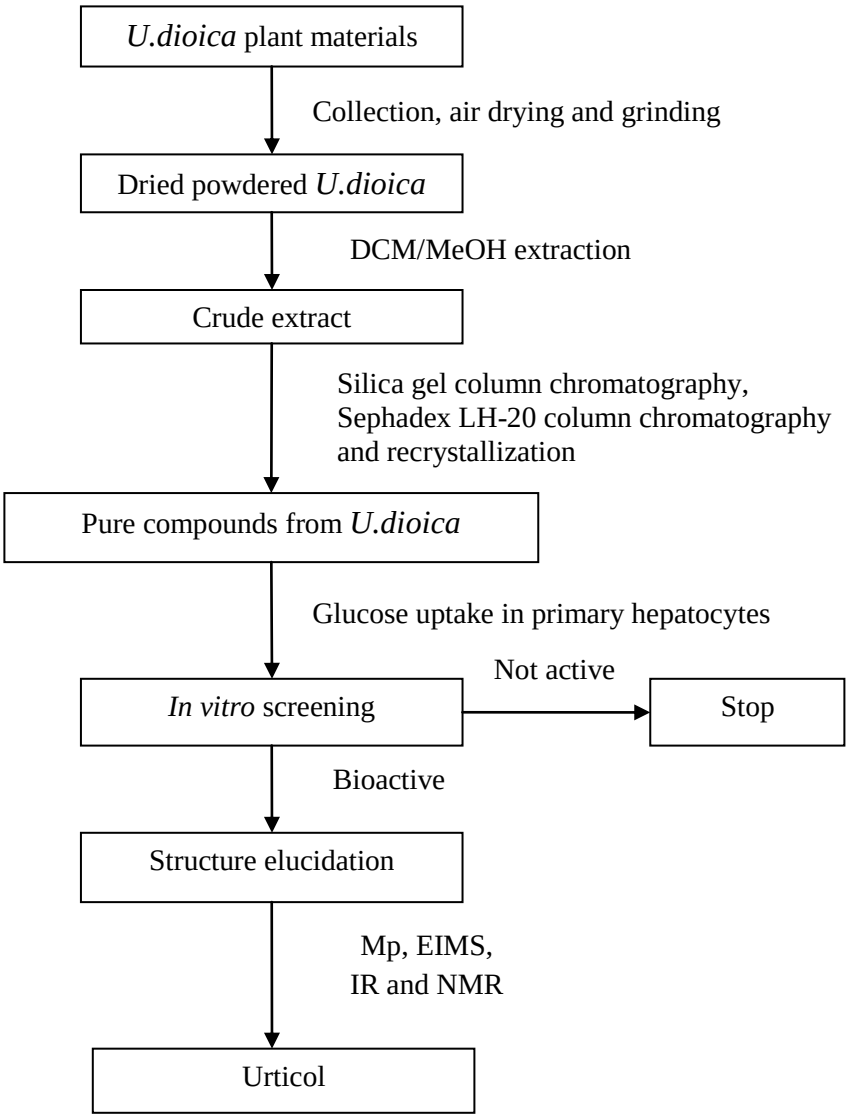


Figure 3.1: Flow chart for isolation and bioassay of the compounds from *U.dioica.* (Source: Author, 2011)

3.2 Collection and identification of plant materials

The plant material was collected from Kamara village, Total along Eldoret-Nakuru highway. Fresh, green leaves were picked from *U.dioica,* commonly known as the stinging nettle, and taxonomically identified at the Department of Biological Sciences, University of Eldoret (UOE), by Mr. Dennis Odhiambo. It was then given a voucher number **MUFT137** and deposited at the herbarium of UOE.

3.3 Extraction and purification of the plant components

Fresh leaves of the plant were air dried for three weeks, then chopped into pieces and finely ground. The powdered leaves (1.5 kg) were soaked/ percolated in 50:50 dichloromethane (DCM): methanol for 72 hours and filtered using cotton wool followed by Whatman No. 2 filter paper. The extract was concentrated under a vacuum using rotary evaporator to yield 40 g of the crude extract. The crude extract (35 g) was adsorbed on silica gel (35 g) and charged onto a column packed with 150 g of silica gel in petroleum ether. The column was eluted using increasing gradient of dichloromethane in petroleum ether which gave fractions 1-392. This was followed by increasing gradient of ethyl acetate in dichloromethane which gave fractions 393-1018 and finally, EtOAc-MeOH solvent system (9:1) gave fractions 1019-1198. The fractions were then pooled according to their TLC profiles to give four semi-pure fractions labeled F_A (fractions 1-60), F_B (fractions 61-120), F_C (fractions 121-800) and F_D (fractions 801-1118).

The semi-pure fraction F_A was concentrated using a rotary evaporator to yield 10 g of the fraction which was adsorbed on 10 g of silica gel and charged onto a flash column packed with 80 g of silica gel using petroleum ether. The column was eluted using increasing gradient of DCM in petroleum ether which gave fractions 1-139 followed by EtOAc in DCM (1:9 v/v) which gave fractions 140-186 and finally, DCM-EtOAc solvent system (1:1 v/v) gave fractions 187-204. TLC experiments on fractions 87-117 displayed two pigments; a yellow and a red pigment whose TLC spots turned purple and blue respectively when sprayed with *p*-anisaldehyde/sulfuric acid in methanol reagent. The two compounds overlapped in their TLC profiles (developed in petroleum ether-DCM solvent gradient). The fractions were pooled together and concentrated to yield 20 mg of the mixture. The yellow compound readily dissolved in hexane and DCM while the red compound readily dissolved in DCM. 3 mL of hexane was added to the mixture to dissolve the yellow compound completely and was pipetted into a clean vial leaving the red solid behind. Then, 3 mL of DCM was added to the red solid to completely dissolve it followed by a few drops of EtOAc. The red solid solution was recrystallized to obtain 12 mg of a pure compound, named compound **1,** as red-brown prismatic crystals. Fractions 23-86 contained a yellow pigment which turned purple when sprayed with *p*-anisaldehyde/sulfuric acid reagent. TLC examination showed one spot similar to that of the yellow compound obtained from fractions 87-117. The fractions were pooled together and concentrated to yield 200 mg of compound **2** as a yellow solid.

Fractions 61-120 from flash column chromatography of crude extract pooled as semi-pure fraction F_B were concentrated to dryness using a rotary evaporator to yield 8 g of the fraction. Fraction F_B (8 g) was adsorbed in 8 g of silica gel and charged onto a flash column packed using hexane. The column was eluted using increasing gradient of DCM in hexane which gave fractions 1-210 followed by 10 % EtOAc in DCM which gave fractions 211-214. Fractions 1-17 yielded 10 mg of an impure compound. TLC profiles of fractions 21-42 were found to be similar with only one blue/violet spot in the fractions when sprayed with *p*-anisaldehyde/sulfuric in methanol reagent, thus were pooled together and concentrated to yield 100 mg of compound **3** which was recrystallized in hexane/DCM (94:6 %) to yield 98 mg of the product as a yellow solid. Fractions 43-100 were pooled together according to their TLC profiles developed in increasing gradient of DCM in hexane which displayed two spots in each of the fractions. The pooled fractions were concentrated to yield 192 mg of the mixture. One of the spots was found to be similar to that of compound **3** while the other was a new spot. Hexane was then added to just cover the solid mixture and the yellow compound (**3**) was found to dissolve while the other white compound remained in solid form. The solution was pipetted into a clean dry vial and recrystallized in hexane/DCM (94:6 %) to give 64 mg of compound **3**. DCM was also added just to cover the white solid and it was found that the white solid was insoluble in cold DCM but soluble in warm DCM. Besides, the white solid was found to dissolve readily in methanol. That led to recrystallization of the solid in

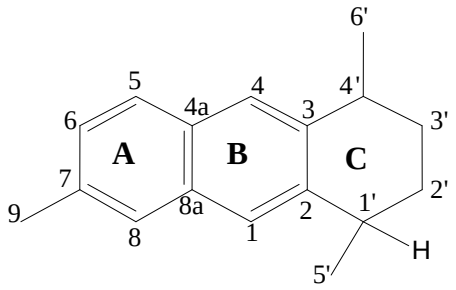
DCM/MeOH solvent system to yield 127 mg of compound **4** as white amorphous solid.

The pool of the semi-pure fraction F_C was concentrated to yield a gummy dark green of the fraction. The semi-pure fraction contained mainly chlorophyll and little consideration was given, but was dried and stored at 4 ^{o}C. The final fractions from the first flash column chromatography pooled as F_D were concentrated using rotary evaporator to yield 3 g of the semi-pure fraction. F_D was subjected to gel filtration using Sephadex LH-20 in DCM-MeOH (1:1) solvent system to remove chlorophyll which prevailed mainly in fractions 1-20 while fractions 21-38 were pooled together to yield 1.4 g of solid semi-pure fraction named F_E. F_E was dissolved in 10 mL of DCM and loaded onto a column packed with 10 g of silica gel using hexane. The column was eluted using DCM/EtOAc and EtOAc/MeOH solvent gradients yielding two pure compounds (<5 mg each) whose spectroscopic data were not obtained because of their small quantities.

3.4 Spectroscopic and physical data of the compounds

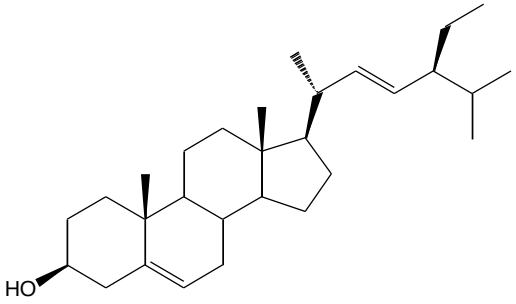
The isolated compounds were subjected to spectroscopic studies including EIMS, ^{1}H NMR, ^{13}C NMR and IR. The data are as follows:

Compound 1: 1'-hydroxy-1', 4', 7-trimethylcyclohex-2-ylnaphthalene



Red-brown crystalline (prismatic) solid. Mp 178-179 ^{o}C. IR (KBr): ν_{max} 1456 (m) (C=C), 1172 (C-H bend in-plane, aromatic) and 968 (C-H bend out-of-plane). ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.62 (2H, m, H-2'), 1.49 (2H, m, H-3'), 2.03 (1H, s, H-4'), 6.27 (1H, s, H-5), 6.15 (1H, s, H-6), 6.36 (1H, d, *J* = 8 Hz, H-8), 6.17 (1H, s, H-1), 6.64 (1H, d, *J* = 8 Hz, H-4), 1.73 (3H, s, H-5'), 1.04 (3H, s, H-6'), 1.98 (3H, s, H-9)). ^{13}C NMR (100 MHz, $CDCl_3$): δ 34.3 (C-1'), 19.3 (C-2'), 39.7 (C-3'), 33.1 (C-4'), 129.3 (C-3), 132.4 (C-5), 130.8 (C-6), 137.2 (C-7), 136.0 (C-8), 136.4 (C-8a), 126.6 (C-1), 137.9 (C-2), 130.0 (C-4), 137.7 (C-4a), 21.7 (C-5'), 29.0 (C-6'), 12.7 (C-9). EIMS: *m/z* 90 for $[C_7H_6]^{+\cdot}$, *m/z* 125 for $[C_{10}H_5]^{+\cdot}$**,** *m/z* 140 for $[C_{11}H_8]^{+\cdot}$

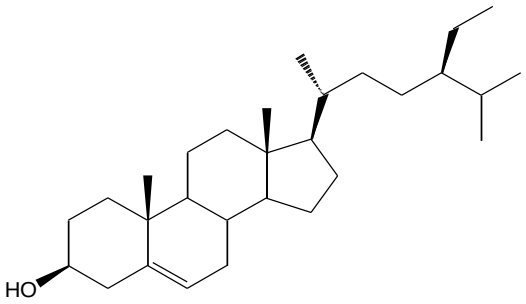
Compound 2: stigmasterol derivative



stigmasterol

Yellow solid. IR (KBr): ν_{max} 3400 (br) (C-H stretch), 1720 (s) (C=O), 1640 (m) (C=C), 1420 (m) (C-H bend), 1150 (m) (C-O). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.07 (t, *J* = 4 Hz), 0.86 (t, *J* = 4 Hz), 1.23 (s), 1.27 (m), 2.05 (d, *J* = 4 Hz), 3.66 (s), 4.05 (m), 4.59 (s), 5.10 (s), 5.35 (br, s).

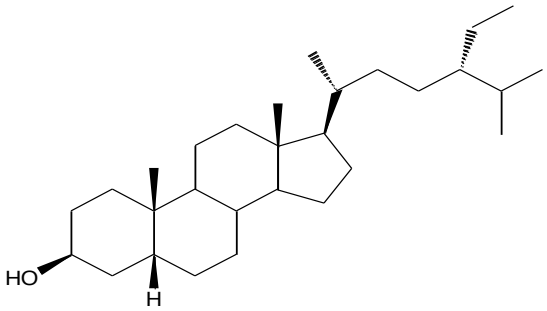
Compound 3: sitosterol derivative



sitosterol

Yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.82 (d, *J* = 4 Hz), 0.88 (q, *J* = 8 Hz), 1.07 (d, *J* = 4 Hz), 1.23 (s), 1.30 (t, *J* = 12 Hz), 1.55 (s), 1.62 (s), 1.70 (s), 2.01 (s), 2.30 (t, *J* = 8 Hz), 4.60 (d, *J* = 8 Hz).

Compound 4: ethyl cholestanol



Ethyl cholestanol

White powdered solid. IR (KBr): ν_{max} 3313 (br, weak) (OH), 1465 (m) (C=C), 1062 (cycloalkane). ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.07 (s), 1.26 (d, *J* = 8 Hz), 1.47 (s), l.78 (s), 1.96 (s), 3.65 (t, *J* = 8 Hz).

3.5 *In vitro* model for the assay of the compounds from *U.dioica*

In order to determine the antihyperglycemic effects of *U.dioica* compounds isolated, an *in vitro* model was designed to investigate the effects of these compounds on hepatic glucose uptake using freshly isolated primary rat hepatocytes. The hepatocytes were isolated according to the protocol by Prajapati and Patel (2011) and River (2012) and the cells were treated in the presence or absence of *U.dioica* compounds and the conventional drug for diabetes (pioglitazone). The amount of cellular glucose was determined according to the protocol by Heim and co-workers (2002) using DNSA colorimetric method of assay.

3.5.1 Reagents for the assays

Bicarbonate buffer (BB) with glucose, HEPES buffer pH 7.4 and without calcium chloride suitable for cell culture; 1000 mL bubbled in 5 % CO_2 for 20 minutes, sterile filtered and stored at 4 ^{o}C - 50 mL at 37 ^{o}C per rat in two 25 mL aliquot, 50 mM EDTA solution; 500 mL, 0.11 M $CaCl_2$; 250 mL, MEM (100 mL) and DMEM (500 mL) each supplemented with 10 % FBS, 4 mM Glutamine and Pen/strep, 0.25 % Trypsin solution; 200 mL, Phosphate buffered saline (PBS) pH 7.4; 500 mL, Perfusion buffer 1; 250 mL BB + 0.5 mL 50 mM EDTA, Perfusion buffer 2; 250 mL

BB + 343 µl 0.11 M $CaCl_2$, D-Glucose. 250 mL of lysis buffer (50 mM Tris HCl, pH 7.4, 2 mM EDTA and 0.1 % SDS).

3.5.2 Animals

Two twelve-week old male albino rats, and of about the same weight, obtained from Vet labs (Kenya) were used for the study. They were acclimatized for at least two weeks with free access to food and water in well ventilated room (25 ^{o}C) under a 12 hour light 12 hour dark cycle, and were fasted for 12 hours prior to the experiments (Portha *et al.*, 2001).

3.5.3 Isolation of rat hepatocytes

The rats were anesthetized using chloroform and sprayed with 70 % ethanol. A horizontal incision was then made on the ventral side near to the diaphragm and perfusion carried out. Briefly, the portal vein was exposed and the catheter hooked up to a syringe filled with 30 mL of perfusion buffer 1 at 37 ^{o}C was pinned down, angling upwards towards the head, into the left ventricle of the heart. Then, the portal vein was quickly clipped and perfusion started. During perfusion, the abdominal area was washed carefully with PBS at 37 ^{o}C to flush clotting blood pools and to keep the hepatocytes warm. The liver changed colour from red to light. The second syringe filled with perfusion buffer 2 was exchanged with syringe 1 just before the perfusion buffer 1 ran out, in order to prevent air bubbles from getting in lines, and perfusion continued with regular washing of the abdominal area with PBS at 37 ^{o}C. After

perfusion, the liver was excised, placed in chilled Petri dishes containing PBS and minced with scissors in the tissue culture hood. The small pieces were transferred into a 250 mL conical flask where 20 mL 0.25 % Trypsin solution was added to cover them. The content in the flask was magnetically stirred for about 1 h (until turbid), filtered using muslin cloth and the filtrate transferred into two chilled 25 mL conical tubes where they were centrifuged (50 g) for 4 minutes at 4 ^{o}C. The supernatant was decanted and the pellet washed three times with chilled PBS to remove trypsin. Thereafter, 10 mL of cold bicarbonate buffer was added and the cells redispersed by gently pipetting in up and down fashion using a 25 mL pipette. The 25 mL conical tubes were filled to 20 mL with cold bicarbonate buffer and centrifuged (50 g) for 3 minutes. The supernatant was decanted and 20 mL of bicarbonate buffer added to wash the cells, and resuspended by gently pipetting. Again, centrifuged for 3 minutes and the supernatant decanted. Finally, the cells were resuspended in 10 mL low glucose minimum essential media (MEM) at 37 ^{o}C and the cell viability determined.

3.5.4 Assay of cell viability

Cell viability was determined using Trypan blue test at the ratio of 1:3, cells to trypan blue reagent in a 2 mL vial. The mixture was then loaded onto the hemocytometer and the cells counted using inverted microscope. The test revealed that 92 % of the cells were viable while 8 % were not viable.

3.5.5 Assay of glucose uptake

The isolated rat hepatocytes were seeded in 96-wells microtitre plates from corning at the density of 2.0×10^6 cells/mL and incubated in humidified 5 % CO_2 incubator at 37 ^oC for 3 h to allow for cells attachment. Then, the media in each well was replaced with 200 μl/well complete DMEM supplemented with the compounds from *U.dioica* leaves and pioglitazone each at the concentrations of 50 μM and 100 μM. The media was collected and the cells washed two times with ice cold PBS. 0.2 mL of lysis buffer (50 mM Tris HCl, pH 7.4, 2 mM EDTA and 0.1 % SDS) was added and the cell suspension pipetted up and down ten times, and then centrifuged at 1000 g for 15 minutes. The amount of glucose in each cell was determined spectrophotometrically, as described by Heim group (2002), using dinitrosalicyclic acid colorimetric method (λ = 540 nm) (Miller, 1959) at intervals of 15, 30, 45 and 60 minutes and the results recorded.

3.5.6 Statistical analysis

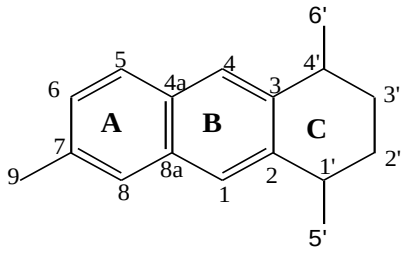
All data are presented as mean ± SD of the mean. Statistical analysis was performed using SPSS V16. One-way ANOVA was used to determine the effect of treatment over time on hepatic glucose uptake. The differences between experimental groups were analyzed by two-tailed unpaired t-test. The experimental groups which differ were determined by Tukey's post-hoc test. Differences between groups were considered significant at $p<0.05$.

CHAPTER FOUR

RESULTS AND DISCUSSIONS

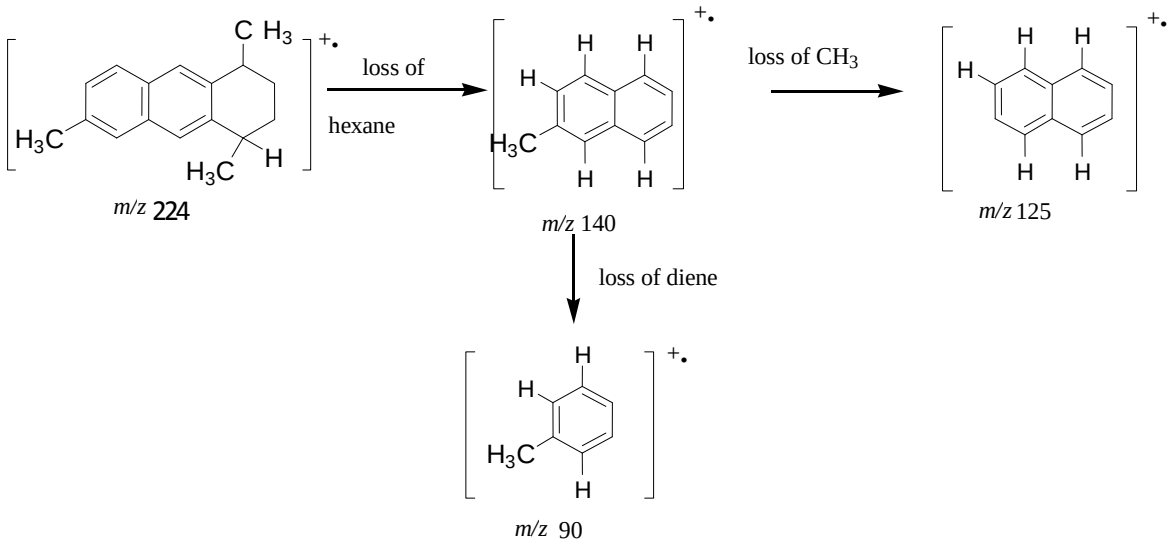
The leaves of *U.dioica* were extracted with a mixture of DCM/MeOH in the ratio of 1:1 ratio. The gummy extract was subjected to flash chromatography on silica gel followed by gel filtration using sephadex LH-20. This study generated structures of four compounds; compounds **1**, **2**, **3** and **4**. Compound **1** is novel while **2**, **3** and **4** are derivatives of the sterols previously isolated from the flowers and roots extract of *U.dioica*.

4.1 1',4',7-trimethylcyclohex-2-ylnaphthalene



Compound **1** was obtained as a red-brown crystalline (prismatic) solid. Its melting point was found to be 178-179 ^{o}C. The EIMS spectrum for the compound is shown in Figure 4.1. The mass spectrum did not reveal the molecular ion peak. The signal at *m/z* 140 which corresponds to 7-methylnaphthalene ion fragment $[C_{11}H_8]^{+\cdot}$ is due to the loss of hexane ion fragment from the molecular ion $[C_{17}H_{20}]^{+\cdot}$. The peak at *m/z* 125 is as a result of demethylation of the fragment with *m/z* 140 and corresponds to the naphthalene ion fragment $[C_{10}H_5]^{+\cdot}$. Furthermore, the peak at *m/z* 90 is due to the

loss of the diene ion fragment from the 7-methylnaphthalene ion fragment and is compatible with $[C_7H_6]^{+\cdot}$ ion fragment. The fragmentation pattern for the molecule is illustrated in Scheme 4.1.



Scheme 4.1: Plausible mass spectrum fragmentation pattern for compound 1. (Source: Author, 2012)

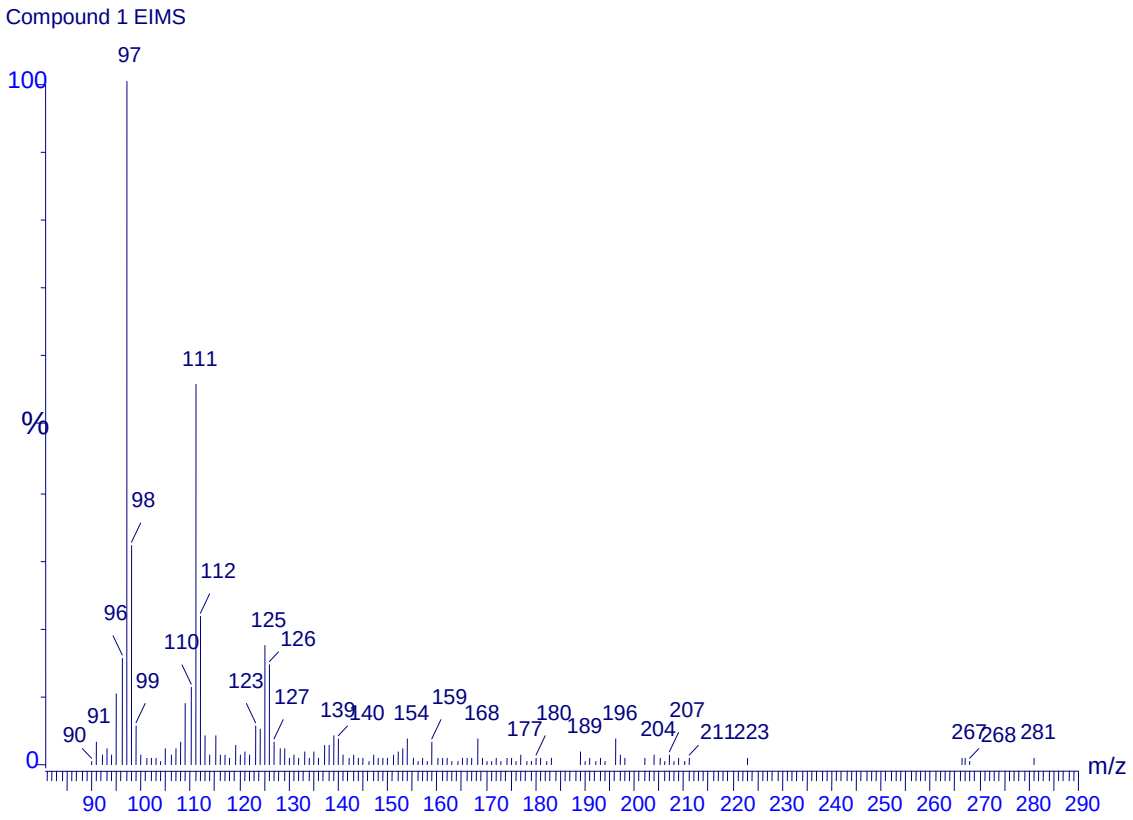


Figure 4.1: Mass spectrum of compound 1 obtained by electron impact mass spectrometry (Source: Author, 2012)

The IR spectrum of compound **1** displayed a broad absorption band at 3452 cm^{-1}, and sharp peaks at 2924 cm^{-1} and 2858 cm^{-1} for CH_3 and CH_2 respectively. It further displayed medium absorption bands at 1719 cm^{-1} and 1456 cm^{-1} for an aromatic ring C=C stretch. Finally, it revealed the C-H in-plane bending bands in the region 1172 cm^{-1} and the C-H out-of-plane bending in the region 968 cm^{-1} (Figure 4.2) which confirmed the presence of the aromatic ring.

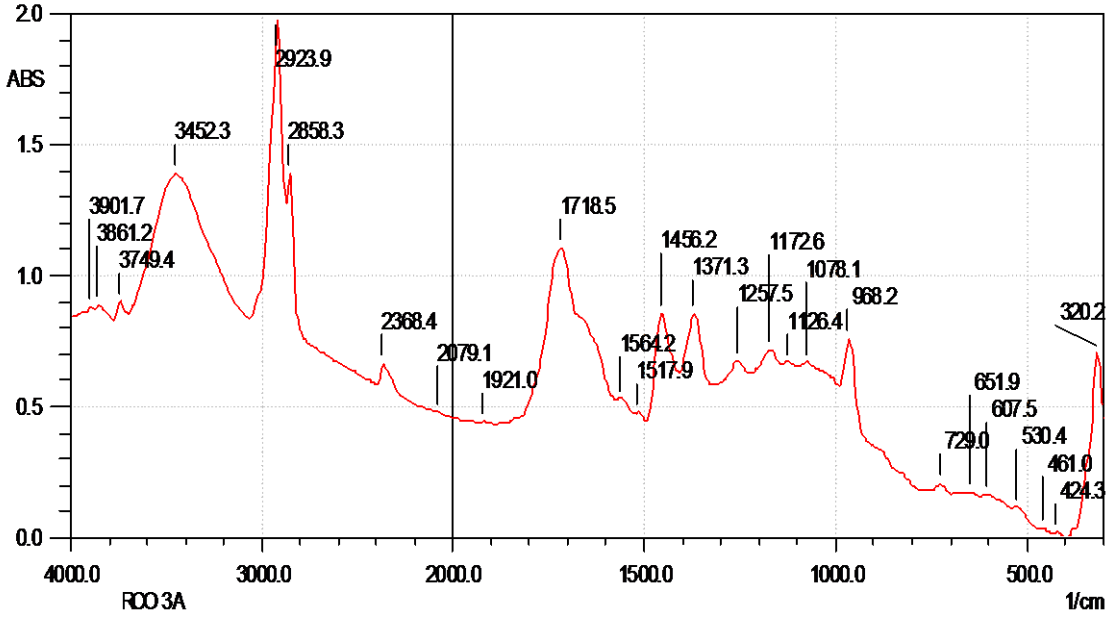


Figure 4.2: FTIR spectrum of compound 1 (Source: Author, 2012)

The ^{1}H-NMR spectrum of compound **1** showed four aromatic protons resonating at δ_H 6.14, δ_H 6.27, δ_H 6.36 and δ_H 6.64 (Figure 4.3). A multiplet absorption peaks at δ_H 1.49 ppm corresponded to methylene proton, H-3', directly correlated to δ_C 39.7 as shown in Table 4.2 below. H-3' is adjacent to methine proton, H-4', at δ_H 2.03 on one side and methylene protons, H-2', at δ_H 1.62 on the other side.

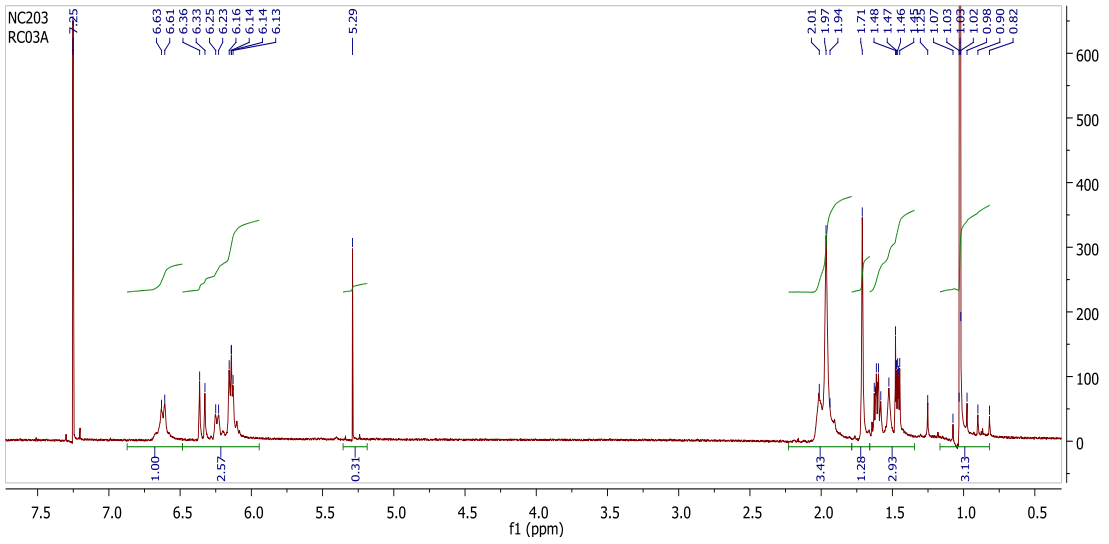
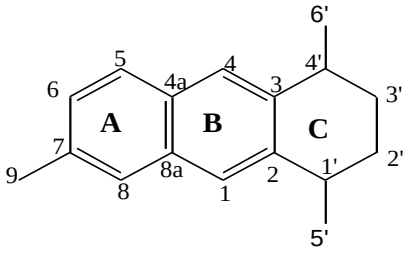


Figure 4.3: ^{1}H NMR spectrum of compound 1. (Source: Author, 2012)

Table 4.1: Positions of protons and carbons in compound 1

Position	δ_H	δ_C
1	6.17 (s)	126.6
2		137.9
3		129.3
4	6.64 (s)	130.0
4a		137.7
5	6.27 (d, *J* = 8 Hz)	132.4
6	6.15 (d, *J* = 8 Hz)	130.8
7		137.2
8	6.36 (s)	136.0
8a		136.4
9	1.98 (s)	12.7
1’	2.03 (m)	34.3
2’	1.62 (m)	19.3
3’	1.49 (m)	39.7
4’	2.03 (m)	33.1
5’	1.73 (s)	21.7
6’	1.04 (d, *J* = 4 Hz)	29.0



The proton NMR spectrum also showed singlet absorption peak at δ_H 1.73 which corresponds to the methyl H-5'. The ^2J correlation between this proton and the carbon at δ_C 34.3 confirmed the methyl is attached to C-1'. The singlet peak asserted the argument that the C-5' methyl group is attached to a quaternary carbon C-1'. The proton NMR spectrum displayed a doublet at δ_H 1.04 (J = 4 Hz) corresponding to H-6' methyl group while a singlet peak at δ_H 1.98 corresponded to the H-9 of the methyl group. The ^{1}H NMR, HSQC and HMBC data for compound **1** are shown in Table 4.2 below.

Table 4.2: ^{1}H NMR, HSQC and HMBC spectra of compound 1

Position	**HSQC**		**HMBC**
	δ_H	**δ_C**	
1	6.17 (s)	126.6	136.0, 136.4, 137.2, 137.9
4	6.64 (s)	130.0	132.4
5	6.27 (d, J = 8 Hz)	132.4	137.7
6	6.15 (d, J = 8 Hz)	130.8	136.0, 12.7
8	6.36 (s)	136.0	130.8, 132.4
9	1.98 (s)	12.7	130.8, 132.4, 136.0, 136.4, 137.2
2'	1.62 (m)	19.3	34.3, 137.9
3'	1.49 (m)	39.7	29.0, 33.1, 34.3, 129.3
4'	2.03 (m)	33.1	19.3, 29.0, 39.7, 129.3, 137.9
5'	1.73 (s)	21.7	34.3, 137.9
6'	1.04 (d, J = 4 Hz)	29.0	33.1, 39.7

^{13}C-NMR spectrum of compound **1** revealed the presence of 18 carbon atoms, 11 of which were aromatic carbon atoms which corresponded to two fused aromatic ring systems present in the compound (Figure 4.4). The signals at δ_C 12.7, 21.7 and 29.0 corresponded to saturated carbon atoms of the methyl groups at C-9, C-5' and C-6' respectively.

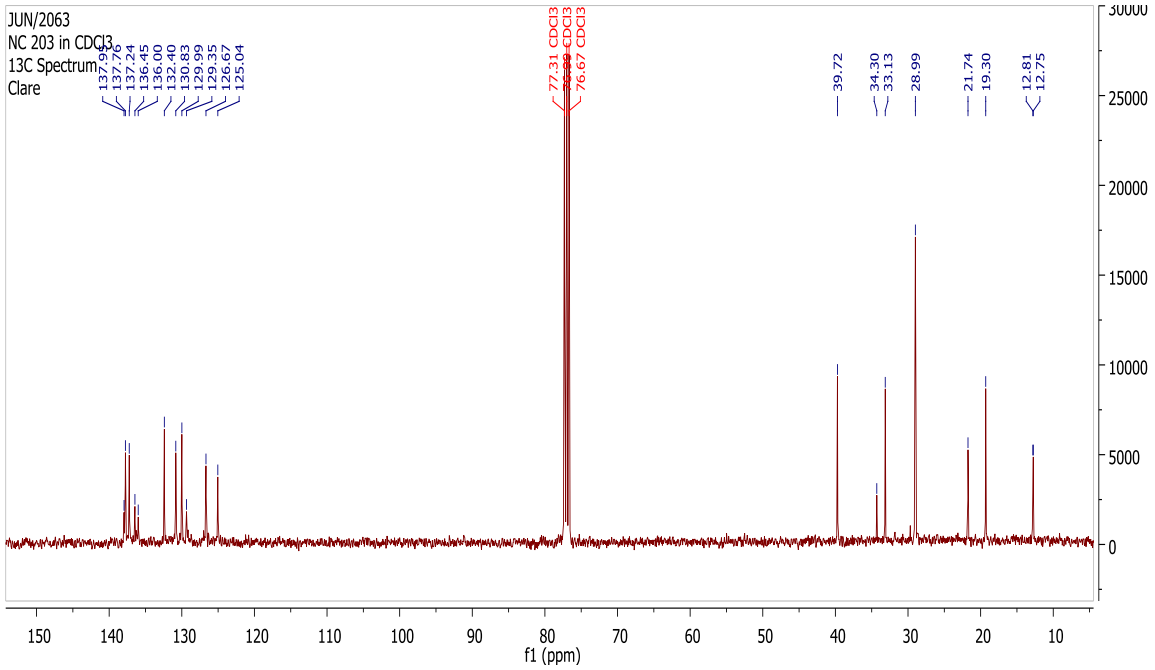


Figure 4.4: ^{13}C NMR spectrum of compound 1 (100 MHz, $CDCl_3$). (Source: Author, 2012)

Furthermore, HSQC spectrum did not display any proton correlation with C-1'. The two fused rings contained 10 aromatic carbons (Figure 4.5). It also displayed that the aromatic carbons at δ_C 126.7, 130.0, 130.8, 132.4 and 136.0 had directly attached proton H-1, H-4, H-6, H-5 and H-8 as shown in Figure 4.6 (a) below. The position of H-1 at δ_H 6.17 was confirmed by its HMBC correlations to 136.0, 136.4, 137.2 and

137.9. HMBC spectrum revealed a ^{3}J correlation of H-6 to δ_C 136.0 and 12.7. The strong ^{3}J correlations methyl proton H-9 to δ_C 130.8 and 136.0 and a ^{2}J correlation to δ_C 137.2 confirmed the position of the methyl group at C-7. C-5, C-6, C-7 and C-8 were aromatic carbons belonging to ring **A** while C-8a was one of the shared carbon atoms in the fused rings **A** and **B** of compound **1**.

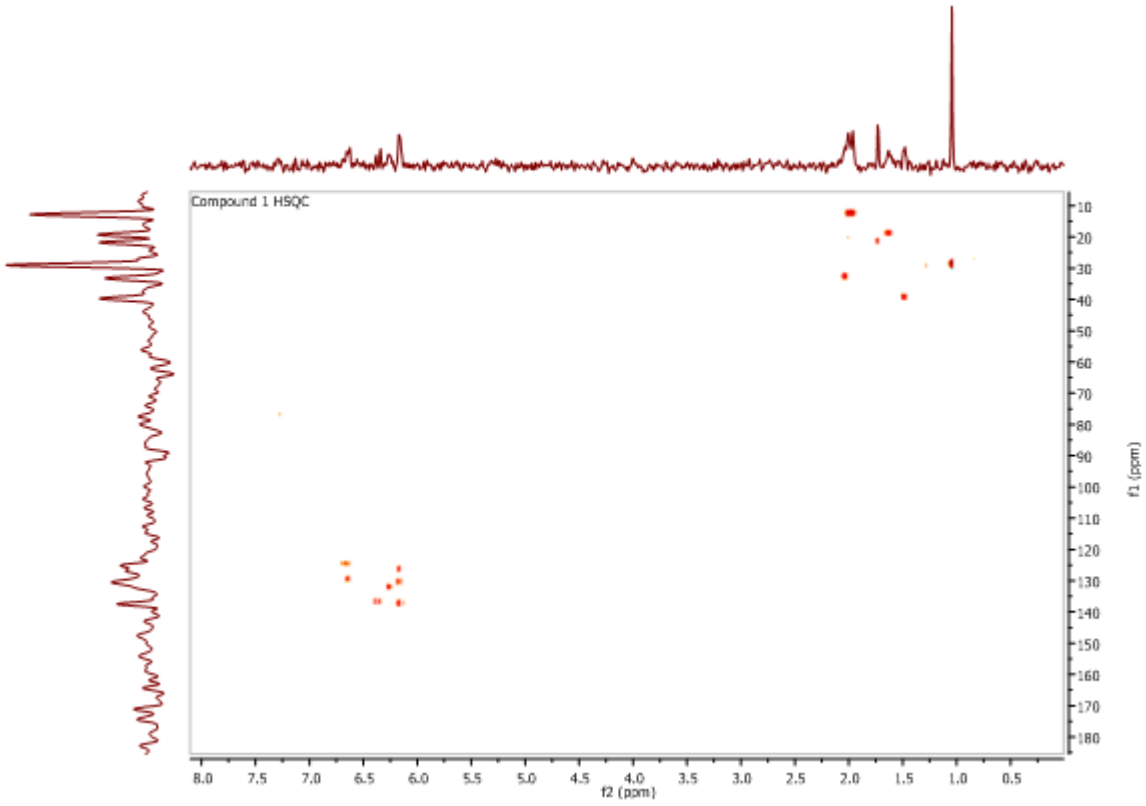


Figure 4.5: HSQC spectrum of compound 1 (Source: Author, 2012)

Moreover, the HMBC data (Figure 4.6 (b)) indicated a strong ^{3}J correlation between H-6 and the methyl group carbon at δ_C 12.7 which again ascertained the position of the methyl group at C-7. The methyl group at C-1' connectivity was based on its HMBC correlation between its protons and δ_C 34.3. The position of H-3' was further

confirmed by a strong ^{3}J correlation to the methyl carbon δ_C 29.0, weak ^{2}J correlation to δ_C 33.1 and strong ^{3}J correlations to δ_C 129.3 and 34.3. Therefore, compound 1 was characterized as 1',4',7-trimethylcyclohex-2-ylnaphthalene and given the trivial name urticol.

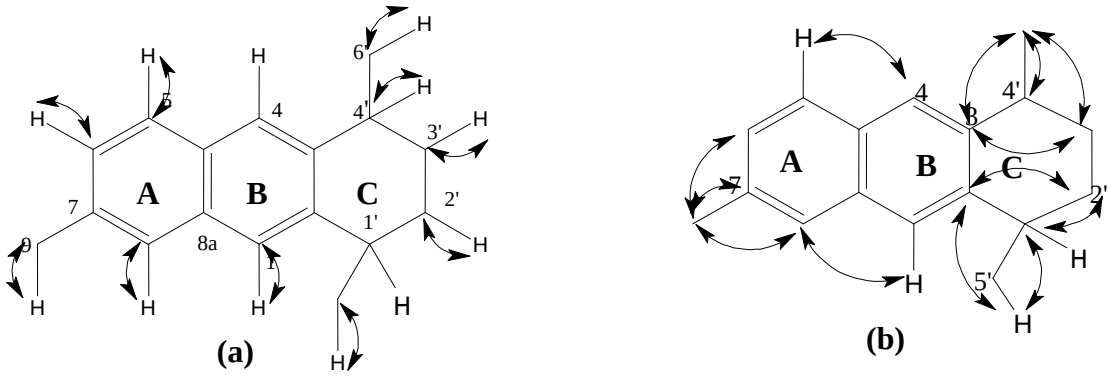


Figure 4.6: Important (a) HSQC and (b) HMBC correlations observed for compound 1. (Source: Author, 2012)

4.2 Proton NMR spectral data for compounds 2, 3 and 4

These compounds were found to be terpenoids and non-aromatic as compared to urticol. The ^{1}H NMR spectral data for these compounds are summarized in Table 4.3 below.

Compound 2:

Compound **2** was isolated as a yellow amorphous solid. Salkowski reaction (Harborne, 1998) of compound **2** revealed that the compound contained unsaturated steroidal nucleus. Its proton NMR (Figure 4.7) displayed five olefinic protons

resonating at δ_H 3.66 (s), 4.05 (m), 4.59 (s), 5.10 (s) and 5.35 (br, s). Also, the compound was found to be non-aromatic as revealed by the proton NMR.

Table 4.3: ^{1}H NMR data for compounds 2, 3 and 4 (400 MHz, $CDCl_3$)

δ_H		
2	**3**	**4**
0.07 (t, *J* = 4 Hz)	0.82 (d, *J* = 4 Hz)	1.07 (s)
0.86 (t, *J* = 4 Hz)	0.88 (q, *J* = 8 Hz)	1.26 (d, *J* = 8 Hz)
1.23 (s)	1.06 (s)	1.47 (s)
1.27 (m)	1.23 (s)	1.78 (s)
2.05 (d, *J* = 4 Hz)	1.30 (t, *J* = 12 Hz)	1.96 (s)
3.66 (s)	1.55 (s)	3.65 (t, *J* = 8 Hz)
4.05 (m)	1.62 (s)	
4.59 (s)	1.70 (s)	
5.10 (s)	2.01 (s)	
5.35 (br, s)	2.30 (t, *J* = 8 Hz)	
	4.60 (d, *J* = 8 Hz)	

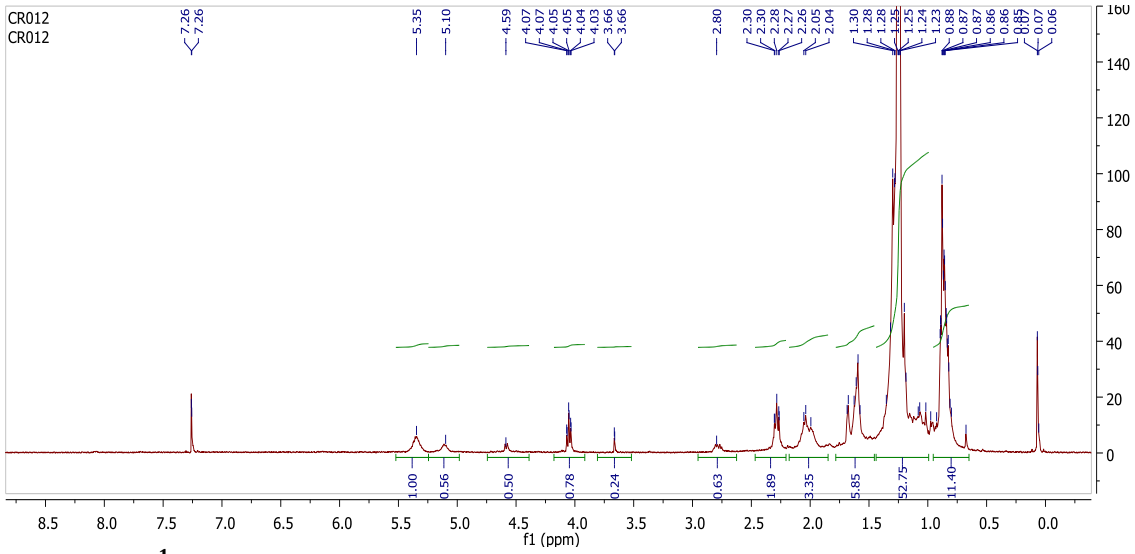


Figure 4.7: ^{1}H NMR spectrum of compound 2. (Source: Author, 2012)

Furthermore, the comparison of its proton NMR data (Table 4.4) with the literature values of steroidal compounds revealed that it is a stigmasterol derivative (Pateh *et al.*, 2009; Isah *et al*., 2012).

Compound 3:

Compound **3** was isolated as a yellow amorphous solid. Its proton NMR (Figure 4.8) displayed the presence of olefinic protons resonating at δ_H 4.60 and a signal at 5.35 ppm corresponding to a proton on a carbon atom next to oxygen.

Table 4.4: ^{1}H NMR spectral data for compounds 2, 3 and 4 and the literature values for the corresponding compounds.

δ_H					
Stigmasterol derivative (2) Experimental Values	**Stigmasterol Literature values (Pateh *et al.*, 2009)**	**Sitosterol derivative (3) Experimental Values**	**Sitosterol Literature values (Pateh *et al.*, 2009)**	**Ethylcholestanol (4) Experimental values**	**Ethylcholestanol Literature Values (Luo *et al.*, 2009)**
0.86	0.85	0.82	0.83	0.91	0.90
1.23	1.23	0.90	0.92	3.65	4.10
2.05	2.01	1.06	1.01		
3.66	3.57	1.23	1.23		
4.59	4.96	2.01	2.01		
5.10	5.10	4.60	4.97		
5.35	5.36	5.35	5.36		

Salkowski reaction (Harborne, 1998) revealed that compound **3** contained unsaturated steroidal nucleus. The NMR data analysis of the experimental compound displayed seven different kinds of protons whose chemical shift positions tally with the literature values of β−sitosterol. Key absorptions like singlet peak at δ_H 2.01 and broad singlet at δ_H 5.35 were in agreement with the reported values in Table 4.4 (Pateh *et al.*, 2009). This comparison of experimental values with the literature values of β−sitosterol allowed the determination of compound **3** as β−sitosterol

derivative (Rahman *et al.,* 2009). In fact, β–sitosterol was previously isolated by Chaurasia and Witchl (1987) from the roots and flowers of the plant.

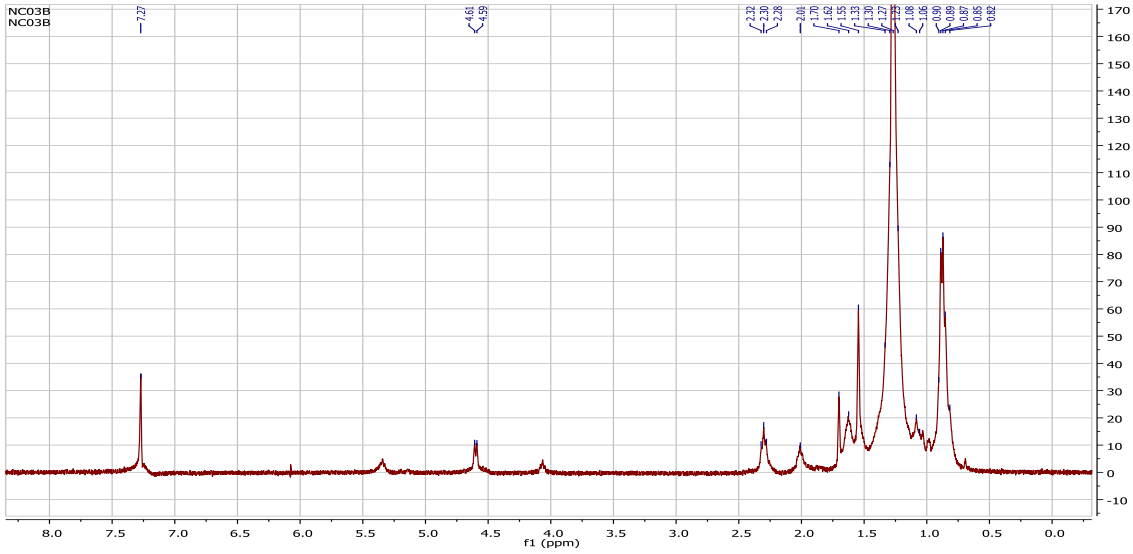


Figure 4.8: ^{1}H NMR spectrum of compound 3. (Source: Author, 2012)

Compound 4

Compound **4** was isolated as a white amorphous solid. Its proton NMR spectrum (Figure 4.9) revealed the presence of a signal at δ_H 3.65 (t, J = 8 Hz). Salkowski reaction tested slightly positive with the compound implicative of its saturated steroidal nature. Furthermore, the proton NMR of compound **4** revealed that olefinic protons were absent and the compound is non-aromatic but the peak at δ 3.65 corresponded to a proton on carbon next to oxygen. Analysis of its FTIR, proton NMR data (Table 4.4) and comparison with literature values of stanols (Luo *et al.,* 2009) led to the determination of the compound as ethylcholestanol. This compound

was previously isolated by Chaurasia and Witchl (1987) from the roots and flowers of the *U.dioica*.

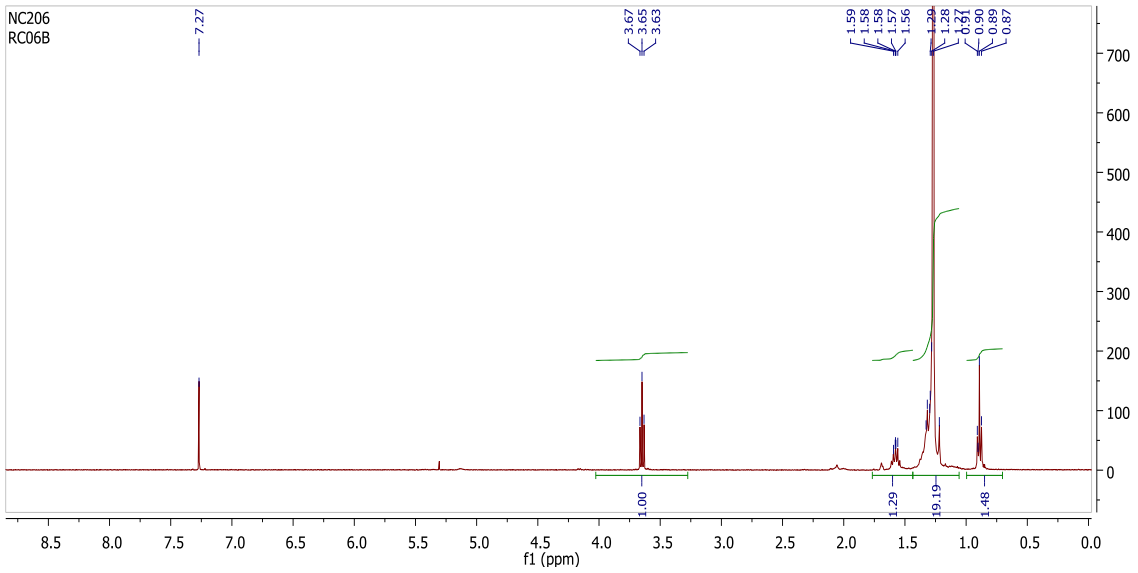


Figure 4.9: ^{1}H NMR spectrum of compound 4. (Source: Author, 2012)

4.3 Bioassay of the isolated compounds

The amount of cellular glucose in both control and treatment groups for 50 μM dose of the compounds from *U.dioica* and pioglitazone for various experimental time intervals. Table 4.5 shows the mean cellular glucose values for hepatocytes cultured in DMEM (untreated control), DMEM supplemented with compound **1**, DMEM + compound **2**, DMEM + compound **3,** DMEM + compound **4,** and DMEM + pioglitazone. Glucose uptake increased with time in all the treatment groups and controls, and in both treatment doses of the compounds from *U.dioica* and pioglitazone.

Table 4.5: Effects of *U.dioica* compounds at 50 μM dose on upregulation of hepatic glucose uptake in primary rat hepatocytes for various experimental time intervals.

Time (min)	Glucose concentration (x 10^{-3} mmol/L)					
	untcontrol	cpd1	cpd2	cpd3	cpd4	pioz
0	0.23±0.02	0.23±0.02	0.23±0.02	0.23±0.02	0.23±0.02	0.23±0.02
15	0.47±0.06	0.44±0.02	0.30±0.06*	0.44±0.02	0.26±0.08*	0.43±0.02
30	0.52±0.03	0.52±0.02	0.39±0.05*	0.54±0.03	0.36±0.04*	0.49±0.02
45	0.56±0.03	0.55±0.02	0.44±0.04*	0.64±0.03	0.43±0.01*	0.51±0.03
60	0.63±0.04	0.59±0.03	0.48±0.04*	0.67±0.02	0.48±0.01*	0.58±0.02

The values were presented as Mean ± SD of the mean for n=3. *Mean values considered statistically different at $p<0.05$ versus untcontrol. Untcontrol – abbreviates untreated control, cpd (1-4) – compound (1-4) from *U.dioica* leaves and pioz – pioglitazone.

The treatment dose for the compounds was also administered at 100 μM. Table 4.6 shows the mean cellular glucose values for hepatocytes cultured in DMEM (untreated control), DMEM supplemented with compound **1**, DMEM + compound **2**, DMEM + compound **3,** DMEM + compound **4,** and DMEM + pioglitazone.

Table 4.6: Effects of *U.dioica* compounds at 100 μM dose on upregulation of hepatic glucose uptake in primary rat hepatocytes for various experimental time intervals.

Time(min)	Glucose concentration (x 10^{-3} mmol/L)					
	untcontrol	cpd1	cpd2	cpd3	cpd4	pioz
0	0.23±0.02	0.23±0.02	0.23±0.02	0.23±0.02	0.23±0.02	0.23±0.02
15	0.47±0.06	0.48±0.03	0.24±0.03*	0.43±0.08	0.38±0.04*	0.52±0.03
30	0.52±0.03	0.54±0.02	0.33±0.03*	0.48±0.06	0.44±0.01*	0.60±0.03*
45	0.56±0.03	0.61±0.04	0.42±0.04*	0.58±0.01	0.48±0.02*	0.67±0.04*
60	0.63±0.04	0.81±0.04*	0.50±0.03*	0.64±0.01	0.51±0.03*	0.71±0.04*

The values were presented as Mean ± SD of the mean for n=3. *Mean values considered statistically different at $p<0.05$ versus untcontrol. Untcontrol – abbreviates untreated control, cpd (1-4) – compound (1-4) from *U.dioica* leaves and pioz – pioglitazone.

Glucose uptake increased with time in all the treatment groups and controls, and in both treatment doses of the compounds from *U.dioica* and pioglitazone. At 50 μM dose, compound **2** reduced the uptake of glucose by rat hepatocytes as compared to the untreated control group at all the experimental time intervals (Figure 4.10). The mean values for compound **2** treatment groups differed significantly with those of untreated control at $p<0.05$. For example, compound **2** was found to decrease glucose absorption in hepatocytes by 36.2 % and 25 % at 15^{th} and 30^{th} minutes respectively when compared to the untreated control. Also, compound **4** displayed comparable activity with those of compound **2** as shown in Figure 4.10 below. It significantly reduced the uptake of glucose by hepatocytes with respect to untreated control ($p<0.05$). Compound **1** and pioglitazone treatment groups were found to have mean values comparable to those of untreated control at all experimental time intervals as shown in Figure 4.10. Therefore, compound **1** and pioglitazone at 50 μM doses were found to be non-bioactive.

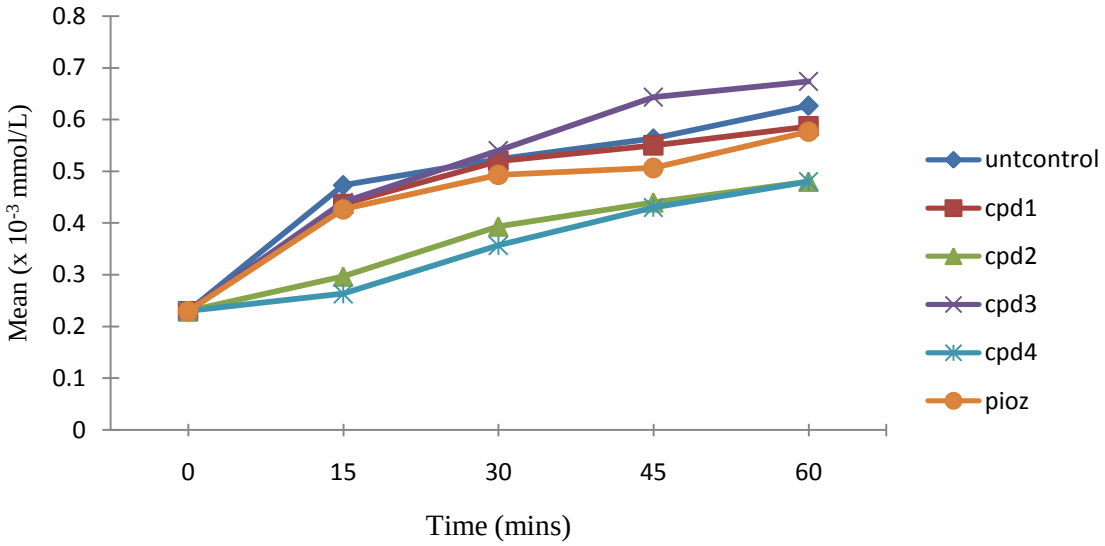


Figure 4.10: Glucose variations with time for treatment groups at 50 μM dose.

(Source: Author, 2012)

However, when the dosage was increased to 100 μM, all the treatment compounds (**2**, **3** and **4**) except compound **1,** did not show any potency to stimulate glucose uptake in primary rat hepatocytes. Compounds **2** and **4** significantly reduced glucose uptake in rat hepatocytes with respect to the untreated control although glucose uptake increased significantly between experimental time intervals within each of the treatment groups as shown in Table 4.6 above. Compound **3** mean values were comparable with those of the untreated control.

Compound **1** was found to significantly stimulate the uptake of glucose in primary rat hepatocytes when compared to the untreated control. Figure 4.11 below shows that the uptake of glucose in hepatocytes increased significantly with time for compound **1** treatment group.

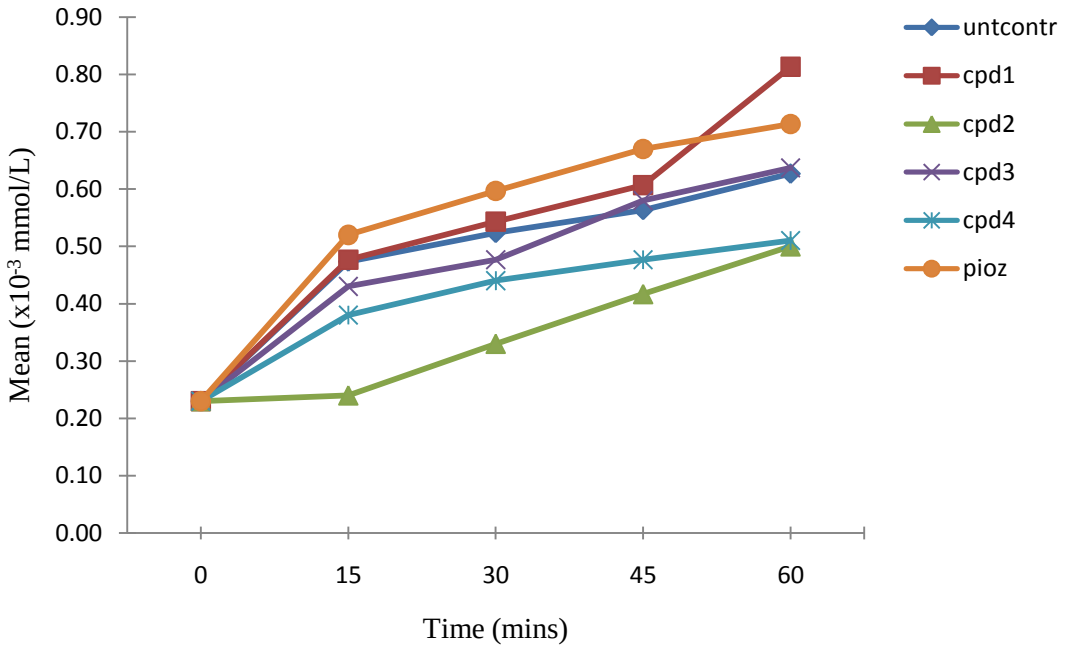


Figure 4.11: Glucose variations with time for treatment groups at 100 μM dose. (Source: 2012)

It was observed that compound **1** stimulated the uptake of glucose by 2.13 %, 3.84 %, 8.93 % and 28.57 % at 15th, 30th, 45th and 60th minute intervals respectively in comparison to the untreated control (Figure 4.12). Although the difference between urticol and pioglitazone at the active dose (100 μM) was insignificant at $p<0.05$, the mean difference at the 60th minute interval shows that compound **1** increased glucose uptake in hepatocytes by 11.45 % versus pioglitazone.

The results obtained demonstrate that the bioactivity of urticol is stronger than that of pioglitazone (member of thiazolidinediones) in stimulation of glucose absorption in rat hepatocytes. Therefore, *U.dioica* in part improves diabetes by enhancing glucose

uptake in hepatocytes. The observed effect for compound **1** serves to increase liver glucose storage and reduce blood glucose level and hence could possibly reverse/prevent insulin resistance.

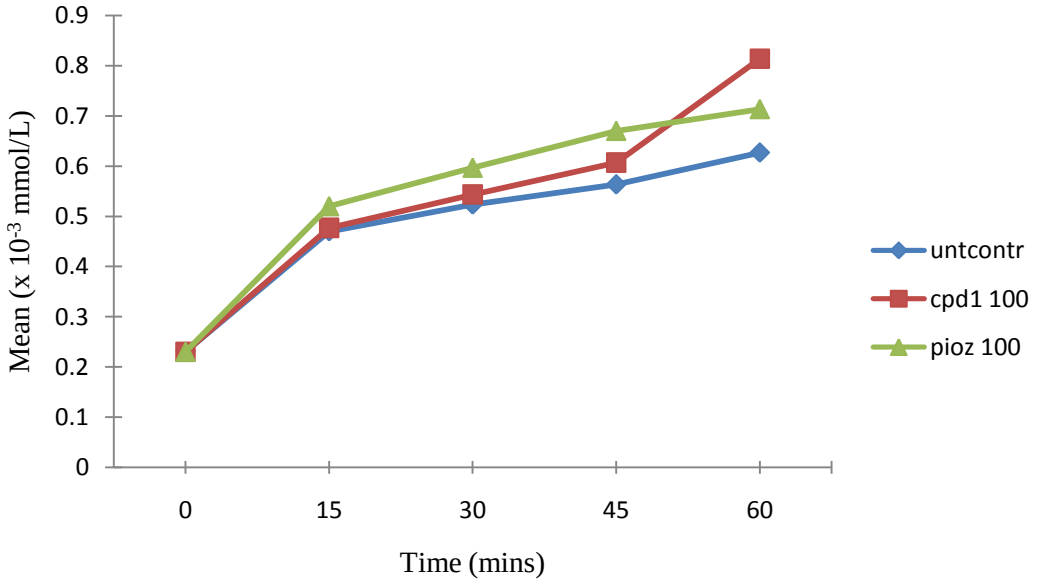


Figure 4.12: Glucose variations with time for the bioactive treatment groups. (Source: 2012)

As stated earlier, insulin resistance in the liver reduces glucose storage and this effect serves to elevate blood glucose level and hence progression to T2DM. Also, increased free fatty acids are associated with hepatic insulin resistance resulting in increased gluconeogenesis (Boden *et al.*, 2002; Heek *et al.*, 2001; Kim and Ahn, 2004). The improved glucose uptake observed in this study due to compound **1** could possibly reduce free fatty acids in the liver and hence hepatic insulin sensitivity which would decrease endogenous glucose production.

Insulin stimulates glucose uptake in the liver of both insulin-sensitive and insulin-resistant subjects. Besides, insulin has been shown to upregulate glucokinase transcription (Iynedjian *et al.,* 1989, Nouspikel *et al.,* 1992) and glycogen synthase activity (Ortmeyer *et al.,* 1997). It has, however, been found to inhibit glucose-6-phosphatase (Gardner *et al.,* 1984) and glycogen phosphorylase (Ortmeyer *et al.,* 1997) in hepatocytes *in vitro*. Compound **1** may mimic insulin action (insulin mimetic) *in vitro* and could possibly upregulate glucokinase transcription and other previously mentioned enzymes just as insulin does.

The liver plays a crucial role in glucose metabolism and is an important regulator of glucose levels in plasma (Heim *et al.,* 2002). The liver and the pancreatic β−cells, as compared to other tissues involved in glucose homeostasis, can sense and respond to blood glucose levels (Kim and Ahn, 2004). After glucose ingestion, the liver suppresses its basal rate of glucose production and takes up approximately one-third of the glucose in the ingested meal. Suppression of hepatic glucose production and augmentation of hepatic glucose uptake, collectively, account for the maintenance of nearly one-half of the rise in plasma glucose concentrations following ingestion of a carbohydrate meal (Cersosimo *et al.,* 2011). As previously mentioned in the literature, hepatic glucose uptake serves to decrease postprandial glucose and hence prevent progression of hyperglycemia (in the state of hyperinsulinemia) to insulin resistance.

CHAPTER FIVE

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

From the results, purification of *U.dioica* leaves extract by silica gel chromatography and recrystallization led to isolation of four compounds, three of them were terpenoids previously isolated from the roots of *U.dioica* and the leaves of other herbal plants and one novel compound. Physical and spectroscopic data led to characterization of the novel compound as 1',4',7-trimethylcyclohex-2-ylnaphthalene and was given the trivial name urticol. Biological assay of urticol based on its stimulatory effect on hepatic glucose uptake by freshly isolated primary rat hepatocytes model showed that the compound has a relatively higher potent antihyperglycemic activity than the conventional drug pioglitazone at 100 μM dose level. The findings show that the compound stimulated glucose uptake in hepatocytes cultured in DMEM by 11.45 % versus pioglitazone after 1 h of treatment. Besides, its stimulatory effect on glucose uptake was found to be 28.57 % in comparison to the untreated control group of hepatocytes. The difference between pioglitazone and urticol was statistically insignificant at $p<0.05$ showing that the novel compound had comparable glucose upregulatory effect in rat hepatocytes with pioglitazone. This enhanced glucose uptake activity of urticol *in vitro* explains, in part, the role of *U.dioica* in improving the state of hyperglycemia and insulin resistance. In general, there is induction of glucose uptake by urticol, and so

U.dioica, in freshly isolated primary rat hepatocytes in improving the state of hyperglycemia and hence T2DM.

5.2 Recommendations

From the findings, the following recommendations can be drawn. There is need for synthesis of the compound for large scale production to allow further investigations of the compound for its therapeutic potential and the exploration of its mechanisms of action in improving various metabolic disorders. It was observed that only 0.08 % of urticol was isolated from 1.5 kg of the powdered *U.dioica* material and that the compound displayed potent antidiabetic activity *in vitro*; however this quantity is too small to rely on the plant material for the therapeutic agent. Since diabetes is a metabolic disorder that could be controlled by lifestyle changes, individuals should be encouraged to plant the herbs in their farms as the plant can be cultivated with good harvest, as shown in previous work on cultivation of the plant, for use as food supplement which would then boost self-oriented health care. Further work needs to be done to fully understand the exact mechanism of action of urticol in inducing glucose uptake. It is hypothesized that urticol may work by inducing Glut-4 expression on hepatocytes but this remains to be determined. Also, studies need be done to establish additional mechanism of the compound other than glucose uptake. It is suggested that the compound could be responsible for the antihyperglycemic effects earlier seen on the crude extract of the plant which include induction of insulin secretion by a component of *U.dioica* extract in perfuses islets of Langerhans, inhibition of liver α– glucosidase activity and its increased expression of PPAR–γ by

a component of *U.dioica* extract. Furthermore, investigative work needs be done based on *in vivo* studies in order to determine if the compound has similar results as *in vitro*. The observed effect of the compound while using freshly isolated primary rat hepatocytes could be similar to the observations which will be made in rat hepatocytes *in vivo*. Apart from the antihyperglycemic activity of *U.dioica*, the extract from *U.dioica* has been shown to have antihyperlipidemic activity. It is hypothesized that urticol may be responsible for this therapeutic activity of *U.dioica*. Studies need to be done in order to determine whether urticol could be actually the likely hypolipidemic agent and also whether its effect on the expression of PPAR−α could be the likely mechanism of action. Finally, cytotoxicity studies (LD_{50}) and dose response studies need be done on urticol. This may pave way for clinical trials and hence the usefulness of the compound as a drug for diabetes in the medical field.

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