

**IN VITRO ASSAY FOR THE ANTI-DIABETIC EFFECT
OF OCIMUM CANUM AND OTHER MEDICINAL PLANTS**

A THESIS SUBMITTED

BY

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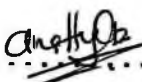
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DECLARATION

THE WORK DESCRIBED IN THIS REPORT WAS CARRIED OUT BY ME AT THE DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF GHANA, LEGON, UNDER THE SUPERVISION OF PROF. MARIAN E. ADDY AND DR. ALEX K. NYARKO.

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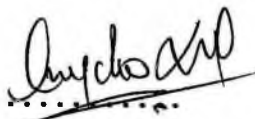
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DEDICATION


TO MY WIFE JOYCE AND CHILDREN (BAFFOUR AND OWURAKU), FOR
THEIR PATIENCE AND SUPPORT.



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ABSTRACT

The major objective of this work was to establish an *in vitro* assay system for evaluating *Ocimum canum*, an anti-diabetic plants, using insulin release from isolated pancreatic β -islet cells of rats. An *in vivo* assay was carried out to confirm the previous work by Hogarh (1996) indicating the anti-diabetic activity of *O. canum*. The effect of the aqueous extract of fresh leaves of *O. canum* on lipid profile of genetically diabetic mice and their lean littermates was also evaluated.

In vivo, the aqueous extract of fresh leaves of *O. canum* reduced fasting blood glucose levels and body weight of genetically diabetic mice and their non-diabetic lean littermates confirming Hogarh's result. The extract lowered serum total cholesterol and LDL-cholesterol levels and raised HDL-cholesterol levels of the experimental animals, but did not affect their triacylglycerol levels. However, aqueous extract from dried leaves lowered, fasting blood glucose levels but not body weight.

The anti-diabetic property of "mirabetic" a plant preparation used for the management of diabetes mellitus was also evaluated *in vivo* using STZ-induced diabetic mice and

their non-diabetic lean littermates. The "mirabetic" extract did not prevent the rise in plasma glucose levels in STZ-induced diabetic mice. In addition, the extract did not lower plasma glucose concentrations of the littermates at the end of the period, and did not affect the body weight of the littermates either.

From *In vitro* experiments, the freeze dried extracts of fresh leaves of *O. canum* significantly, enhanced insulin release from pancreatic β -islet cells. The effect was concentration-dependent up to a concentration of 0.03 mg/ml and thereafter decreased. The *in vitro* system established with *O. canum* was used to evaluate "mirabetic" and *Desmodium adscendens*, a non anti-diabetic medicinal plant. "Mirabetic" significantly enhanced insulin release while *D. adscendens* decreased insulin release, with higher concentrations completely inhibiting insulin release.

The results indicate that glucose-induced insulin release which is the mode of action of *O. canum*, could be used effectively as an *in vitro* assay system to evaluate other anti-diabetic plants.

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

Herbal medicine has been in use for thousands of years by the majority of the world's population. Until the beginning of the 19th century, all medicines were traditional (Jelliffe and Jelliffe, 1977). According to Brancho, (1990) "Two hundred years ago, anyone wishing to be a physician had to study botany, because most medical prescriptions were made from plants".



In the industrialized countries, medicinal plants constitute about 25% of prescribed medicines (Principe, 1989). In Africa, herbal medicine is the first, if not the only health care system available to more than 70% of the population in the poor rural and urban areas. The World Health Organization, in recognition of this fact, underlined the potential role that herbal medicine may play in reinforcing the health care system through the primary health care in developing countries (WHO, 1978).

The resilience of herbal medicine in spite of advances in

modern medicine, shows the effectiveness of herbal medicine. Another contributing factor is that there are some shortcomings of Western medicine in meeting the health needs of the people, especially in relation to diseases such as cancer, cardiovascular ailments and Acquired Immune Deficiency Syndrome (AIDS). With regard to cost, more especially in Africa where there is dramatic worsening of economic situation and almost total dependence on the importation of modern pharmaceutical drugs and technology from the developed countries, the renewed interest in herbal medicine will enable these countries to save foreign exchange and also make them more self-reliant.



Interestingly, most of the medicinal plants of the world are located in the tropics which contain about two-thirds of the plant species of the world, out of which at least 35,000 are estimated to have medicinal value (Principe, 1989). Accordingly, the rain-forest plants have been considered to be a complex chemical storehouse for modern medicine.

The development of modern pharmaceuticals is based on ancient knowledge of medicinal plants. As at now, about 40% of pharmaceuticals in use are derived from natural sources; plants, fungi, microorganisms and animals (Farnsworth,

1984). These drugs are either used directly as such, or as derivatives.

Quite a number of plants and their derivatives are used in different parts of the world for the treatment of various ailments. For example, the rosy periwinkle, *Catharanthus roseus*, is a source of over 75 alkaloids including vincristine, vinblastine, leurosine and leurosidine two of which, vincristine and vinblastine, have been developed as commercial drugs (Mahunnah and Mshiu, 1990). The major use of vinblastine is the treatment of patients with Hodgkin's disease, lymphomas, adrenal testicular, head and neck cancer. Vincristine is widely used in combination with other anti-cancer agents in the treatment of acute lymphocytic leukemia in childhood, and for certain lymphomas and sarcomas, cervical and breast cancer. Quinine from *Cinchona* bark is still used to cure malaria (Gerzon, 1980).

The alkaloids D-tubocurarine, extracted from a liane, *Chondradendon tomentosum*, is widely used as a muscle relaxant in surgery. Saponins, extracted from *Dioscorea*, are chemically altered to produce sapogenins, materials for the manufacture of steroidal drugs. Furthermore, cocaine from the plant *Erythroxylum coca* serves as a model for the syn-

party collectors. For example, Bionics Company from the United Kingdom provided Glaxo Pharmaceuticals, also in the United Kingdom, with plants from Ghana (Mshiu, 1990).

In this regard, a consultancy report by McAlpine and Warriar a United Kingdom firm, indicated that the market potential for herbal drugs in the Western World could range from \$4.9 billion to \$47 billion by the year 2000, if the AIDS epidemic continued unchecked (Mshiu, 1990). Thus herbal medicine, in addition to becoming economic potential through crop export, will also serve as a resource for developing local industries which can substitute for the costly pharmaceutical imports.



The practice of herbal medicine in Ghana dates back to pre-historic times and it is still practiced both in the rural and urban areas. About 70% of the population of Ghana are estimated to depend on herbal medicine. In addition, the country exports some plant materials. For example, Ghana exports an estimated 75-80 tones of *Griffonia simplicifolia* (Kagya) seeds per annum to Germany. The seed contains BSII lectin, a compound which has affinity for type III polyagglutinable red cells (Judd, 1977). Other plants exported include *Hunteria eburnea* (Kanwen-akoa), *Strophanthus*

gratus (Omaatwa) and *Duparquetia orchidacea* (Pikeabo).

To boost the use of medicinal plants in Ghana, the government of Ghana established the Center for Scientific Research into Plant Medicine (CSRPM) in 1973. The Center was charged with screening plant materials reputed to have medicinal properties, as well as their preparation and also verifying their efficacy.

The fact that herbal medicine has been in use since pre-historic times does not mean that it is always safe to use as, a number of toxic compounds have been isolated from some plant extracts. Studies carried out by Atta-Ur-Rahman and Khurshid (1989), showed that, many plant extracts which contain tannin, also contain alkaloids whose side effect is toxic to man. The most important contribution scientists in this field can make would be to carry out proper scientific investigations not only into efficacy but also into possible toxicity of these plant extracts. *In vivo* and *in vitro* assays could be used for these investigations.

Scientific investigations to establish the efficacy of herbal plants have been carried out at the Department of Biochemistry, University of Ghana, Legon. In one of such

investigations Addy and Nyarko (1988), reported that extracts of *Indigofera arrecta*, a plant used locally to treat diabetes, lowered plasma glucose levels in diabetic patients but was unable to return levels to normal. *I. arrecta* also prevented the development of hyperglycemia in young genetically diabetic mice of the C57BL/KsJ db/db strain (Addy *et al.*, 1992). The plant extracts, however, did not respond to increase glucose loads (Nyarko *et al.*, 1993). Another plant that has received much attention in the Department is *Desmodium adscendens* (Papilionaceae).

The extract from this plant was shown to modulate both cyclooxygenase and monooxygenase activities (Addy and Schwartzman, 1992). The plant extract also had both antispasmodogenic and spasmolytic actions on smooth muscles (Addy and Burka, 1987, 1989; Addy, 1989). The Department has also investigated the insecticidal properties of *Piper guineense* (Gbewonyo and Candy, 1992a and 1992b; Gbewonyo *et al.*, 1993).

At the CSRPM, *I. arrecta* Hochst ex A., and *Bridelia ferruginea* Wild. Corr. Spreng., are the two most common local plants used for the treatment of diabetes mellitus. Iwu, (1980) reported that aqueous and methanolic extracts of the

leaves of *B. ferruginea*, significantly lowered plasma glucose levels of fasting albino rats. Further work on flavonoids of *B. ferruginea* indicated that the flavonoids were not hypoglycemic but reduced postprandial plasma glucose levels in diabetic patients (Mills-Robertson, 1993). Earlier work by Addae-Mensah and Munenge (1989) revealed flavonoids as the active hypoglycemic agent in *B. ferruginea*.

Moswa, (1995) had indicated that an extract of *Ocimum gratissimum* Linn (nunun), significantly lowered blood glucose concentrations 7 days after administration, using streptozotocin-induced diabetic rats. However, the extracts did not lower the blood glucose levels in normoglycemic rats. The whole herb and leaf of *O. gratissimum* are popular treatments for diarrhoea (Sofowora, 1993). *In vitro*, aqueous decoctions of *O. gratissimum* relax guinea pig ileum and rat jejunum (El-Said et al., 1969) The plant is also rich in volatile oil which contains up to 75% of thymol known to have antimicrobial activity (El-Said et al., 1969).

Working on *Ocimum canum* Sims, Hogarh, (1996) observed that aqueous extract of the leaves was hypoglycemic in both genetically diabetic mice, representing the non-insulin de-

pendent diabetes mellitus (NIDDM) model of diabetes and their non-diabetic lean littermates. The extract, however, did not cause hypoglycemia in insulin-dependent diabetes mellitus (IDDM model). Kamtchouing *et al.*; (1996), evaluated the hypoglycemic property of *Anacardium occidentale* leaf extract in streptozotocin induced diabetic rats. The leaf decoction decreased the blood glucose levels by 46%.

The scientific investigations into the anti-diabetic activity of the medicinal plants so far discussed made use of whole animals, that is, *in vivo* assays. However, having established the anti-diabetic activity of the medicinal plant through *in vivo* assays, it is important to further investigate its mode of action. This could be better achieved through *in vitro* assays which are usually more convenient and require lesser quantities of the plant extract. Fractionation to obtain the compound responsible for the activity is also important in these scientific investigations. Again, the active fraction could be more efficiently and conveniently assayed using *in vitro* assays.

Having an *in vitro* assay system which would provide a convenient method of screening plants claimed by herbalists to be anti-diabetic would really be beneficial in developing me-

dicinal plants.

The present study was therefore, aimed at establishing an *in vitro* assay for evaluating the anti-diabetic property of *Ocimum canum* using glucose-induced insulin release from pancreatic β -islet cells isolated from rats. The assay was to be used in evaluating other medicinal plants.

The specific objectives were to:

1. confirm the anti-diabetic property of *Ocimum canum* using an *in vivo* assay system.
2. isolate pancreatic β -islet cells from normal rats.
3. determine the amount of insulin released from the pancreatic β -islet cells when challenged with glucose.
4. compare glucose-induced insulin release in the presence and absence of varying concentrations of *O. canum*.
5. evaluate other medicinal plants using this *in vitro* assay system.



1.2 LITERATURE REVIEW

1.2.1 DIABETES MELLITUS

A report by World Health Organization (WHO) indicates that at least 60 million people in the world suffer from diabetes mellitus and that in the Western communities its prevalence is estimated at 2-5%. In Ghana, 2% of the population are diabetic (Gyesie, 1992). In view of the many complications associated with the disease, much attention is being focused on ways of maintaining blood glucose within the reference range so as to promote a healthy lifestyle and to minimize the immediate and long term impact of diabetes on health.

Diabetes mellitus is defined as an acute and chronic metabolic disorder characterized principally by hyperglycemia resulting from an absolute or relative deficiency of metabolically functional insulin or its receptors (Seifter and Englard, 1983). The diagnosis of diabetes mellitus is confirmed if the fasting plasma level is 7.8 mmol/l (142 mg/dl) or more on two occasions, or when the random plasma glucose level is 11.1 mmol/l (202 mg/dl) or more on two occasions or, when fasting level of more than 7.8 mmol/l and the random level of more than 11.2 mmol/l are found (Zilva *et al.*, 1992)

The prominent features of diabetes mellitus are the elevation of blood glucose frequently associated with polyuria, secondary polydipsia and generally accompanied by weight loss (Hall and Evered, 1990). The disease is associated with the development of specific microvascular complication especially in the eye and kidney, and macrovascular disease, such as peripheral vascular and coronary heart diseases.

Neuropathological conditions associated with diabetes mellitus are due to the effect of accumulation of sorbitol (Ononogbu, 1988). Sorbitol (glucitol) is a polyhydroxy alcohol derived from glucose and metabolised through the formation of fructose under normal metabolic condition (fig. 1). In the diabetic state, more sorbitol is produced and not rapidly converted to fructose. Sorbitol does not diffuse through cell membranes easily and therefore accumulates. The osmotic and permeability effects of sorbitol lead to increase in water content in tissues when it accumulates. Sorbitol accumulation in the eye leads to cataracts and other lessions. In addition, increase in sorbitol decreases myo-inositol level and hence, inositol lipids resulting in impaired membrane function (Ononogbu, 1988).

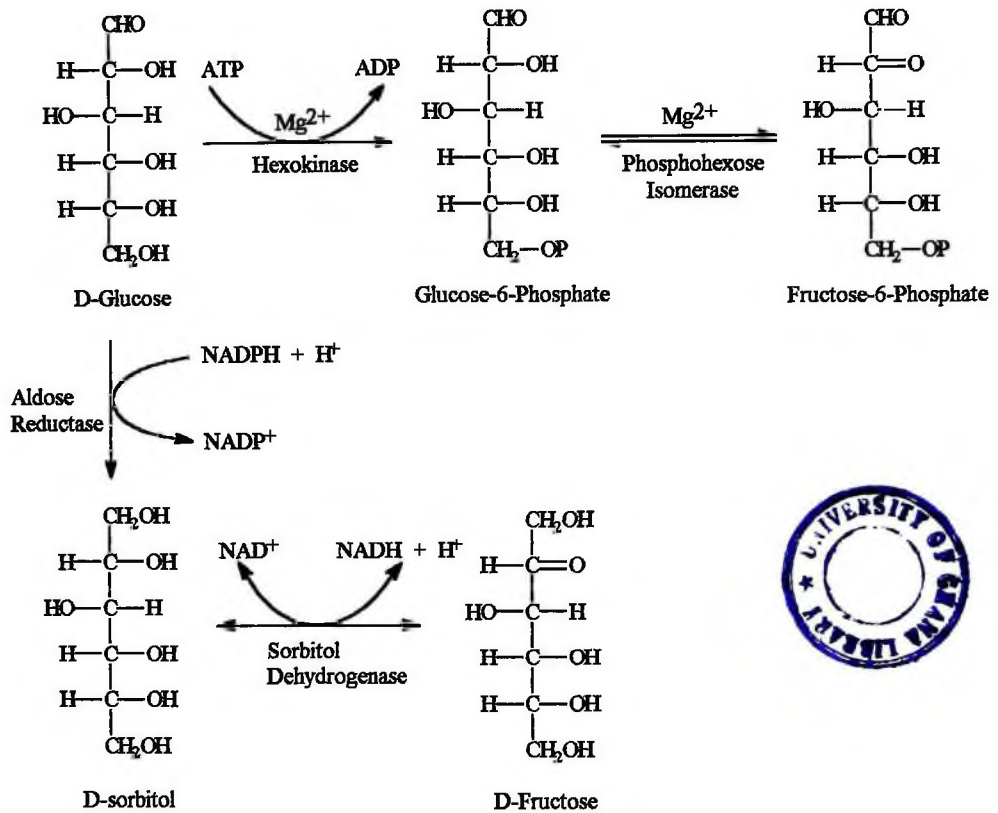


Fig. 1. The Polyol Pathway

The discovery of insulin in 1922 and its rapid application to the successful treatment of diabetes mellitus was a great medical triumph (Wallis *et al.*, 1985). Insulin is a small protein (Mr 5700 Units) consisting of two polypeptide chains, A and B, joined by two disulfide bonds (Lehninger *et al.*, 1993). It is a peptide hormone secreted by the beta cells of the pancreatic islets of Langerhans and is the most important hormone responsible for the control of carbohydrate metabolism and one of the two hormones responsible for the moment-to-moment regulation of blood glucose concentration, the other being glucagon.

Since insulin controls plasma glucose level, high level of plasma glucose could be due to inadequate insulin secretion, or abnormalities in insulin receptors, or post receptor malfunction. The actual cause of hyperglycemia could be established through *in vivo* and, or *in vitro* assays.

In diabetes mellitus, there is the prevalence of hyperlipoproteinemia. Disorders of lipoproteins are detected by measuring lipids. For example, hypercholesterolaemia with little or no elevation of triacylglycerols is due to raised LDL level (Zilva *et al.*; 1992). Also, hypertriglyceridaemia may be due to increased VLDL or chylomicrons or both.

Hyperchylomicronaemia common in patients with poorly controlled diabetes mellitus, is due to the inability of lipoprotein lipase to catalyse the hydrolysis of triacylglycerols because of insulin deficiency or resistance. Insulin is required for the optimal action of the enzyme. In IDDM patients with diabetic ketoacidosis, the level of chylomicrons and VLDL are elevated although in some instances, LDL is also increased (Eder and Berbgan, 1990). In well controlled IDDM patients as judged by glycated hemoglobin (HbA_{1C}) or blood glucose levels, cholesterol and triacylglycerols levels are normal. IDDM patients on insulin therapy have VLDL cholesterol and LDL cholesterol decreased by 20%. NIDDM patients have higher levels of total cholesterol and triacylglycerol (VLDL) compared with non-diabetic patients (Eder and Bergman, 1990).

Artherosclerosis associated with diabetes mellitus in human is due to increases in lipids and lipoproteins resulting in the glycosylation of lipoproteins. This glycosylation may lead to blood vessel degeneration. It is important therefore, to investigate the effect of any anti-diabetic agent on lipid profile in diabetes. Murshed *et al.*, (1996), reported that methanol extract of the leaves of *Gymnena sylvestre* and fruit pulp of *Momordica charantia* had hypogly-

cemic effect on IDDM and NIDDM rat models respectively. On lipid profile of the respective rat models, *G. sylvestre* group showed significantly lower values of serum TG, total cholesterol and LDL cholesterol in IDDM model rats. The *M. charantia* extract also showed lower values of TG, total cholesterol and LDL cholesterol. Thus, *M. charantia* and *G. sylvestre* may help prevent the dyslipidemia in diabetes mellitus. In this study, the effect of *O. canum* leaf extract on the lipid profile (TG, total cholesterol, LDL and HDL) of diabetic and normoglycemic mice was studied.

Based on WHO classification, diabetes mellitus is divided into i) Insulin-dependent diabetes mellitus (IDDM, Type 1), ii) Non-insulin-dependent diabetes mellitus (NIDDM, Type 2), and iii) diabetes associated with other conditions (Zilva et al., 1992).



Insulin-dependent diabetes mellitus

Insulin-dependent diabetes mellitus (IDDM) is characterized by the abrupt onset of symptoms, insulinopenia, proneness to ketoacidosis (because of excess acetone, acetoacetate and hydroxybutyrate production) and dependence on insulin injection to prevent ketosis and to sustain life. Other symptoms are thirst and weight loss. The onset is most common during

childhood (James and Pearson, 1993). Many of such cases have pancreatic beta cell damage which might have been triggered by environmental causes such as viral infections or chemicals. Also, an ongoing autoimmune process results in beta cell failure, and eventually the clinical symptoms of diabetes. Immunological studies have led to the recognition of antibodies to the pancreatic islet beta cells in the blood of these diabetic patients (Atkinson and Maclaren, 1990). Thus, IDDM is the result of an autoimmune attack in the pancreas. The attack is restricted to the hormone producing cells of the pancreas and not the pancreatic cells which secrete digestive enzymes. The immune system slowly eliminates the beta cells with the symptoms appearing only when at least, 80% of the cells are destroyed. Blood insulin levels fall resulting in hyperglycemia.

Non-insulin-dependent diabetes mellitus

Non-insulin-dependent diabetes mellitus (NIDDM) patients are not insulin-dependent or ketosis-prone. Though insulin may sometimes be needed, it is not essential for survival.

NIDDM is known to involve at least three separate abnormalities. These are i) aberrant pancreatic insulin secretion, ii) insulin resistance in peripheral tissues, particularly

muscles, and iii) hepatic glucose overproduction (Taylor, 1989; Kahn and Porte, 1988; DeFronzo, 1988; and Reaven, 1984). Whereas in some NIDDM patients, impaired insulin secretion appears to be predominant deficit in the disease course (Temple *et al.*, 1989; O'Rahilly *et al.*, 1986), in others peripheral insulin resistance is the primary event (Warran *et al.*, 1990). However, the coexistence of defective insulin secretion with insulin resistance characterizes the overt diabetic state.

For the development of target treatments in NIDDM, it is important to identify specific cellular deficits through *in vitro* assays. NIDDM, like IDDM, is characterized by hyperglycemia. When fasting plasma glucose level becomes greater than 12 mM, the ability of insulin to suppress hepatic glucose production becomes impaired (Kolterman *et al.*, 1981) thus, maintaining the diabetic state. This chronic hyperglycemia makes the beta cells nonresponsive to glucose (Leahy, 1990; Leahy *et al.*, 1992).

Onset of NIDDM is common in obese individuals, but the disease can occur in children and in non-obese adults. The risk of developing diabetes in adults with body mass index (BMI) of >30 is five times that of adults with a BMI of <25.

Physically active individuals, that is, those who engage in exercises, for example dancing, running or swimming have only a 40% chance of developing diabetes compared with inactive individuals (Helmrich *et al.*, 1991). On genetic basis, individuals with one subtype of NIDDM have autosomal dominant inheritance which is linked to an abnormal glucokinase gene on chromosome 7p (Hattersley *et al.*, 1992).

Diabetes has been shown to be associated with other conditions such as absolute insulin deficiency due to pancreatic disease (chronic pancreatitis, hemochromatosis, cystic fibrosis), relative insulin deficiency due to excessive growth hormone (acromegaly) or glucocorticoid secretion (Cushing's syndrome), or increased glucocorticoid levels due to administration of steroids, and finally administration of drugs such as thiazide diuretics.

1.2.2 ANIMAL MODELS OF DIABETES MELLITUS.

Diabetes in man results from genetic mutation caused by viral infections, immunologic abnormalities, β -cell exhaustion, hormonal imbalance, insulin resistance, and chemical destruction of β -cells. In order to discover and understand the basic lesions of diabetes in man and the successful application of anti-hyperglycemic agent, it re-

quires indepth and systematic studies using animal models. The two main animal models are genetically (spontaneously) diabetic and chemically induced diabetic animals.



Spontaneously Diabetic Animals.

Diabetes in these animals is due to genetic mutation and serve as NIDDM models. Two of these animals are *Cricetulus griseus* (Chinese hamster mice) and C57BL/KsJ mice. Diabetes in Chinese hamster is caused by a single recessive gene *dd* plus homozygous modifier genes (Yergnian, 1964). Most diabetic Chinese hamsters have decreased pancreatic insulin levels, hyperglycemia, and relative insulin deficiency. Isolated islets of diabetic Chinese hamsters contain less insulin than islets from non-diabetics. Also, the rate of insulin synthesis decreased in the pancreas of diabetic Chinese hamsters (Chang, 1970).

In the C57BL/KsJ inbred strain of mice, the mutation diabetes designated *db/db* is inherited as an autosomal recessive allele on chromosome IV (Dulin et al., 1983). Diabetes in these mice is characterized by early obesity at 1 month followed by hyperglycemia, weight loss and death within 10 months. At 9-12 weeks, blood glucose level reaches a maximum of 22-27 mmol/l (400-500 mg/dl), and remains at this

levels until early death at approximately 10 months. With regard to plasma insulin, the level is elevated by 10-14 days of age reaching a peak by 6-8 weeks and decreases to near normal level by 16-24 weeks. Glucose stimulated release of insulin is less than normal in older and more severely diabetic animals.

Chemically induced Diabetes Mellitus.

This serves as IDDM animal model and is caused by diabetogenic chemicals such as alloxan and streptozotocin (STZ). Alloxan, 2,4,5,6-tetraoxohexahydropyrimidine, causes permanent hyperglycemia by means of its cytotoxicity to the pancreatic β -islet cells. *In vitro*, alloxan exerts inhibitory effect on glucose metabolism in isolated rat islets. However, pretreatment of the islets with 28 mM (509 mg/dl) glucose completely protects against this effect.

STZ, [2-deoxy-2-(3-methyl-3-nitrosoureido) D-glucopyranose], induces diabetic state in most laboratory animals. Its diabetogenicity is related to pancreatic islet β -cell cytotoxicity. The β -cytotoxic effect of STZ is due to the depletion of NAD^+ through the activation of poly ADP-ribose polymerase, an NAD-degrading enzyme in mammalian cells, which is inhibited by nicotinamide (Ho and Hashim, 1972).

The nitrosourea moiety provides its cytotoxic effects, whereas the deoxyglucose moiety facilitates its entry into the β -cells. Generally, plasma immunoreactive insulin is low in STZ-induced diabetic animal (Dulin *et al.*, 1983).

1.2.3 ASSAYING FOR ANTI-DIABETIC PROPERTY OF MEDICINAL PLANTS.

Medicinal plants are used basically as complex mixture (infusion, essential oils, tinctures, and extracts) containing a broad range of constituents. In the crude extract, active principles are generally present at low concentrations only. The test system has, therefore, to be sensitive enough to reliably detect them.

The effectiveness of anti-diabetic plant could be studied using biological assays, both *in vivo* and *in vitro* assays.

In vivo assays

The *in vivo* technique uses intact, whole organisms or, alternatively parts of the animal subjected to perfusion techniques, to maintain as much as possible the integrity of the tissue. The major advantage of *in vivo* assay is that artefacts are reduced. Hence, results from *in vivo* assays are the true situations in the animals. The disadvantages

associated with *in vivo* assays are that it is sometimes difficult to follow the mode of action of the extract. Also, because *in vivo* assays use intact organism, there could be mutual cell interference, permeability barriers and complexities of metabolism associated with multicellular state. Therefore, precise analysis cannot be made. Furthermore, the use of laboratory animals for *in vivo* biochemical experiments may be expensive. In this study, *in vivo* assay was used only to confirm the hypoglycemic property of the *O. canum* extracts being used and to evaluate the anti-diabetic property of a new plant material under investigation.

Among the *in vivo* assays that could be used to verify the anti-diabetic properties of medicinal plants are i) intestinal glucose absorption ii) rat convulsion experiment iii) plasma insulin and glucose levels from pretreated diabetic mice or rats iv) estimation of hemoglobin A_{1C} and v) pancreatic insulin secretion from pretreated diabetic rats or mice.

Intestinal glucose absorption involves an intestinal perfusion technique (Murillo *et al.*, 1972). This was used to study the hypoglycemic activity of aqueous extract of *Salvia*

lavandulifolia in rats fasted for 36 hours previously and anaesthetized with sodium pentobarbital (Zarzuelo *et al.*, 1990). The extract inhibited intestinal glucose absorption at a dose of 0.5 mg/ml of sample/kg of the rats in the perfusing solution.

The rat convulsion experiment involves injecting different doses of the extract into batches of rats or mice and noting convulsion in them resulting from the lowering of blood glucose levels. The glucose is determined before administering the extract and after convulsion using glucose oxidase.

Another *in vivo* assay is to measure plasma insulin and glucose levels from pretreated diabetic and normoglycemic rats or mice. It involves giving aqueous decoction of the plant orally or by intraperitoneal injections of increasing doses and determining the plasma insulin and glucose levels. This procedure was used in this study to confirm the hypoglycemic property of *O. canum*. The procedure had been used to investigate the basis for the antihyperglycemic activity of *Indigofera arrecta* in the rat (Nyarko *et al.*, 1993).

The linkage between hemoglobin and glucose is referred to as



hemoglobin glycosylation. Glycosylation under non-diabetic condition is low. In diabetes mellitus, glycosylation is increased (Ononogbu, 1988).

The percentage of total hemoglobin (Hb) that is glycated as %Hb A_{1C}, %Hb A₁, or total glycated fraction %GHb, is widely regarded as an important tool in diabetes management since it reflects blood glucose concentration of previous 2-3 months, and is therefore, considered as an objective marker of long-term diabetic control (Ursula *et al.*, 1995). This method for estimating Hb A_{1C} with enzyme linked immunoassay system, is based on micro titer plate technology and yields a reference interval of 2.8-4.9% for Hb A_{1C} in a non-diabetic population. The percentages of hemoglobin that is Hb A_{1C} in diabetic (6.86% ± 2.5%) are significantly greater ($P < 0.001$) than in nondiabetic (3.46% ± 0.52%) (Garry *et al.*, 1993).

In vitro assay

As a result of the cost and ethics involved in *in vivo* experiments, the *in vitro* method is adopted where appropriate. The method involves the use of selected tissue or cells. *In vitro* assays have a number of advantages over *in vivo* assays. The cost of running *in vitro* assays is less

than that for *in vivo* assays. Also, *in vitro* assays are done under reduced interference. There is hardly permeability barriers and complexities of metabolisms associated with multicellular state. Thus, physiological and biochemical constraints associated with contiguous cells are reduced. Another advantage is that *in vitro* assays could be used to explain the mode or mechanism of action of the extract. *In vitro* assays are preferred because they are quick and generally, more reproducible.

The major disadvantage of *in vitro* assay is that, the interfering components eliminated may be critical for biological function or regulation of the tissue or cell studied. Thus, extrapolation of results obtained *in vitro* to situations *in vivo* may be unjustified.

From experimenter's viewpoint, assays using whole animals are the least desirable but, so far, no *in vitro* substitute has been found in cases where the entirety of a living animal is required.

Having established the antidiabetic activity of the plant extract through *in vivo* assays, its mode of action could be investigated by *in vitro* assays. This is because the ex-

tract used for the control of diabetes mellitus could be acting at one or more points in the metabolic pathways that are involved in glucose homeostasis. Therefore, the anti-diabetic activity of the plant extract should involve studies into its effects on some of the steps in the metabolic pathway which are deranged in diabetes mellitus. The extract could work through the potentiation of insulin secretion and synthesis, or tissue uptake of glucose.

Tissue uptake of glucose.

This happens to be the main problem with NIDDM. It can be studied using diaphragms, soleus muscle or epididymal adipose tissue. Tissue uptake of glucose is dependent on the activity of glucose transporter proteins. The tissues are incubated in buffer (KRB) containing radioactive glucose and the extract. To inhibit glycolysis, sodium fluoride is added to the medium. After incubation, the level of radioactive glucose incorporated into the muscle is determined by autoradiography. Glucose oxidase may also be used to determine glucose levels before and after the incubation of the tissues.

Tissue uptake of glucose also involves the activity of insulin receptor. Receptors are isolated from the liver and



purified by affinity chromatography on wheat germ agglutinin agarose. Tyrosine or serine kinase activity using radioactive ATP combined with polyacrylamide gel electrophoresis and autoradiography is used to determine the function of the receptor.

Insulin synthesis.

To determine whether the extract can potentiate the synthesis of insulin, labeled amino acid, for example, leucine is added to the incubating medium containing the extract and the isolated islets. Autoradiography is done on the secreted insulin to determine whether more radioactive leucine has been incorporated into the insulin in the presence of the extract (Keith and Goulding, 1992). Both insulin synthesis and secretion appear to be the major problem associated with IDDM. These may be due to damage to the β -cells of the islets of the Langerhans which is responsible for synthesis and secretion of insulin.

Insulin secretion.

In view of the general interest in the disorders of insulin secretion and glucose homeostasis and the cost of such diseases in terms of treatment and quality of life, much research has been devoted to understanding the physiology and

pathophysiology of insulin secretion. Studies of insulin secretion in diabetes use islets or tumor β -cells from animal models (Flatt, 1990).

The use of islets for such studies involves removing pancreas from fasted (18 hours overnight) anaesthetized (sodium pentobarbitone, 60 mg/ml; dosage 60mg/kg) normoglycemic and diabetic rats or mice after their infusion with buffer. The pancreas is then digested with collagenase, the islets separated with dissecting microscope and incubated in Krebs-Ringer bicarbonate medium (Asamoah *et al.*, 1990). Insulin release is measured by ELISA before and after incubating the islets in the presence and absence of the extract.

Studies by Zarzuelo *et al.*, (1990), have shown that pancreatic incubation in the presence of *Salvia lavandulifolia* extract together with 2.7mmol/l glucose (basal) raised insulin levels. Also, *in vitro* studies with pancreatic β -cells have shown that glucose and chloroquine act synergistically in promoting the secretion of insulin (Asamoah *et al.*, 1990).

The pancreas is made up of two different organs contained in one structure. These are the acinar portion which has exo-

crine function, and the endocrine portion which consists of the islets of Langerhans. The exocrine secretions are made up of the alkaline pancreatic fluid and the digestive enzymes which include proteases (trypsin and chymotrypsin), amylase and lipase. The islet cells are scattered throughout the acinar parenchyma.

A number of methods have been described for the isolation of pancreatic islet cells. Hellerström, (1964) isolated enlarged islets cells present in the pancreas of obese hyperglycemic rats using free-hand dissection. Moskalewiski, (1965) isolated islets of Langerhans by incubating the pancreas in collagenase. Lacy and Kostianovsky, (1967) isolated large number of cells from rat pancreas by disrupting the acinar parenchyma prior to incubation with collagenase. The islet cells were separated by sedimentation and centrifugation methods.

The sedimentation method involved allowing the mixture (containing the digested islets) diluted with 15 to 25 ml of Hanks solution, to settle in a conical graduated cylinder. The islet cells were recognized under a dissecting microscope when viewed against a black background. With the centrifugation method, the digested pancreatic cells were

centrifuged at 250g for one and a half minutes and the tissue layered on the surface of four discontinuous sucrose gradient of concentration 1.4, 1.6, 1.7 and 1.8 molar. The gradients were centrifuged at 2600g for 15 minutes at 4°C. The 1.6 and 1.7 molar layers contained the isolated islet cells. In this research, a method for the isolation of pancreatic islets similar to the method of Lacy and Kostianovsky, (1967) was used.

Radio-immunoassay and ELISA are the two common methods used to measure insulin. These have become the methods for blood insulin measurement due to the limitations associated with biological assays with respect to sensitivity and specificity.

In radio-immunoassay, pork insulin is labeled with I^{131} and an antibody to pork insulin is produced in guinea pigs. Predetermined amount of radio-labeled insulin and anti-insulin is incubated with a serial dilution of standard human insulin and plasma for three to five days at 4°C (Norbert, 1970). Aliquots are removed at the end of the incubation and separated from free insulin and antibody-bound insulin. The radioactivity of each of these two fractions is determined, and the ratio of the radioactive count (I^{131} content)

of bound and free insulin is plotted against the concentration of the standard. The amount of insulin in the plasma is determined by comparing the results with the standard curve.

The ELISA test involves creating an antibody-enzyme fusion that can produce a colour reaction with the cognate antigen by simply mixing the sample, the recombinant antibody and substrates for the enzyme. In this study, the ELISA test was used to measure insulin release from pancreatic β -islet cells of rats.

1.2.4 TREATMENT OF DIABETES MELLITUS.

The methods employed for the treatment of diabetes mellitus are different and depend on the type and severity of the disease. They involve insulin administration, oral hypoglycemic drug administration, dietary control and physical activity. Treatment with medicinal plant preparations is likely to relate to oral hypoglycemic agents.

Insulin administration.

Insulin plays a major role in the management of diabetes mellitus. IDDM patients, for example, are put on daily insulin injection. The amount of injectable insulin they

require depends on the extent of islet cell damage, the amount of exercise taken and the amount, type, absorption and the metabolism of food eaten. In IDDM, soluble insulin is usually given 2-3 times daily. In patients with NIDDM, insulin may be given during periods of stress.

The prime target cells of insulin actions are muscles, liver and adipose cells. In the adipose tissue, heart and skeletal muscles, insulin acutely stimulates glucose entry by recruiting glucose transporter proteins, for example, GLUT-4 (Birnbaum, 1989; and Kaetner *et al.*, 1989) from an intracellular pool to the plasma membrane. It has been reported that chronic insulin treatment enhances the synthesis of GLUT-4 transporters (Garvey *et al.*, 1989). In the liver, GLUT-2 glucose transporter plays a key role in glucose homeostasis by mediating bidirectional transport of glucose during opposing physiological states for glucose storage or release (Bell *et al.*, 1990).

As a result of antigenicity problems that have become common with intramuscular injection of insulin (Dolger and Seeman, 1976; Berhanu and Olefsky, 1981) a more convenient way of administering insulin by upper gastrointestinal absorption has been done (Nishihata *et al.*, 1981). This involves the

protection of genetically engineered human insulin molecule by wrapping the outside with fatty cells that breakdown to release the insulin in the small intestine where it is absorbed into the blood (Mennum, 1990).

Insulin therapy in NIDDM patients associated with insulin resistance require doses which are greatly in excess of all estimates of β -islet cell response in nondiabetic individuals (Olefsky and Kolterman, 1981). This results in hyperinsulinemia. The potential complications associated with hyperinsulinemia include atherosclerosis (Stout, 1985) and retinal neovascularization (King *et al.*, 1985). Insulin therapy also results in sodium and water retention. This provides a partial explanation for the prevalence of hypertension in diabetic population (DeFronzo, 1981). Furthermore, the problems associated with weight gain in diabetes may be compounded by insulin acting as an appetite stimulant (Booth and Brookover, 1968; Mackey *et al.*, 1940; Steffens, 1969). This has led to resurgent interest in alternative to insulin monotherapy.

Oral hypoglycemic agents.

Oral hypoglycemic agents are mainly used in treating NIDDM patients. In designing an effective treatment strategy for

NIDDM patients, it is important to develop a regimen that will i) stimulate muscle glucose uptake, so that the disposal of ingested glucose load could be promoted, and ii) inhibit hepatic glucose production, thus lowering fasting glucose levels. Two of such agents, sulphonylureas and biguanides, are widely used as adjuncts to dietary measure in the treatment of NIDDM. Generally, sulphonylureas are well tolerated.

The principal pharmacological effect of sulphonylureas (chlorpropamide and glibenclamide) is the sensitization of the islet beta-cells to glucose, resulting in enhanced endogenous insulin secretion as well as stimulating deposition of glycogen by its action on hepatic enzymes. The main adverse effect associated with sulphonylureas is hypoglycemia. Protracted hypoglycemia due to sulphonylurea-induced suppression of hepatic glucose production is found especially in elderly patients, individuals with intercurrent illnesses and reduced caloric intake, or when taken in combination with compounds having hypoglycemic potential, example ethanol. Sulphonylureas have longer duration of action and, therefore, induce serious hypoglycemia more especially when drug elimination is reduced by renal impairment.



Biguanide therapy is associated with alterations in lactate homeostasis which may result in fatal lactic acidosis (Lebovite and Feinglos, 1983). All biguanides must be avoided in patients with renal impairment, hepatic dysfunction and cardiac failure conditions where drug accumulation or disordered lactate metabolism may predispose to lactic acidosis (Krentz *et al.*, 1994).

In the United States, nearly 40% of the 12 million people afflicted with NIDDM (Gerich, 1989) are being treated with oral hypoglycemic agents (Kennedy *et al.*, 1988).

Dietary Control.

Dietary control forms a major part in the control of diabetes mellitus. In patients with NIDDM, plasma glucose concentrations can often be controlled by diet with weight reduction in the case of obese diabetic patients. Weight reduction, possibly through exercise, improves abnormal glucose metabolism in such patients (Salans *et al.*, 1983). The benefits of weight loss are significant in reducing the risk of heart disease and improving the life expectancy of NIDDM patients. Furthermore, soluble fibre is beneficial as it promotes a slower absorption of food, helping to prevent large swings in blood glucose levels. It may also help to

control blood fat levels. High fibre foods also give a feeling of fullness which is useful for those trying to lose weight. A diabetic patient should increase intake of fruits and vegetables. However, the amount of fat in the diet should be reduced and saturated fats exchanged for polyunsaturated and mono-unsaturated fats. Reducing the amount of saturated fats in a diet helps prevent the onset of heart disease (Wells, 1992).

Physical Activity

Physical activity improves the sensitivity of tissues to insulin independent of any body weight, thereby promoting glucose uptake into muscle even with modest levels of circulating insulin. Thus, with a steady decline in physical activity and progressive weight gain with age, insulin resistance usually arises. Physical activity can therefore, control plasma glucose concentrations in patients with NIDDM.

Anti-diabetic herbal preparations.

Anti-diabetic agents, especially those of plant origin, have received much attention of late. This is as a result of the potential complications associated with hyperinsulinemia which has led to resurgent interest in alternative to insu-

lin monotherapy for patients who have failed on a regimen of diet and sulphonylureas. Another factor is the limited efficacy and questionable safety of the sulphonylurea drugs as well as the risk of lactic acidosis with biguanide type agents. More than 400 traditional plants are reported to possess hypoglycemic properties and investigations have affirmed the potential value of some of these plants (Bailey and Day, 1989). Thus, it is important to research into traditional medicinal plants as possible sources of new hypoglycemic agents.

Based on the work of Farnsworth and Segelman (1971), the following plants showed experimental hypoglycemic activity. *Cocos nucifera* (coconut), *Rauvolfia serpentina* (snakeroot), *Carica papaya* (papaya), *Brassica oleracea* (cabbage), *Spinacia oleracea* (spinach) and *Ipomonea batatas* (sweet potato). The hypoglycemic action of *O. gratissimum* known in Akan as "nunum" has also been reported (Moswa, 1995). Other anti-diabetic medicinal plants are *Salvia lavandulifolia* Vahl, ssp. *Oxyodon* (Zarzuelo et al., 1990) and *C. roseus* (Sofowora, 1982).

In Ghana, a number of medicinal plants have been documented to have anti-diabetic property. These include *Indigofera*

arrecta, *Bridelia ferruginea*, unripe fruit of *Musa paradisiaca*, bark of *Myrianthus arboreus*, *Momordia charantia*, *Costus schlechteri* and *O. canum* (Hogarh, 1996; Ayensu, 1978; Addy et al., 1992, 1995; Addy and Nyarko, 1988; Mills-Robertson, 1993).

1.2.5 OCIMUM CANUM

Ocimum canum Sim, an annual herb which is endogenous to tropical regions of Africa, possesses scented leaves. *O. canum* belongs to the family Lamiaceae. In Rwanda, whole leaves of *O. canum* are usually added to stored foodstuff for the protection against post-harvest insect damage (Dunkel et al., 1986). In folk medicine, the whole plant is reputed to exhibit analgesic and rubefacient properties (Ekundayo et al., 1989). The plant is rich in volatile oil such as linalol (Ntezurubanza, 1987) and eugenol (Ekundayo et al., 1989).

In Ghana, *O. canum* is cultivated around houses. It has several local names. The Gas call it "Kowe" and the Akan "Akoko besa". The plant can grow to a height of about 65 cm. It has small whitish flowers and simple oval leaves that grow opposite to each other. The seeds are tiny and black in color. The mint leaves are used as flavor enhancer

in stews and soups. The plant has been shown to have hypoglycemic activity (Hogarh, 1996). However, its mode of action is not known. The aim of the research reported here was to investigate the mode of action using an *in vitro* method which can be used to screen other anti-diabetic plants.

CHAPTER TWO

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Plant material

Fresh leaves of *O. canum* were collected around various houses in the University of Ghana during the month of November, 1996. Authentication was done at the Herbarium Section, Department of Botany, University of Ghana. Freeze-dried extract of *Desmodium adscendens* was provided by Prof. Marian Ewurama Addy of the Department of Biochemistry, University of Ghana. "Mirabetic", a trade name for an anti-diabetic medicinal plant was from Miracream Products, Tema, Ghana.

2.1.2 Animals

Male Wistar rats, ten-weeks old, genetically diabetic mice of strain C57BL/KsJ db/db and their non-diabetic lean littermates, obtained from the Animal Unit of the Noguchi Memorial Institute for Medical Research (NMIMR), University of Ghana, Legon, were used for the study. The animals were fed *ad libitum* on pelleted animal feed obtained from Ghana Agro Food Company Limited (GAFCO) Tema, Ghana. The composition of the feed was 40% wheat bran, 40% maize, 2% fish meal, 17% cod

liver oil and 1% sodium chloride.

2.1.3 Reagents and Chemicals

Potassium dihydrogen phosphate, potassium chloride, magnesium sulphate, sodium chloride, sodium hydrogen carbonate (NaHCO_3), calcium chloride, anhydrous glucose, sodium pyruvate, glutamic acid, sodium hydroxide, and trisodium citrate were obtained from Fluka Switzerland. Bovine serum albumin (BSA), streptozotocin (STZ), collagenase Type XI (EC 3.4.24.3), specific for the preparation of islets from rat pancreas, and sodium pentobarbitone were from Sigma Chemical Company MO. U.S.A. Fumaric acid was purchased from BHD Chemical Ltd., England. Glucose, HDL-cholesterol, LDL-cholesterol, triglycerides and cholesterol test kits were from Randox Laboratories Ltd., Ireland. Citric acid monohydrate was from Eastman Kodak Company, U.S.A. Enzyme-immunological test for insulin was from Boehringer, Germany. Thiopentone sodium BP was from Rotexmedica GmbH, Germany and Medi-Test Combi 9 (Urine strips) was obtained from Macherey-Nagel Düren, Germany.

2.2 METHODS

2.2.1 Preparation of plant extract.

Fresh leaf extract of *O. canum* was prepared by boiling 120 g



of the leaves in a litre of tap water for 45 minutes. The aqueous extract was filtered and made up to a litre. This was stored in a brown bottle at 4°C and used for *in vivo* studies. Freeze-dried extract was prepared by evaporating some portion of the aqueous extract to a small volume (20-30 ml) using a vacuum rotary evaporator followed by freeze drying. The freeze dried material was stored at -20°C and used for *in vitro* studies involving pancreatic β -islet cells, when required. The yield of the freeze dried material in terms of fresh weight of starting material was 2.5%

For the preparation of extract from dried leaves of *O. canum*, fresh leaves were dried in a warm air oven at 35-40°C to a constant weight. The dried leaves were milled in a laboratory mill (mesh size 1 mm) into a powder. A weighed amount of the milled powder was added to boiling water and boiled for 30 minutes. The aqueous extract was filtered and made up to a volume giving a concentration of 1.8% w/v.

"Mirabetic" extract was prepared by boiling one sachet (17 g) of "mirabetic" in one and half (1.5) litres of tap water for 15 minutes. The extract was filtered and allowed to cool. This was stored at 4°C and used for *in vivo* assays when

required. Freeze dried material was also prepared for *in vitro* assays.

2.2.2 Induction of diabetes

Male non-diabetic lean littermates of strain C57BL/KsJ db/db mice, weighing between 21-27g were used to provide an insulin-dependent diabetes mellitus model. This was achieved by injecting into the mice, freshly prepared streptozotocin solution in 1 mM citrate buffer pH 4.2, intraperitoneally, in a volume of 0.2 ml for five consecutive days. Each mouse received 60 mg per body weight of STZ per day. Diabetes accompanied with frequent urination, was induced 7 days after STZ injection. The urine tested positive to Combi 9 urine strips for both glucose (>500 mg/dl) and ketone bodies.

2.2.3 In vivo experiments with mice

(a) Treatment Schedules

Male genetically diabetic mice of strains of C57BL/KsJ db/db representing the Type II model of diabetes and their lean littermates, 10 weeks old, were used for the study. Four treatment groups of five mice each were set up. These were

- i) genetically diabetic mice on fresh leaf extract,
- ii) genetically diabetic mice on water,

- iii) lean littermates on fresh leaf extract and
- iv) lean littermates on water.

The study period was 13 weeks. In another study, five such genetically diabetic mice were put on *O. canum* aqueous extract prepared from dried leaves for the same period. Control animals were put on water

In a separate study, three main groups of animals were set up to evaluate the effect of the anti-diabetic preparation "mirabetic". In group one, eight non-diabetic lean littermates of strain C57BL/KsJ db/db mice were pretreated for 21 days and then made diabetic by STZ injection. Thereafter, four of the mice were put on water and the other four on aqueous extract of "mirabetic". Group two comprised eight of the lean littermates of the diabetic mice made diabetic by STZ injection. These were not pretreated with "mirabetic". Four of these were put on water and the other four on the extract. In group three were eight of the non-diabetic lean littermates not injected with STZ. Four of these were put on water and the other four on the extract.

The three main groups are summarized below;



- i. Pretreated, STZ diabetic mice on water or extract,
- ii. STZ diabetic mice (no pretreatment) on water or extract,
- iii. Non-diabetic mice on water or extract,

The mice were weighed weekly or fortnightly. After a 12 hour fast, blood samples were collected for glucose determination.

(b) Determination of Plasma Glucose

Blood samples collected from the tail end of the mice were put into heparinized Beckman microfuge tubes and centrifuged at 4500g for five minutes to obtain plasma. The plasma was pipetted into fresh tubes and its glucose concentration determined by the glucose oxidase method. The procedure used was that provided with the Randox Glucose Test Kit.

Precisely, 10 μ l of plasma were pipetted into small test-tubes. Into the "reagent blank" and "standard tubes", 10 μ l each of distilled water and standard reagent were respectively pipetted. Into each tube was added 50 μ l of reagent (containing 4-aminophenazone, glucose oxidase and peroxidase). The contents in each tube were mixed and incubated for 25 minutes at 25°C. The absorbances of the standard and the sample were measured against the reagent blank at a wavelength of 500 nm using Shimadzu Micro-flow Spectrophotometer, model

CL-720. The plasma glucose concentration was determined from the relationship;

$$\frac{\text{Absorbance of test sample}}{\text{Absorbance of standard}} \times \text{Conc. of standard}$$

(c) Determination of Serum Lipids

The mice were killed after the thirteenth week by decapitation and the blood collected into plain, non-heparinized tubes. These were made to stand on ice for 30 minutes and then centrifuged at 4500g for 8 minutes. The sera were separated and stored at -20°C. The total cholesterol, triglyceride, LDL-cholesterol and HDL-cholesterol levels were later determined using Randox Test Kit as described below.

(i) Cholesterol

Cholesterol in the serum was determined after enzymatic hydrolysis (with cholesterol esterase) and oxidation (using cholesterol oxidase) leading to the formation of quinoneimine (the indicator) from 4-aminoantipyrine and hydrogen peroxide in the presence of phenol and peroxidase.

Precisely, 10 µl each of serum sample, standard and distilled



water (blank) were pipetted into tubes so labelled followed by the addition of 1.0 ml cholesterol reagent (containing 4-aminoantipyrine, phenol, peroxidase, cholesterol esterase and cholesterol oxidase in Pipes buffer pH 6.8). The tubes were vortexed and incubated at 20-25°C for 10 minutes. Absorbances were measured at 500 nm on Shimadzu micro-flow spectrophotometer model CL-720. The cholesterol levels in the serum were determined from the relationship;

$$\frac{\text{Absorbance of test sample}}{\text{Absorbance of standard}} \times \text{Conc. of standard}$$

(ii) Triacylglycerols

The basis for this reaction is the enzymatic hydrolysis of triacylglycerols with lipases. This results in the formation of the indicator quinoimine from hydrogen peroxide, 4-aminophenazone and 4-chlorophenol under the catalytic influence of peroxidase. The procedure used involved pipetting 10 µl each of sample, standard and distilled water (blank) into their respective tubes followed by the addition of 1.0 ml reagent (composition; 4-aminophenazone, ATP, lipases, glycerol-3-phosphate oxidase and peroxidase in Pipes buffer pH 7.6). The tubes were vortexed and incubated at 20-25°C for 10 minutes. Absorbances were measured at 500 nm on Shimadzu micro-flow spectrophotometer model CL-720. The

concentration of triacylglycerol in the sample was calculated using a relationship similar to the one above for the determination of cholesterol.

(iii) HDL-Cholesterol

Chylomicron and low density lipoproteins in the serum were precipitated quantitatively by the addition of diluted precipitant containing phosphotungstic acid in the presence of magnesium ions. The cholesterol concentration in the HDL fraction which remained in the supernatant after centrifugation was determined as described below.

Two hundred microlitres of serum were added to 500 μ l of diluted precipitant. This was vortexed and allowed to sit for 10 minutes at room temperature, then centrifuged at 4,500 g for 10 minutes. The supernatant was separated from the precipitate and its HDL-cholesterol content determined by pipetting into test tubes labelled blank, standard and test 100 μ l distilled water, 100 μ l standard and 100 μ l sample respectively. This was followed by the addition of 1.0 ml of cholesterol reagent into each tube. The HDL-cholesterol level was determined according to the method described previously.



(iv) LDL-Cholesterol

The concentration of LDL-cholesterol in the serum was determined by first, precipitating the low density lipoproteins using precipitation reagent which contained heparin and sodium citrate. The cholesterol in HDL and the VLDL which remained in the supernatant were determined as described below.

The procedure involved pipetting 100 μ l serum into 1.0 ml precipitation reagent, vortexing and leaving to stand for 10 minutes at 15 to 25°C. The mixture was then centrifuged at 4,500g for 15 minutes. Precisely, 50 μ l each of distilled water, standard and supernatant were pipetted into test tubes labelled reagent blank, standard and test respectively. This was followed by the addition of 1.0 ml cholesterol reagent. The mixture was vortexed and incubated for 10 minutes at 20 to 25°C. The absorbances at 500 nm were read against reagent blank and the concentration of LDL-cholesterol in the supernatant was calculated from the relationship;

$$\text{LDL-cholesterol} = \text{Total cholesterol} - \text{Cholesterol in the supernatant}$$

The cholesterol levels were determined as described before.

2.2.4 In vitro experiments.

(a) Digestion, Isolation and Incubation of β -Islet Cells

Pancreas from two fasted (12 hr) rats, anaesthetised with thiopentone sodium BP (60 mg/Kg), were removed after cannulating the common bile duct with a polyethylene catheter near the hilus of the liver. This was followed by clamping the distal end of the common bile duct adjacent to the duodenum and disrupting acinar tissue by infusion with Krebs Ringer bicarbonate (KRB) buffer (Appendix 1a). The lymph nodes and fatty tissues were removed from the pancreas after which it was cut into small pieces with scissors and digested with 10 mg collagenase. The mixture was resuspended in cold buffer, centrifuged for 2 minutes at 3500g, 3000g and twice at 1500g. The buffer was removed after each centrifugation and fresh cold buffer added. The β -islet cells were separated with the aid of a dissecting microscope when viewed against a black background. The β -islet cells appeared as free, ovoid or round structures with cream colour.

In another study, ten male Wistar rats, eight weeks old, were pretreated for 21 days with extract from fresh leaves of *O. canum* prior to the digestion of the pancreas and subsequent isolation of the β -islet cells for *in vitro* assay.

Preliminary work was done to determine the incubation period and the number of β -islet cells suitable for the *in vitro* assay. In the determination of the incubation period, groups of six β -islet cells were incubated for 15, 30, 60, 90, and 120 minutes respectively. An incubation period of 60 minutes was found to be suitable based on the amount of insulin produced and the number of β -islet cells seen after incubation. Thereafter, groups of β -islet cells containing different number of β -islet cells (1, 3, 6, and 9) were incubated for 60 minutes. Groups of six β -islet cells incubated for 60 minutes were found to be suitable and therefore, used in the subsequent assays.

In the main study, groups of six β -islet cells were randomly distributed into vials (4×2 diameter) and preincubated for 30 minutes in 1 ml Krebs-Ringer bicarbonate medium (Appendix 1b) containing 3 mM glucose and 3 mg/ml bovine serum albumin, pH 7.4 in a shaking water bath (66 cycles per minute) at 37°C under a 95%O₂/5%CO₂ gas phase. The islets were further incubated for 1 hr in a fresh 1 ml KRB (Appendix 1c) containing 3 mg/ml BSA, 16.7 mM glucose under 95%O₂/5%CO₂ gas phase. The buffer was removed and its insulin concentration determined by an enzyme-linked immunosorbent assay (ELISA) procedure.



Insulin determination

An ELISA method used was that of Boehringer, (1994). In this assay, insulin in the sample is bound by the monoclonal anti-insulin antibodies coated onto the inside wall of plastic tubes. The addition of incubation solution (containing anti-insulin antibody-peroxidase [POD] conjugate) leads to the binding of insulin-POD conjugate to the other side of the sample insulin not occupied by the insulin antibodies. In a separation step, the anti-insulin antibody-POD conjugate not bound by sample insulin is removed by a washing solution. The final step, that is, an indicator reaction, involves the addition of substrate and chromogen di-ammonium 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonate) (ABTS®). The substrate reacts with the anti-insulin-POD conjugate leading to the generation of hydrogen peroxide which then reacts with the chromogen resulting in the development of colour whose intensity is determined spectrophotometrically at 420 nm.

For the procedure, 0.1 ml each of sample, different concentrations of standard were put into plastic tubes followed by the addition of 1 ml solution 1a (containing anti-insulin-POD conjugate, pH 7.0). The tubes were incubated for 120 ± 10 minutes after which the contents were aspirated and the tubes rinsed immediately by filling to the brim with

Enzymum-Test® Washing Solution and the contents aspirated within 3-5 minutes. This was followed by the addition of 1 ml substrate-chromogen solution to each tube and the tubes incubated for 60 ± 10 minutes. The contents of the tubes were mixed and their absorbances read at 420 nm. The concentration of insulin in the sample was determined from a calibration curve (appendix 6)

(c) Evaluation of anti-diabetic property of plant extract

To evaluate the anti-diabetic properties of *O. canum*, *D. adscendens* and "Mirabetic" based on glucose-stimulated insulin release, varying concentrations of the extracts were added to Krebs-Ringer bicarbonate buffer containing groups of six β -islet cells and incubated for 60 minutes during the second incubation as described in the main study. Insulin release was measured by (ELISA) procedure as already described.

(d) Different concentrations of glucose on insulin release

The β -islet cells were incubated in either 5 mM or 16.7 mM glucose in the presence or absence of 0.03 mg/ml *O. canum* extract and incubated for 60 minutes. Insulin released was measured by (ELISA) procedure as already described.

2.2.6 Statistics

Statistical analyses were conducted using the SigmaStat, Version 1.01 (Copyright Jandal Corporation, 1986-1992) or Quick CSS, Version 3.1 (Copyright, StatSoft, Inc., 1991) software packages. Analyses performed included descriptive statistics, tests of homogeneity and comparison of group means using either parameter (Student's t-test or analysis of variance (ANOVA) or the corresponding non-parametric procedures. The Student's Newman-Keul's Post hoc test was used for all statistical analyses. Graphs were plotted with Microsoft excel for window 95 Version 7.5 (Copyright Microsoft Corporation 1985-96).



CHAPTER THREE

RESULTS

3.1 In vivo Assays

3.1.1 Effect of aqueous extract of fresh leaves of *O.canum*.

Plasma Glucose Concentration.

The blood glucose concentration of genetically diabetic mice on extract decreased steadily during the 13-week period of study. By week 13, the extract had lowered the concentration of fasting blood glucose the most ($62.13 \pm 0.34\%$) a value similar to that obtained at week 11. The reduction was statistically significant compared to the initial concentration ($P < 0.05$). The percentage decreases in fasting blood glucose levels, calculated for the 2-weekly intervals, were as follows: 0.0, 21.26 ± 2.17 , 39.74 ± 3.64 , 50.23 ± 2.64 , 57.77 ± 0.81 , 62.08 ± 0.29 , and 62.13 ± 0.34 . Statistically, no significant difference was observed between weeks 11 and 13 (Figure 2).

A decrease in fasting blood glucose concentration was also observed during the 13-week period for the genetically diabetic mice on water. The decrease was, however, not statistically significant.

Pairwise comparison between genetically diabetic mice on extract and those on water showed that the percentage decrease in fasting blood glucose concentrations in the two groups of mice were statistically significant ($P < 0.05$) throughout the 13-week period.

Percentage decrease in fasting blood glucose concentrations were observed in lean littermates on extract. Optimum decrease was observed at week 9. Statistically significant differences ($P < 0.05$) were observed between week 1 and weeks 5, 7, 9 and 13, and also between week 3 and 7. However, values, obtained from weeks 5 to 13 were statistically not significant (Figure 2). The percentage decreases in fasting blood glucose concentrations for lean littermates on water during the 13-week period were statistically not significant. Between the two groups, that is, littermates on extract and those on water, statistically significant differences ($P < 0.05$) were observed from weeks 7 to 13.

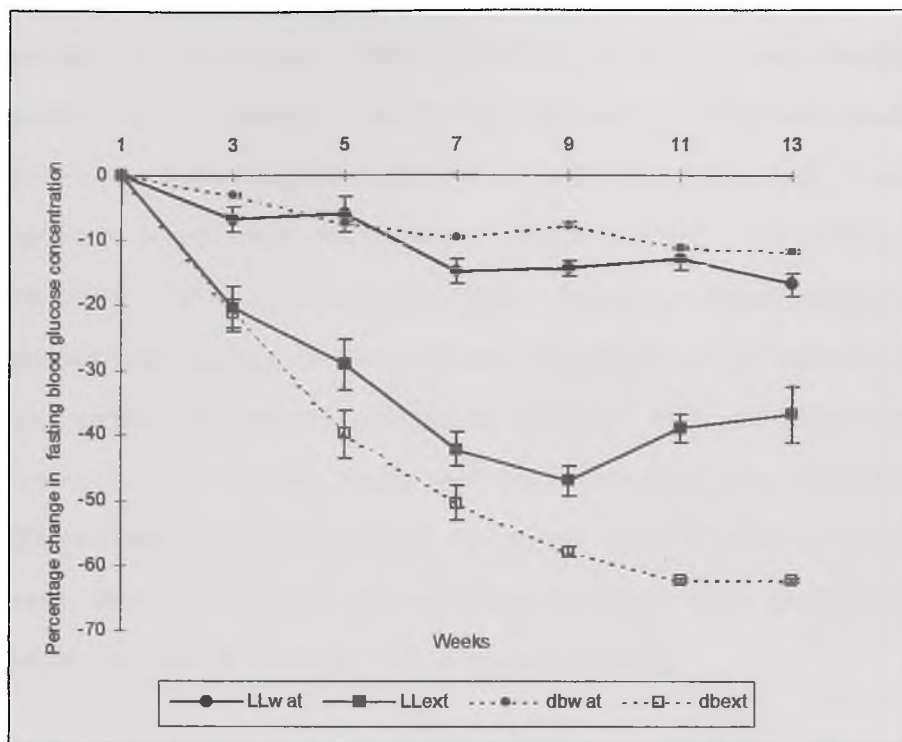


Figure 2. Effect of aqueous extract of fresh leaves of *O. canum* on fasting blood glucose concentration in genetically diabetic mice and their lean littermates, calculated as percentage change in blood glucose concentrations from the raw data in appendix 4, Table 1. Values are means of 5 determinations. Error bars indicate standard error of the mean (SEM). LLwat= littermates on water, LLezt = littermates on extract, dbwat = diabetic mice on water, dbext = diabetic mice on extract.



Body Weight

Figure 3 is a graphical representation of percentage in body weight of the mice. There were decreases in body weights of genetically diabetic mice on extract. The decrease was statistically significant ($P < 0.05$) only at week 7 with an optimum percentage decrease of 9.69 ± 4.33 . Only this group of mice had negative percentage change in body weight. The percentage change in body weight of genetically diabetic mice on water increased slightly during the 13-week period. However, the weight gain was statistically not significant. Comparing, the two groups of mice, significant differences were observed between genetically diabetic mice on extract and those on water during the 13-week period.

Slight increases in percentage weight gain were observed in lean littermates on extract. The observed weight gain was, however, not significant. A steady gain in body weight was observed in lean littermates on water. The weight gain was significant between week 3 and weeks 7, 9, 11, and 13. The weight gain from weeks 7 to 13 were, however, not significant. Pairwise comparison between lean littermates on extract and those on water were statistically significant ($P < 0.05$). However, percentage increases in weight over the experimental period for LLExt dbwat were the same.

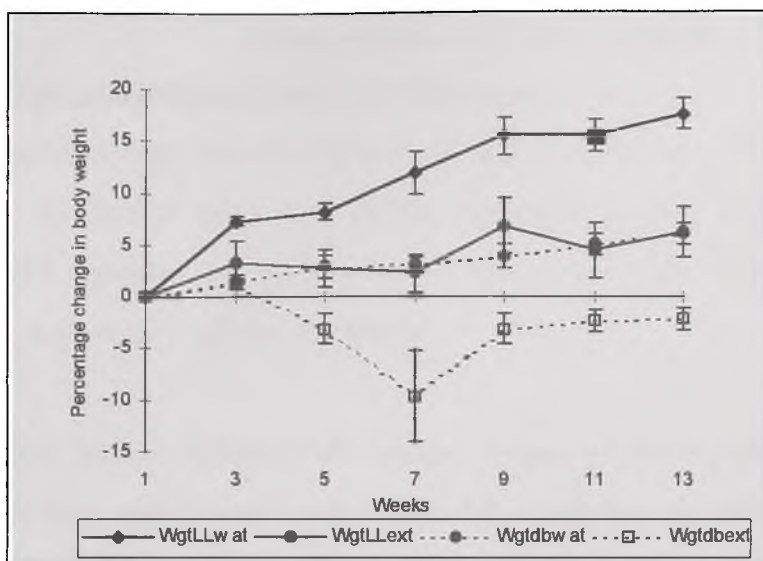


Figure 3. Effect of aqueous extract of fresh leaves of *O. canum* on body weight of genetically diabetic mice and their lean littermates, calculated as percentage change in body weight using the data in appendix 4, Table 2. Values are means of 5 determinations. Error bars indicate standard error of the mean (SEM). WgtLLwat = weight of littermates on water, WgtLLext = weight of littermates on extract, Wgtdbwat = weight of diabetic mice on water, Wgtdbext = weight of diabetic mice on extract.

Serum Lipids and LipoproteinsSerum cholesterol and triacylglycerols

Figure 4 depicts the effect of aqueous extract of fresh leaves of *O. canum* on total serum cholesterol and triacylglycerol (TG) levels in genetically diabetic mice and their lean littermates, after 13 weeks.

Total serum cholesterol levels in genetically diabetic mice on extract were lower than levels of those on water. One way analysis of variance showed that there was a statistically significant difference ($P < 0.05$) between these levels in genetically diabetic mice on extract and those on water (4.34 ± 0.15 vs. 4.75 ± 0.07). Though total cholesterol levels were lower in littermates on extracts (4.22 ± 0.55) than those on water (4.53 ± 0.05), the difference was not significant.

With respect to triacylglycerols the values calculated for genetically diabetic mice on extract and those on water were 1.21 ± 0.15 vs 1.26 ± 0.15 respectively. The results for the lean littermates on extract and those on water were 0.93 ± 0.13 vs 1.15 ± 0.05 respectively. No statistically significant differences ($P = 0.305$) were observed within or between groups of genetically diabetic mice and their lean littermates on extract and those on water.



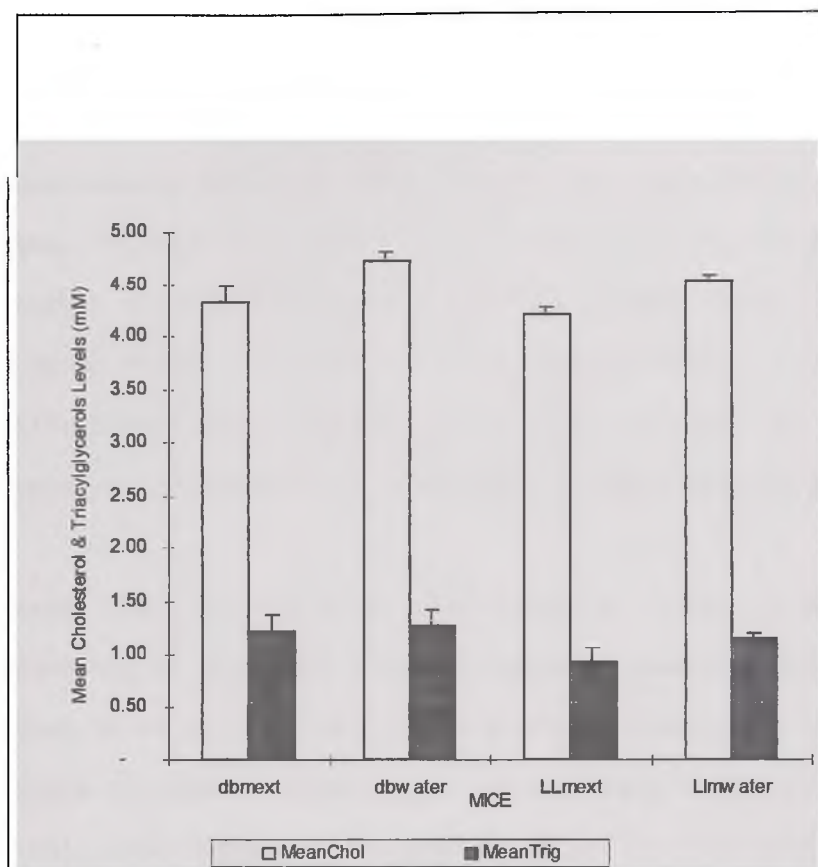


Figure 4. Effect of aqueous extract of fresh leaves of *O. canum* on total cholesterol and triacylglycerol levels of genetically diabetic mice and their lean littermates. Values are means of 3 determinations. Error bars indicate standard error of the mean (SEM). LLwater = littermates on water, LLmext = littermates on extract, dbwater = diabetic mice on water, dbmext = diabetic mice on extract.

Serum HDL-Cholesterol and LDL-Cholesterol

Serum HDL-C, determined after 13 weeks of treatment with aqueous extract of fresh leaves of *O. canum* was higher in genetically diabetic mice on extract compared to those on water ($2.34 \pm 0.02\%$ vs $2.22 \pm 0.04\%$) For lean littermates on extract and those on water, the mean (\pm SEM) HDL-C levels were $2.52 \pm 0.06\%$ and $2.39 \pm 0.03\%$ respectively. Significant differences were observed ($P < 0.05$) between the levels in genetically diabetic mice on extract and those on water.

Serum LDL-C levels were significantly lower ($P < 0.05$) in genetically diabetic mice on extract compared to those on water ($1.45 \pm 0.06\%$ vs. $1.80 \pm 0.10\%$). Similarly, serum LDL-C levels in lean littermates on extract were significantly ($P < 0.05$) lower compared to those on water ($1.23 \pm 0.08\%$ vs. $1.57 \pm 0.08\%$) Results showing the effect of the extract on HDL-C and LDL-C levels are represented in figure 5.

3.1.2 The effect of aqueous extract of dried leaves of *O. canum*.

Fasting blood glucose level

Having shown that aqueous extract of fresh leaves of *O. canum* significantly lowered fasting blood glucose levels in geneti-



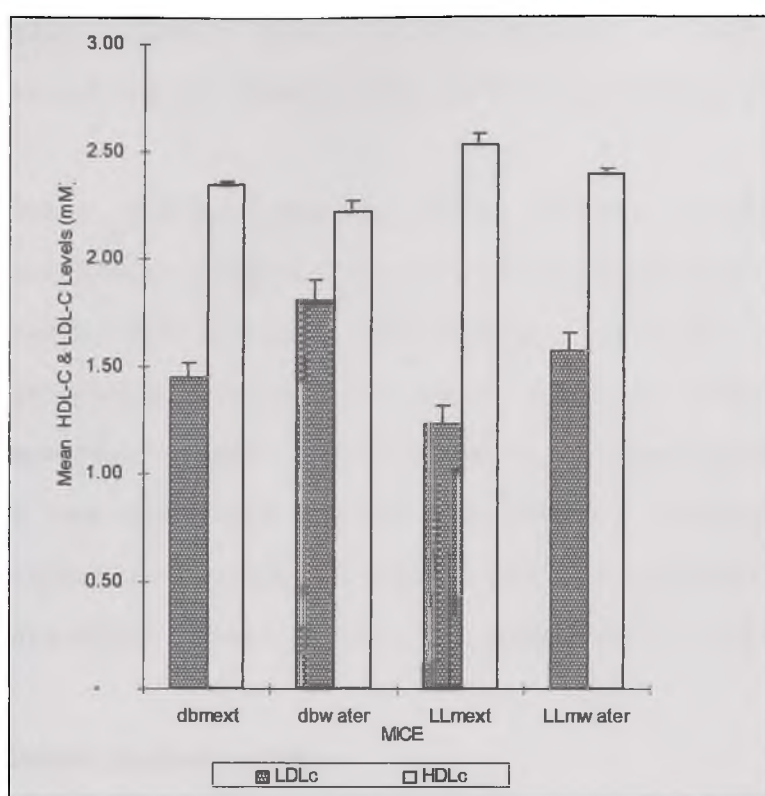


Figure 5. Effect of aqueous extract of fresh leaves of *O. canum* on HDL-C and LDL-C levels of genetically diabetic mice and their lean littermates. Values are means of 3 determinations. Error bars indicate standard error of the mean (SEM). LLmw water = littermates on water, LLmext = littermates on extract, dbw water = diabetic mice on water, dbmext = diabetic mice on extract.

cally diabetic mice, it was decided to test the aqueous extract of the dried leaves on the genetically diabetic mice.

Within groups, fasting blood glucose concentrations of genetically diabetic mice on the extract were significantly lowered during the 13-week period ($P < 0.05$). There was no significant change in blood glucose concentrations in genetically diabetic mice on water. Comparing the two groups at the same time period, the extract decreased the blood glucose concentration significantly ($P < 0.05$) at all time intervals. These results are presented in figure 6.

Change in body weight

The percentage change in body weight of genetically diabetic mice on extract and those on water are shown in figure 7. No significant differences were observed within groups, that is, mice on extract those on water. However, gain in weight in mice on water was higher compared to those on extract at all time intervals.



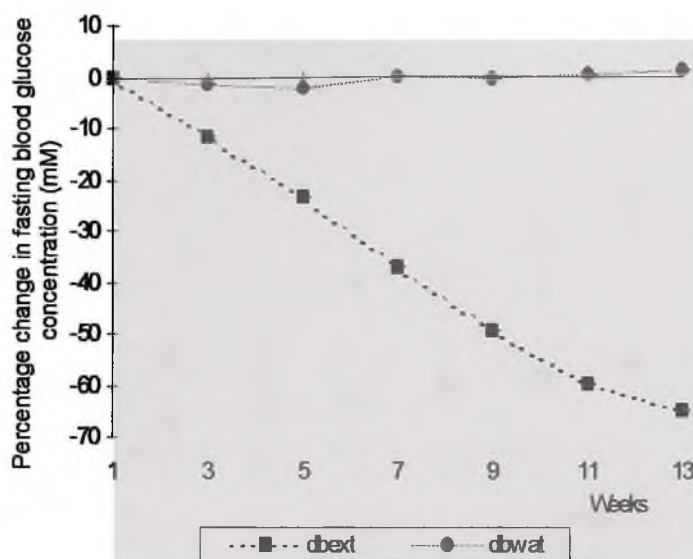


Figure 6. Effect of aqueous extract of dried leaves of *O. canum* on fasting blood glucose levels of genetically diabetic mice calculated as percentage change using the data from appendix 5. Values are means of 5 determinations. Error bars indicate standard error of the mean (SEM). dbwat = diabetic mice on water, dbext = diabetic mice on extract.

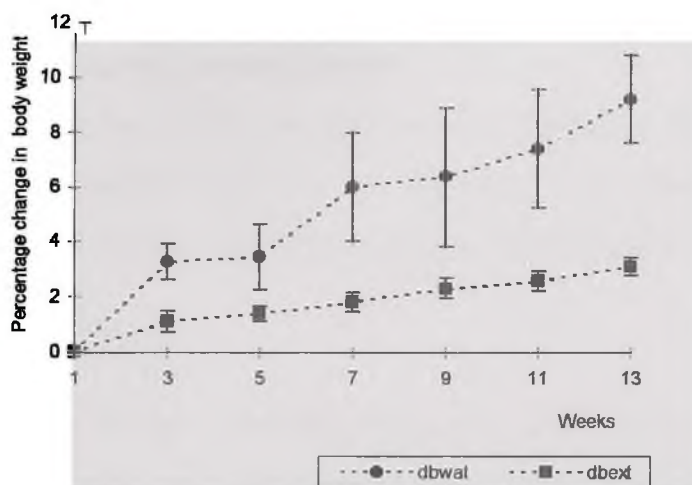


Figure 7. Effect of aqueous extract of dried leaves of *O. canum* on body weight of genetically diabetic mice calculated as percentage change using the data from appendix 5. Values are means of 5 determinations. Error bars indicate standard error of the mean (SEM). dbwat = diabetic mice on water, dbext = diabetic mice on extract.



3.1.3 Effect of aqueous extract of "Mirabetic" on STZ-induced diabetes.

Plasma glucose concentration

Lean littermates of the genetically diabetic mice pretreated with "mirabetic" extract for 21 days were injected with STZ and put on either extract or water for the rest of the period of study.

After the 21 day pretreatment, that is, before the mice were injected with STZ, the fasting blood glucose levels decreased (16.32%) significantly ($P < 0.05$). The fasting blood glucose concentrations in the pretreated STZ-induced diabetic mice on extract (PSTZEx) increased significantly ($P < 0.05$) throughout the study period (Table 1a). The fasting blood glucose concentration calculated for pretreated STZ-induced diabetic mice on water (PSTZWt) also increased significantly ($P < 0.05$) during the same time period.

Comparing the two groups of mice, that is, PSTZEx and PSTZWt at the same period, the increase in blood glucose in PSTZEx mice was less than in PSTZWt mice. A statistically significant difference ($P < 0.05$) was observed between PSTZEx

and PSTZWt during the period of study. PSTZEx died at week 8 and PSTZWt died at week 7.

In another set-up, STZ-induced diabetic mice not pretreated, were also put either on extract or on water during the same period of study. Within each group, the increase in blood glucose concentration was significant ($P < 0.05$). However, the increase in the STZ-induced diabetic mice on water (STZWt) was significantly ($P < 0.05$) greater than that in STZ-induced diabetic mice on extract (STZEx). Whereas, STZ-induced diabetic mice (not pretreated) on water died at week 5, those on died at week 6 (Table 1a).

Comparison between the pretreated STZ-induced diabetic mice on water and STZ-induced diabetic mice (without pretreatment) on, showed that the percentage increase in blood glucose concentrations was significantly higher ($P < 0.05$) in mice without pretreatment than in pretreated STZ-induced diabetic mice on water during the same time period. Also, blood glucose concentrations in STZ-induced diabetic mice (without pretreatment) put on extract was significantly ($P < 0.05$) higher than in pretreated STZ-induced diabetic mice on extract during the same time period.

Table 1. Effect of "Mirabetic" on fasting blood glucose levels in mice

1a. STZ-induced diabetic mice				
Blood glucose levels (mmol/l)*				
Weeks	Pretreatment		No pretreatment	
	Extract	Water	Extract	Water
0	4.78 ± 0.17**	4.73 ± 0.23**		
1	4.00 ± 0.09***	4.00 ± 0.12***	4.44 ± 0.13'	4.43 ± 0.17'
2	8.18 ± 0.37	8.93 ± 0.19	10.55 ± 0.25	11.55 ± 0.23
3	10.28 ± 0.38	12.15 ± 0.22	11.95 ± 0.30	12.85 ± 0.31
4	10.98 ± 0.49	13.18 ± 0.11	14.00 ± 0.20	16.90 ± 0.20
5	12.18 ± 0.44	15.95 ± 0.23	15.88 ± 0.23	23.50 ± 0.40
6	14.25 ± 0.45	19.15 ± 0.36	19.45 ± 0.26	
7	18.33 ± 0.39	21.50 ± 0.28		
8	21.08 ± 0.38			

* Given as mean ± standard of the mean

** Blood glucose levels before pretreatment

***Blood glucose levels 21 days after pretreatment and just before STZ injection

' Blood glucose levels just before STZ injection

1b. Lean littermates		
Blood glucose levels (mmol/l)*		
Weeks	No pretreatment	
	Extract	Water
1	4.38 ± 0.05	4.33 ± 0.05
2	4.05 ± 0.06	4.20 ± 0.07
3	3.25 ± 0.13	4.08 ± 0.03
4	3.58 ± 0.09	4.13 ± 0.07
5	4.03 ± 0.02	4.15 ± 0.06
6	4.18 ± 0.07	4.33 ± 0.09
7	4.30 ± 0.04	4.30 ± 0.06



During the same study period, lean littermates of genetically diabetic mice were also put either on extract or on water. Significant difference in blood glucose concentrations were observed at weeks 3 and 4 within littermates on extract. No significant differences in blood glucose concentrations were observed between littermates on extract and those on water after week 5 (Table 1b).

Change in body weight

The effect of aqueous extract of "mirabetic" on changes in body weight of STZ-induced diabetic mice is presented in table 2a. Within groups, significant ($P < 0.05$) decreases in weight were observed in all STZ-induced diabetic mice during the experimental period. Comparing the two groups of pretreated mice, the decreases in weight between PSTZEx and were statistically significant ($P < 0.05$) with weight loss greater in PSTZWt than PSTZEx (Table 2a).

In the STZ-induced diabetic mice without pretreatment, weight loss in STZWt was significantly ($P < 0.05$) greater than in STZEx at all time intervals. In the littermates of genetically diabetic mice, there were no significant differences in weight gain within groups and between groups (Table 2b).

Table 2. Effect of "Mirabetic" on body weight of STZ-induced diabetic mice.

2a. STZ-induced diabetic mice				
Body weight*				
Weeks	<u>Pretreatment</u>		<u>No pretreatment</u>	
	Extract	Water	Extract	Water
0	23.78 ± 0.70**	24.08 ± 0.72**		
1	23.25 ± 0.67***	24.00 ± 0.57***	21.75 ± 0.96'	21.70 ± 1.14'
2	20.52 ± 0.67	22.16 ± 0.48	19.65 ± 1.30	18.86 ± 0.82
3	18.83 ± 0.52	20.15 ± 0.25	18.22 ± 0.94	17.01 ± 0.54
4	18.25 ± 0.38	18.18 ± 0.14	17.02 ± 0.66	15.15 ± 0.36
5	17.84 ± 0.39	16.89 ± 0.29	15.37 ± 0.48	14.38 ± 0.35
6	16.97 ± 0.40	15.65 ± 0.19	14.15 ± 0.46	
7	15.91 ± 0.25	15.16 ± 0.13		
8	15.55 ± 0.26			

* Given as mean ± standard of the mean

** Blood glucose levels before pretreatment

***Blood glucose levels 21 days after pretreatment and just before STZ injection

' Blood glucose levels just before STZ injection

2b. Lean littermates		
Body weight *		
Weeks	<u>No pretreatment</u>	
	Extract	Water
1	19.37 ± 0.83	20.33 ± 0.35
2	19.17 ± 0.83	20.13 ± 0.26
3	18.42 ± 0.83	20.10 ± 0.44
4	18.35 ± 0.88	21.00 ± 0.62
5	18.55 ± 0.96	21.03 ± 0.41
6	18.33 ± 0.74	21.41 ± 0.46
7	18.50 ± 0.94	20.97 ± 0.53

3.2 In vitro Assay

3.2.1 Time Course of Insulin Release

The time course of insulin release from pancreatic β -islet cells incubated in Krebs Ringer bicarbonate buffer at various time intervals is presented in figure 8. The amount of insulin released was approximately linear during the first 60 minutes of incubation. Statistically significant differences ($P < 0.05$) were observed in the amount of insulin released between the different time periods. Calibration curve for determining insulin release is presented in appendix 6.

3.2.2 Effect of β -islet cell number on insulin release

Figure 9 illustrates the effect of β -islet cell numbers on insulin release. Statistically significant differences ($P < 0.05$) were observed among the cell groups in the amount of insulin released from the different groups representing different numbers of β -islet cells with the greatest release (per unit cell) in the group with 6 cells. Therefore, 6 was chosen for the subsequent *in vitro* assays.



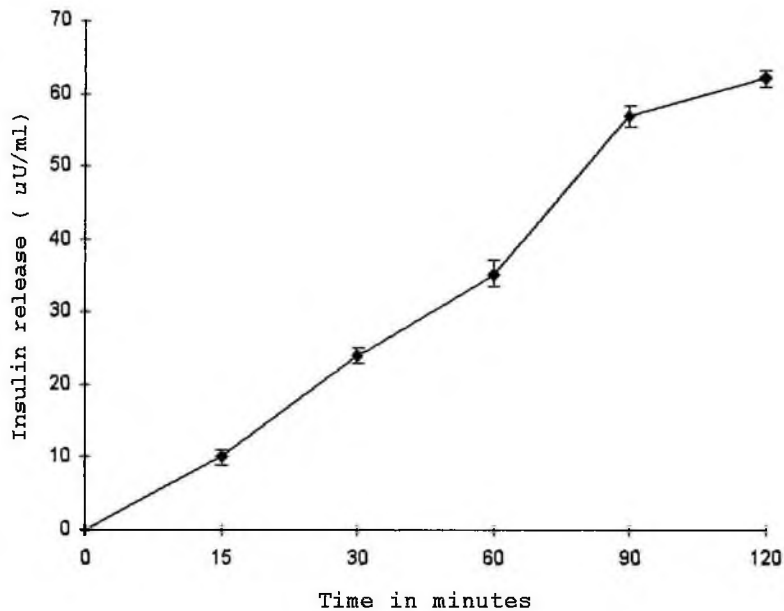


Figure 8. Time course of insulin release from pancreatic β -islet cells of rat. Values are means of 3 determinations. Bars indicate standard error of the mean.

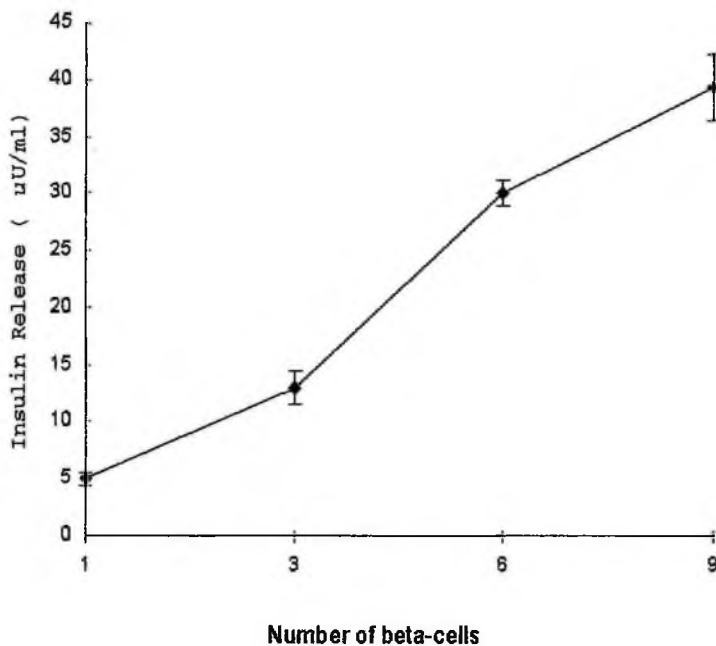


Figure 9. Effect of β -islet cell concentrations on insulin release. Values are means of 3 determinations. Bars indicate standard error of the mean.

3.2.3 Effect of freeze-dried extract of *O. canum* on glucose-stimulated insulin release

Table 3a shows the amounts of insulin released from pancreatic β -islet cells of rats, incubated in varying concentrations of *O. canum*. Among the various extract concentrations, the difference between the amounts of insulin released were statistically significant ($P < 0.001$). The amount of insulin released increased with increasing extract concentration. The extract concentration which gave maximum insulin release was 0.03 mg/ml. However, the amount of insulin released decreased after 0.03 mg/ml.

Administering the extract to the rats (pretreatment) for 21 days before isolating the β -islet cells increased the amount of glucose-induced insulin release in the presence of the various extract concentrations significantly ($P < 0.05$). The highest amount of insulin release ($144.6 \pm 10.1 \mu\text{U/ml}$) also occurred at 0.03 mg/ml of extract.

A comparison of the effect of various concentrations of freeze dried extract of *O. canum* on insulin release from pancreatic β -islet cells of rats pretreated and those not pretreated with the extract is illustrated in figure 10. The results show



that the amounts of insulin released at 0.03 mg/ml extract concentration which happened to be the maximum in both groups of rats, was significantly greater ($P < 0.05$) in non pretreated rats than in pretreated rats. At 0.005 mg/ml and 1.0 mg/ml, the amounts of insulin released were significantly greater ($P < 0.05$) in pretreated rats than in nonpretreated rats. However, at 0.1 mg/ml and 0.5 mg/ml no significant differences were observed in the amounts of insulin released between the pretreated and nonpretreated rats.

Table 3. Effect of freeze-dried extract of fresh leaves of *O. canum* on glucose-stimulated insulin release from pancreatic β -islet cells of rats.

3a. No pretreatment		
Conc. of extract (mg/ml)	Conc. Of insulin release (μ U/ml)	%change in insulin release (μ U/ml)
0.0	30.67 \pm 3.57	0.0 \pm 0.0
0.001	38.67 \pm 1.76	29.17 \pm 1.46
0.005	44.33 \pm 2.33	50.00 \pm 2.50
0.01	96.40 \pm 3.72	218.06 \pm 3.14
0.03	134.33 \pm 1.85	350.28 \pm 5.29
0.1	84.33 \pm 1.20	182.17 \pm 3.12
0.316	68.66 \pm 2.90	130.79 \pm 3.05
0.5	59.67 \pm 2.73	99.19 \pm 2.94
1.0	49.33 \pm 3.33	64.82 \pm 1.93

3b. Pretreatment		
Conc. of extract (mg/ml)	Conc. Of insulin release (μ U/ml)	% change in insulin release (μ U/ml)
0.0	41.00 \pm 4.00	0.0 \pm 0.0
0.005	68.35 \pm 6.65	69.91 \pm 3.28
0.03	144.60 \pm 10.10	258.51 \pm 5.96
0.1	112.10 \pm 11.90	178.91 \pm 5.62
0.5	81.60 \pm 4.20	101.95 \pm 2.99
1.0	95.45 \pm 2.45	134.45 \pm 1.690

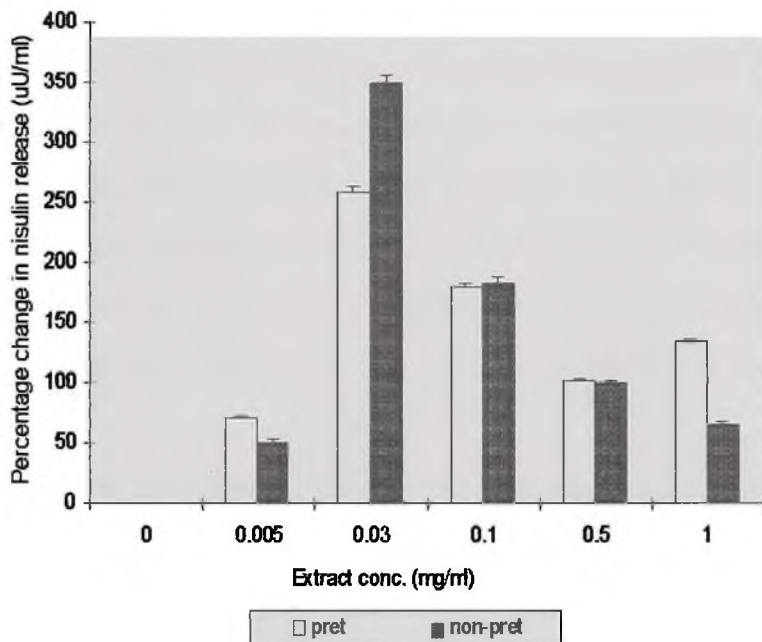


Figure 10. Comparison of insulin release from pancreatic β -islet cells of rats receiving pretreatment and those not receiving *O. canum* pretreatment. Bars indicate standard error of the mean.

3.2.4 Effect of Glucose Concentration on Insulin Release

The effect of glucose concentration on insulin release in the absence and presence of 0.03 mg/ml *O. canum* extract was studied using a lower glucose concentration, 5.0 mM, in addition to the 16.7 mM concentration of glucose used in the other experiments. In the presence of the extract, the amount of insulin released when the β -islet cells were challenged with the two concentrations of glucose was significantly ($p < 0.05$) greater than the amount of insulin released in the absence of the extract. The result is shown in figure 11.

In addition, the amount of insulin released in the presence of 0.03 mg/ml *O. canum* when the β -islets were challenged with 5.0 mM of glucose was higher than the amount released when the β -islet cells were challenged with the higher glucose concentration (16.7 mM) in the absence of the extract although, the difference was not statistically significant.

3.2.5 Effect of other Medicinal Plant Extracts (*Desmodium adscendens* and "Mirabetic") on Insulin Release

The effects of "Mirabetic" and *Desmodium adscendens* on insulin release from pancreatic β -islet cells of rats were

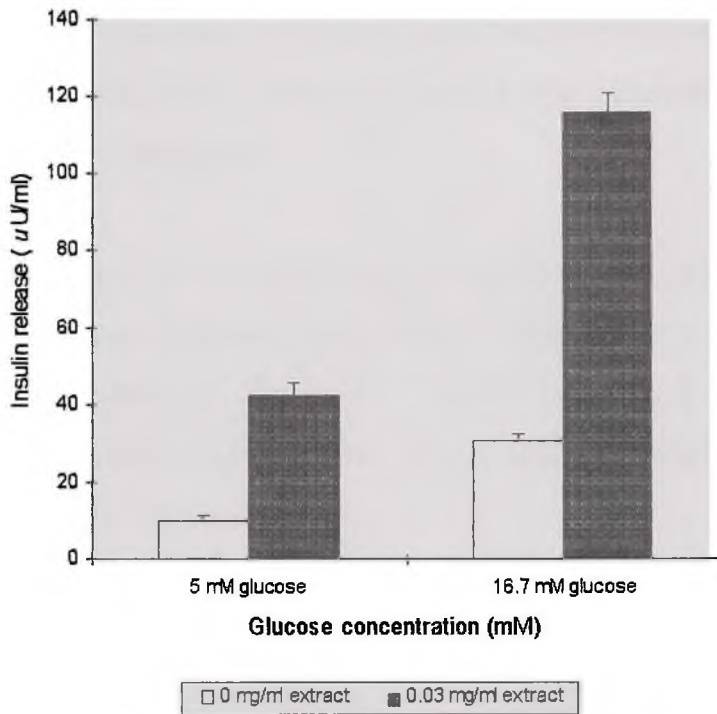


Figure 11. Effect of glucose concentration on insulin release from pancreatic β -islet cells of rats in the presence and absence of *O. canum* extract. Bars indicate standard error of the mean.



evaluated. As indicated in Table 4, insulin release in the presence of *D. adscendens*, a medicinal plant not used for the treatment of diabetes, decreased with increasing concentration of the extract, with higher values of the extract inhibiting the release completely.

Insulin release in the presence of "Mirabetic" extract at all concentrations, increased and the increases were statistically significant ($P < 0.05$), (Table 5). The dosage which effectively potentiated insulin release the most, giving a value of $100.90 \pm 3.23 \mu\text{U/ml}$, was 1.0 mg/ml.

Comparing "Mirabetic" to *O. canum*, the amounts of insulin released were not significantly different at extract concentrations of 0.001 mg/ml. At extract concentrations of 0.01 mg/ml and 0.1 mg/ml, the amounts of insulin released in the presence of *O. canum* were significantly greater ($P < 0.05$) than in "mirabetic". However, at concentrations of 1.0 mg/ml and 0.001 mg/ml the amounts of insulin released in the presence of "mirabetic" were significantly greater ($P < 0.05$) than in the presence of *O. canum* (see figure 12.)

Table 4. Effect of *Desmodium adscendens* on insulin release from pancreatic β -islet cells of rats.

Extract conc. (mg/ml)	Insulin release* (μ U/ml)	Percentage change in insulin release
0.00	34.88 \pm 1.24	0.0
0.005	26.67 \pm 1.76	-23.69 \pm 3.50
0.03	4.00 \pm 0.58	-88.56 \pm 1.53
0.50	0.0	-
1.0	0.0	-

*Given as mean \pm standard error of the mean. Determinations were done in triplicate

Table 5. Effect of "mirabetic" on insulin release from pancreatic β -islet cells of rats.

Extract conc. (mg/ml)	Insulin release* (μ U/ml)	Percentage change in insulin release
0.00	34.88 \pm 1.24	0.00
0.001	53.38 \pm 3.38	54.26 \pm 14.47
0.01	67.48 \pm 3.78	95.05 \pm 5.10
0.1	92.75 \pm 1.94	168.92 \pm 1.03
1.0	100.90 \pm 3.23	192.62 \pm 16.53
10.0	84.30 \pm 2.99	144.19 \pm 2.48

*Given as mean \pm standard error of the mean. Determinations were done in triplicate

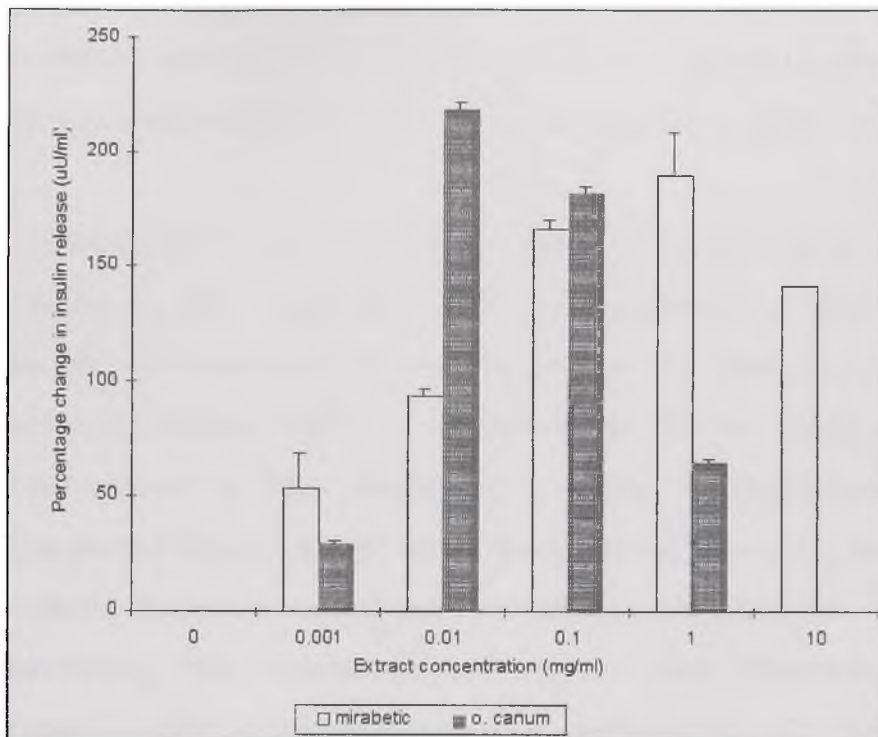


Figure 12. Comparison of insulin release from pancreatic β -islet cells of rats receiving various concentrations of "mirabetic" or *O. canum* extracts. Bars indicate standard error of the mean.

CHAPTER FOUR

DISCUSSION AND CONCLUSION

The overall aim of this study was to establish an *in vitro* assay for evaluating the anti-diabetic property of *O. canum*.

The hypoglycemic activity of *O. canum* had previously been evaluated *in vivo* (Hogarh, 1996) using genetically diabetic mice, STZ-induced diabetic mice, and lean littermates of the genetically diabetic mice. In that study, it was shown that aqueous extract of fresh leaves of *O. canum* was hypoglycemic in the genetically diabetic mice representing the non-insulin dependent diabetic mellitus, and their non-diabetic lean littermates. The aqueous extract did not show anti-diabetic activity in STZ-induced diabetic mice representing the insulin dependent diabetic mellitus.

The *in vitro*, assay involved isolating and determining insulin release from pancreatic β -islet cells of rats incubated in the presence or absence of varying concentrations of *O. canum* extract. The assay was to be used in evaluating other medicinal plants (*D. adscendens* and "mirabetic"). *In vivo* studies were included in order to confirm the previous work of Hogarh, (1996) and also, to evaluate the effect of the extract

on serum lipids and lipoproteins which are deranged in diabetes mellitus. Abnormalities in lipid metabolism is secondary to diabetes mellitus. In type I diabetes mellitus with diabetic ketoacidosis, the levels of chylomicrons and very low density lipoproteins are elevated. However, high density lipoproteins are usually low (Eder and Berbgan, 1990). In type II diabetes mellitus, total cholesterol and triacylglycerol levels are also elevated.

As the results indicate, the aqueous extract of fresh leaves of *O. canum* was able to lower fasting blood glucose concentration significantly ($P < 0.05$) in both groups of mice at the end of a 13-week period of study. The effect of the extract in genetically diabetic mice was noticed from week 3. The slow effect of the extract might be due to the stabilization of β -islet cells of the genetically diabetic mice, a process which may take time. The effect may also be due to the fact that the concentration of the active agent in the extract responsible for the hypoglycemic action may be low and that the extract must reach a threshold concentration in the tissue before effects manifest themselves.

The goals of the treatment of NIDDM and IDDM are to optimize blood glucose control and also normalize the associated lipid

disturbances since changes in lipids contribute to cardiovascular complication in diabetes mellitus. Therefore, effects of any anti-diabetic agent on lipid metabolism need to be investigated. Thus, the effect of aqueous extract of fresh leaves of *O. canum* on lipid levels was evaluated in genetically diabetic mice and their lean littermates.

The results showed that the extract significantly lowered the cholesterol levels in genetically diabetic mice and their lean littermates. No significant differences were observed in triacylglycerol levels in all groups of mice on extract and those on water, though, triacylglycerol levels were lower in mice on extract than those on water. The extract also lowered significantly, serum LDL-c in genetically diabetic mice and their lean littermates. However, serum HDL-c levels increased significantly in genetically diabetic mice and their littermates on extract compared to those on water.

The ability of the extract to lower total cholesterol and LDL-c levels, and increase HDL-c levels is welcome news since lowering total and LDL-cholesterol levels in blood reduces the risk of cardiovascular diseases which may be secondary to diabetes mellitus. The increased levels of HDL-c in response to the extract would be beneficial since HDL-c is concerned

with reverse transport of cholesterol ester from cells of tissues with a surplus of cholesterol, to liver or cells whose cholesterol requirements exceeds their capacity for synthesis (Zilva *et al.*, 1992). This reverse transport decreases blood cholesterol levels and prevent the development of coronary heart diseases. The results suggest that *O. canum* may help prevent dyslipidemia associated with diabetes mellitus, possibly by inhibiting the synthesis of lipids or activating lipoprotein lipase thereby increasing the rate of clearance of lipids from the plasma (Zilva, *et al*; 1992).

In Hogarh's work, the extract was also found to suppress weight gain in all the three groups of mice, that is, genetically diabetic mice, their non-diabetic lean littermates and STZ-induced diabetic mice. In this study, the extract also suppressed weight gain in both the genetically diabetic mice and their lean littermates.

The significant differences ($P < 0.05$) which were observed in percentage change in body weight of the genetically diabetic mice and their lean littermates in response to the *O. canum* extract compared to the control, meant that the extract is capable of controlling weight gain associated with type II

diabetes. Most type II diabetic patients are obese and obesity, per se, can cause abnormalities in lipoprotein production and removal resulting in hyperlipidemia (Eder and Berbgan, 1990). Also, obesity is frequently accompanied by resistance and or diminished sensitivity to insulin in tissues of man and experimental animals (Salans, et al; 1983). The ability of *O. canum* to reduce weight in genetically diabetic mice and their littermates suggests that fresh leaves of *O. canum* extract could be used in the management of obesity and diabetes secondary to obesity. This is because, weight loss can improve the abnormalities of glucose intolerance, insulin secretion, insulin resistance and hyperlipidemia associated with obesity (Salan, et al; 1983).

The effect of aqueous extract of dried leaves of *O. canum* was also evaluated in genetically diabetic mice. The results showed a significant lowering of fasting blood glucose levels. The efficacy of the dried leaves is of significance because the leaves of the plant are available only during the rainy season. The leaves may therefore, be dried, milled and used in the form of beverage during the dry season, thereby making the plant extract available all year round. In addition, people wishing to travel outside the country could



carry the dried leaves with them.

Comparing genetically diabetic mice on aqueous extract of *O. canum* fresh leaves to those on extract from dried leaves, the differences in percentage decrease in fasting blood glucose were statistically not significant, an indication that the hypoglycemic activity of the extract was not lost upon drying. However, whereas genetically diabetic mice on fresh leaf extract lost weight, those on dried leaf extract did not. The differences in changes in weights between the two groups were significant ($P < 0.05$). Possibly, whatever was responsible for weight loss in the fresh extract might have been destroyed during the drying process, an indication that the active component responsible for the decrease in blood glucose concentration might be different from the active agent responsible for the control of body weight.

The hypoglycemic action of any anti-diabetic agent could be due to one or more of the following; hyperplasia or hypertrophy of the pancreatic β -islet cells, increased peripheral uptake of glucose, decreased intestinal absorption of glucose and potentiation of glucose stimulated insulin release.

Having evaluated the hypoglycemic activity of *O. canum* in genetically diabetic mice *in vivo*, its mode of action was evaluated through *in vitro* assays using potentiation of glucose-stimulated insulin release from isolated pancreatic β -islet cells of wistar rats.

Preliminary work was done to find the number of β -islet cells and time suitable for the incubation of the islet cells. In the time course of insulin release, the amount of insulin released was approximately linear during the first 60 minutes. After 90 minutes of incubation, some of the beta cells could not be seen under the dissecting microscope indicating that the cells might have lysed. This could give rise to abnormally high insulin release, and therefore, a time period less than 90 minutes was chosen as the incubation time. Inferring from the results of the effect of β -islet cell numbers on insulin release, a group of 6 β -islet cells was chosen for the *in vitro* assays.

Incubation of the β -islet cells in the presence of various concentrations of extract constituted from freeze dried sample of fresh leaves of *O. canum* raised the amount of insulin released significantly, with maximum insulin release occurring



at 0.03 mg/ml extract concentration. Higher doses above 0.03 mg/ml decreased insulin release. The decline at higher doses might be due to the fact that the extract, being crude, may contain other components which become inhibitory, at higher concentration, to the β -islet cells. But the results clearly indicate potentiation of insulin release as one mode of action of the extract. Similar results were obtained when rats were pretreated with aqueous extract of fresh leaves of *O. canum* for 21 days.

Comparing the pretreated rats with the non treated rats, the percentage change in the amounts of insulin released were significantly greater at 0.005 mg/ml and 1.0 mg/ml extract concentrations in pretreated rats than in non treated rats.

Increase in insulin released after pretreatment may be due to the extract sensitizing the pancreatic β -islet cells to glucose, or to hypertrophy and/or hyperplasia of the pancreatic β -islet cells, a case which can be confirmed through histological studies seen as enlargement of the islets of Langerhans together with increased cell density (Zarzuelo *et al.*, 1990). However, the results of the experiment in which 5mM and 16.7 mM glucose concentrations were used in the presence and absence of the extract suggest that the extract

sensitized the islet cells to glucose and potentiated insulin release from the isolated pancreatic β -islet cells, irrespective of the glucose concentrations. The ability of the extract to potentiate insulin release even at higher glucose concentration is significant since, chronic hyperglycemia makes the beta cells unresponsive to glucose (Leahy, 1990; Leahy et al., 1992)

The *in vitro* assay established with *O. canum*, was used to evaluate the mode of action of "Mirabetic", a trade name for a plant preparation claimed to be anti-diabetic. Prior to the *in vitro* assay, the anti-diabetic property of "mirabetic" was evaluated *in vivo* using STZ-induced diabetic mice (an IDDM model). At the time of study, there were no genetically diabetic mice hence, the NIDDM model could not be used.

The results of the *in vivo* effect of "mirabetic" extract on STZ-induced diabetic mice showed that mice pretreated for 21 days before induction of the diabetes with STZ lived longer compared to STZ-induced diabetic mice without pretreatment. The pretreated, STZ-induced diabetic mice put on extract also lived longer compared to pretreated STZ-induced diabetic mice on water (8wk vs 7wk). Furthermore, mice receiving no

pretreatment before the induction of the diabetes but put on extract after the induction of the diabetic state, lived longer than those put on water (6wk vs 5wk). All these results could be due to the fact that the mice which lived longer took in more extract.

STZ-induced diabetic mice pretreated with the extract for 3 weeks but put on water lived longer compared to non-pretreated STZ-induced diabetic mice put on extract for five weeks (7wk vs 5wk). This suggests that the pretreatment, not the period on the extract, had the effect of reducing the severity of the β -cytotoxic effect of the STZ. Taken together, the extract might probably be preventing the destructive effect of the STZ against the pancreatic β -islet cells by stabilizing these cells during pretreatment.

STZ is taken up by β -islet cells by means of the glucose transporter 2. It then reduces the cellular levels of NAD^+ through the activation of poly(ADP-ribose) synthetase, an NAD-degrading enzyme (Thulesen et al., 1997) leading to the destruction of the β -islet cells resulting in insulin dependent diabetes mellitus. The extract could be inhibiting the poly ADP-ribose synthetase.

During the same time period, the increase in fasting blood glucose concentration was greater in the pretreated, STZ-induced diabetic mice put on water than in those put on extract. Likewise, the increase in fasting blood glucose concentration was greater in mice receiving no pretreatment before the induction of the diabetes but put on water after the induction of the diabetic state than in mice put on extract. From the results, there is an indication that the extract is anti-hyperglycemic, probably because of its effect of protecting the β -islet cells against STZ, thereby reducing the severity of the diabetes. The fact that mice in both groups, that is, non-pretreated and pretreated, developed extreme hyperglycemia just before death suggests that the extract might require functional beta cells for activity.

The extract did significantly lower fasting blood glucose levels in the lean non-diabetic littermates by the third and fourth weeks. Thereafter, the fasting blood glucose levels gradually increased such that there were no significant differences in blood glucose levels between weeks 1 and 7. This suggests that the extract is not hypoglycemic in non-diabetic mice. This property is an added advantage since severe hypoglycemia progresses to coma and if not treated, may result in death.

At week 5, weight loss in pretreated, STZ-induced diabetic mice put on water was significantly greater than in those put on extract. Similarly, weight loss in mice receiving no pretreatment before the induction of diabetic state but put on water, was significantly greater than in those put on extract. The β -cytotoxic effect of STZ results in absolute insulin deficiency. Insulin facilitates the storage of all ingested nutrients and insulin deficiency enhances mobilization and or production of metabolic fuels (Felig, 1983) resulting in weight loss and appearance of ketone bodies in the urine. Therefore, the ability of the extract to reduce weight loss in these mice indicates another type of anti-diabetic property. Weight loss was one of the parameters used to monitor the induction of diabetes following the injection of STZ. Other indicators were urine testing positive to Combi 9 urine strips for both glucose and ketone bodies. All of these were used to indicate the severity of the diabetes.

The *in vitro* assay developed with extract of *O. canum* was used to evaluate (the mode of action of) "mirabetic" extract. The results showed that insulin release from β -islet cells incubated in "mirabetic" extract was increased significantly. The results indicate that the assay system developed with *O. canum* extract gives positive results with a different anti-

diabetic plant, the anti-diabetic nature established *in vivo*.

In contrast to the stimulatory effects of "mirabetic" on insulin release from pancreatic β -islet cells of rats, *D. adscendens* (a non anti-diabetic medicinal plant) extract decreased insulin release, shutting the release completely at higher concentrations. Therefore, the assay developed could discriminate between anti-diabetic and non anti-diabetic plant extracts.

In summary, the results of the *in vivo* studies using aqueous extract of fresh leaves of *O. canum* have confirmed Hogarh's results that aqueous extract is hypoglycemic in genetically diabetic mice (NIDDM model) and their lean littermates (Hogarh, 1996). The aqueous extract of fresh leaves of *O. canum* also lowered cholesterol and triacylglycerol levels but raised HDL-c levels. Dried leaves of *O. canum* have also been shown to be hypoglycemic in genetically diabetic mice. However, unlike the fresh leaf extract, the dried leaf extract did not significantly change body weight of genetically diabetic mice.

In vivo experiments on "mirabetic" suggest that the extract



did not have any hypoglycemic activity in STZ-induced diabetic mice. However, the severity, both in hyperglycemia and weight loss, was less in mice pretreated with the extract, indicating amelioration of the diabetic state. The results of the *in vitro* studies indicate that both *O. canum* and "mirabetic" stimulate glucose-induced insulin release from pancreatic β -islet cells of rats, probably through the sensitization of the pancreatic β -islet cells to glucose, suggesting this to be the mode of action of these plant extracts. The extract of *D. adscendens*, a non-diabetic medicinal plant did not increase insulin release.

In conclusion, an *in vitro* assay system for the anti-diabetic activity, has been established with *O. canum* using glucose-stimulated insulin release from isolated pancreatic β -islet cells of rats. This assay has been used effectively to evaluate one anti-diabetic plant which gave a positive result (true positive) and another non anti-diabetic plant which gave a negative result (true negative). However, more medicinal plants, both anti-diabetic and non anti-diabetic, need to be evaluated to ascertain the reliability of the assay, as a method to screen plants for anti-diabetic property.

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APPENDIX 1**Preparation of Krebs Ringer Buffer (KRB)**

- 1a Krebs Ringer bicarbonate buffer (KRB) for digestion and isolation.

<u>Stock solution</u>		<u>Working solution</u>
1.6%	KH_2PO_4	10ml
3.5%	KCl	10ml
1.1%	MgSO_4	10ml
22%	NaCl	25ml
4.2%	NaHCO_3	50ml
14.7%	CaCl_2	2.5ml
Glucose		1g

The working solution was made up to 1 litre with doubled distilled water.

- 1b Pre-incubation KRB

All the above in (1a) without glucose initially, then supplemented with

BSA 3mg/ml

Glucose 3mM

Pyruvate 5mM

Fumarate 5mM

Glutamate 5mM

- 1c Incubation KRB

Same as pre-incubation KRB with 16.7mM

APPENDIX 2**Preparation of 1 mM Citric Buffer pH 4.2 for STZ- Injection****Stock Solution**

0.1 M citric acid monohydrate and 0.1 M trisodium citrate stock solutions were prepared by dissolving 21.02 g citric acid monohydrate and 29.41 g trisodium citrate respectively in a litre of demineralized water.

Solution A

1 ml of 0.1 M sodium citrate stock solution was made to 100 ml with demineralized water to give a 1 mM solution.

Solution B

1 ml of 0.1 M citric acid stock solution was made up to 100 ml with demineralized water to give a 1 mM solution.

A 1 mM buffer solution, pH 4.2 was obtained on adding 46.0 ml of solution A to 54.0 ml of solution B.

APPENDIX 3**STZ Injection (60 mg/kg)**

Sample calculation:

Volume of STZ given per mouse 0.2ml

Weight of a mouse e.g. 26 g

STZ required $(26/1000) \times 60$

= 1.56 mg in 0.2 ml buffer.



APPENDIX 4

Table 1. Effect of aqueous extract of *O. canum* fresh leaves on fasting blood glucose levels of genetically diabetic mice and their lean littermates.

1a. Genetically diabetic mice on extract. (N=5)

Weeks	Blood glucose levels (mmol/l)					
	1	2	3	4	5	mean*
1	14.5	13.2	14.2	15.9	16.6	14.88 ± 0.61
3	11.4	10.1	8.8	13.9	14.8	11.80 ± 1.13
5	7.0	7.9	5.3	13.0	12.3	9.10 ± 1.51
7	5.2	5.8	5.9	10.5	10.2	7.50 ± 1.16
9	5.5	5.4	5.6	7.2	7.9	6.30 ± 0.52
11	5.4	5.2	5.2	6.3	6.1	5.60 ± 0.23
13	5.4	5.3	5.4	6.1	5.9	5.60 ± 0.16

*Given as mean ± standard error of the mean

1b. Genetically diabetic mice on water (N=5)

Weeks	Blood glucose levels (mmol/l)					
	1	2	3	4	5	mean*
1	13.3	14.8	12.2	15.6	10.1	13.20 ± 0.97
3	13.4	13.4	12.1	15.0	10.0	12.78 ± 0.83
5	12.1	12.9	11.0	14.4	9.7	12.02 ± 0.80
7	12.1	13.1	10.3	14.5	9.6	11.97 ± 0.90
9	12.0	13.4	10.4	14.7	10.3	12.16 ± 0.85
11	11.7	12.6	10.4	13.8	9.8	11.66 ± 0.72
13	12.0	12.4	10.3	13.4	10.0	11.62 ± 0.64

1c. Lean littermates on extract. (N=5)

Weeks	Blood glucose levels (mmol/l)					mean*
	1	2	3	4	5	
1	5.8	5.7	5.4	5.6	6.2	5.74 ± 0.13
3	4.4	4.4	5.0	4.4	4.5	4.54 ± 0.12
5	4.6	4.3	3.7	4.2	3.5	4.06 ± 0.20
7	3.2	3.5	3.0	3.7	3.2	3.22 ± 0.12
9	3.3	3.3	3.2	2.8	3.0	3.12 ± 0.10
11	3.8	3.6	3.5	3.2	3.4	3.50 ± 0.10
13	4.1	4.2	3.2	3.5	3.1	3.62 ± 0.23

*Given as mean ± standard error of the mean

1d. Lean littermates on water. (N=5)

Weeks	Blood glucose levels (mmol/l)					mean*
	1	2	3	4	5	
1	5.7	5.5	6.3	5.8	6.1	5.88 ± 0.14
3	5.3	5.3	6.0	5.0	5.8	5.48 ± 0.18
5	5.6	5.4	6.2	5.0	5.4	5.52 ± 0.20
7	4.8	4.6	5.6	4.6	5.5	5.02 ± 0.22
9	4.9	4.7	5.6	4.7	5.3	5.04 ± 0.18
11	5.0	5.1	5.5	4.8	5.2	5.12 ± 0.12
13	4.4	4.5	5.6	4.9	5.1	4.90 ± 0.22

Table 2. Effect of aqueous extract of *O. canum* fresh leaves on body weights of genetically diabetic mice and their lean littermates

2a. Genetically diabetic mice on extract. (N=5)						
Weeks	Body weight (g)					mean*
	1	2	3	4	5	
1	52.0	54.5	53.0	57.0	53.5	54.0 ± 0.85
3	55.0	55.0	53.0	54.0	53.0	54.0 ± 0.45
5	53.0	53.5	50.5	52.0	52.0	52.2 ± 0.51
7	49.0	50.0	48.0	48.0	49.0	48.8 ± 0.37
9	52.0	53.0	51.0	50.5	52.0	51.7 ± 0.44
11	52.0	53.5	51.5	51.0	52.5	52.1 ± 0.4
13	51.5	54.0	51.0	52.0	53.0	52.3 ± 0.54

*Given as mean ± standard of the mean

2b. Genetically diabetic mice on water. (N=5)

Weeks	Body weight (g)					mean
	1	2	3	4	5	
1	51.0	52.0	51.5	55.0	53.0	52.5 ± 0.71
3	52.5	53.0	52.0	56.0	52.5	53.2 ± 0.72
5	54.5	53.5	52.5	56.5	53.0	54.0 ± 0.71
7	54.0	53.5	53.0	56.5	53.5	54.1 ± 0.62
9	55.0	53.5	53.5	56.5	54.0	54.5 ± 0.57
11	55.5	54.5	54.0	57.0	54.5	55.1 ± 0.53
13	56.0	55.0	54.5	57.5	55.0	55.6 ± 0.53

2c. Lean littermates on extract. (N=5)

Weeks	Body weight (g)					mean*
	1	2	3	4	5	
1	18.5	16.0	17.5	18.0	15.5	17.1 ± 0.58
3	20.0	15.5	19.0	18.5	15.5	17.7 ± 0.93
5	19.5	16.0	19.0	18.5	15.0	17.6 ± 0.89
7	20.0	16.0	18.5	17.5	15.5	17.5 ± 0.82
9	21.0	16.0	20.0	18.5	16.0	18.3 ± 1.02
11	20.5	15.5	19.5	18.5	15.5	17.9 ± 1.03
13	20.5	16.0	20.0	18.5	16.0	18.2 ± 0.96

Given as mean ± standard error of the mean

2d. Lean littermates on water. (N=5)

Weeks	Body weight (g)					mean*
	1	2	3	4	5	
1	24.0	23.5	24.5	23.5	22.0	23.5 ± 0.42
3	26.0	25.0	26.5	25.0	23.5	25.2 ± 0.51
5	26.0	26.0	26.0	25.5	23.5	25.4 ± 0.48
7	27.5	27.0	27.0	27.0	23.0	26.3 ± 0.83
9	28.5	27.5	28.0	27.0	24.0	27.1 ± 0.80
11	28.0	28.0	27.5	27.5	24.5	27.1 ± 0.66
13	28.5	28.5	28.0	28.0	25.0	27.6 ± 0.66

APPENDIX 5

Table 3 Effect of aqueous extract of *O. canum* dried leaves on fasting blood glucose of genetically diabetic mice

1a. Genetically diabetic mice on extract. (N=5)

Weeks	<u>Blood glucose levels (mmol/l)</u>					mean*
	1	2	3	4	5	
1	14.2	17.3	16.8	15.4	19.0	16.54 ± 0.82
3	13.6	16.4	15.8	10.7	16.6	14.62 ± 1.11
5	11.7	15.1	13.4	9.3	13.3	12.56 ± 0.97
7	1.05	13.1	9.3	7.3	11.4	10.32 ± 0.97
9	7.5	9.9	7.7	6.8	9.6	8.30 ± 0.61
11	5.9	7.8	6.6	5.5	7.2	6.60 ± 0.41
13	5.4	5.8	6.1	5.0	6.3	5.72 ± 0.25

*Given as mean ± standard error of the mean

2b. Genetically diabetic mice on water. (N=5)

Weeks	<u>Blood glucose levels (mmol/l)</u>					mean*
	1	2	3	4	5	
1	16.2	14.7	15.8	15.3	17.1	15.82 ± 0.41
3	15.9	14.5	15.8	15.2	16.5	15.58 ± 0.34
5	15.6	15.1	15.1	15.1	16.4	15.46 ± 0.25
7	16.1	14.9	15.6	15.8	16.6	15.80 ± 0.28
9	16.0	14.4	15.7	15.6	16.9	15.72 ± 0.40
11	15.8	15.0	15.8	16.0	16.7	15.86 ± 0.27
13	15.6	14.9	16.0	16.1	17.2	15.96 ± 0.37

APPENDIX 5

Table 2a Effect of aqueous extract of *O. canum* dried leaves on body weights of genetically diabetic mice

4a. Genetically diabetic mice on extract. (N=5)

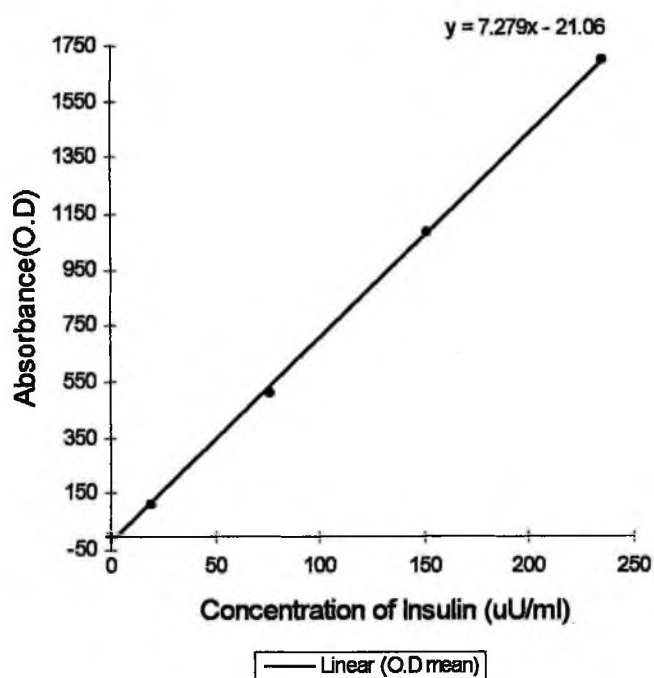
Weeks	Body weight (mmol/l)					mean*
	1	2	3	4	5	
1	55.2	60.6	46.5	49.3	57.4	53.80 ± 2.59
3	56.0	61.0	46.8	50.5	57.6	54.38 ± 2.54
5	56.2	61.2	47.2	50.3	57.7	54.52 ± 2.53
7	56.4	61.1	47.4	50.7	58.1	54.74 ± 2.49
9	57.0	61.6	47.5	50.8	58.2	55.02 ± 2.56
11	57.2	61.7	47.8	50.7	58.4	55.16 ± 2.56
13	57.3	62.0	47.9	51.2	58.8	55.36 ± 2.55

*Given as mean ± standard error of the mean

2b. Genetically diabetic mice on water. (N=5)

Weeks	Body weight (mmol/l)					mean
	1	2	3	4	5	
1	52.7	56.4	48.9	61.7	54.1	54.76 ± 2.11
3	54.7	58.8	51.2	63.0	54.9	56.52 ± 2.01
5	55.7	59.2	51.3	63.0	53.8	56.60 ± 2.05
7	57.3	60.6	54.2	63.2	54.3	57.92 ± 1.76
9	58.3	60.9	54.8	62.6	53.8	58.08 ± 1.69
11	58.1	61.6	55.1	63.6	54.9	58.66 ± 1.73
13	57.9	62.8	55.5	64.9	56.7	59.56 ± 1.92





Appendix 6. Calibration curve for the determination of insulin release from pancreatic β -islet cells of rats