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"I hear, and I forget,

I see, and I remember

I do, and I understand"

(Ancient Chinese Proverb)

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FLAVONOIDS OF BRIDELIA FERRUGINEA: ANTI-DIABETIC PROPERTIES AND THE REDUCTION OF LIVER MICROSOMAL PROTEINS.

A THESIS SUBMITTED

BY

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FACULTY OF SCIENCE
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LEGON

INTEGRI PROCEDAMUS

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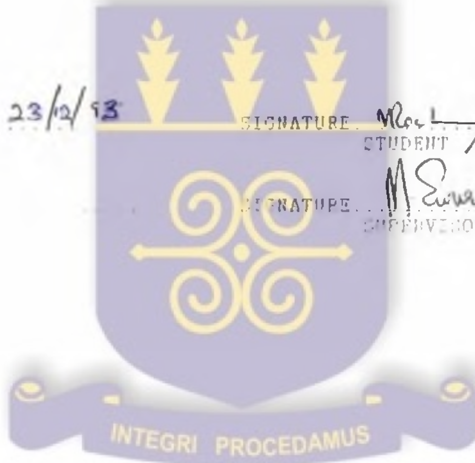
D E C L A R A T I O N

THE WORK DESCRIBED IN THIS REPORT WAS CARRIED OUT BY ME AT THE DEPARTMENT OF BIOCHEMISTRY UNIVERSITY OF GHANA, LEGON, UNDER THE SUPERVISION OF PROFESSOR MARIAN E. ADDY



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D E D I C A T I O N

TO THE LATE NANA ATAADIM-KWEENU VII

(MANKRADO OF NKUSUKUM TRADITIONAL AREA)

WHOSE OFFSPRING HAVE MADE ME WHAT I AM TODAY

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A B S T R A C T

The anti-diabetic properties of flavonoids extracted from Bridelia ferruginea, a plant used at the Centre For Scientific Research Into Plant Medicine (CSRPM) to treat diabetes, were evaluated using streptozotocin-induced diabetic mice to represent insulin dependent diabetes mellitus (IDDM) and genetically diabetic mice to represent non-insulin dependent diabetes mellitus (NIDDM). Also analyzed was the effect of the extract on the reduction of liver microsomal proteins. The possible side-effects of the flavonoid extract on liver and kidney functions were also examined. The test animals were given intraperitoneal injection of the flavonoid extract dissolved in 0.85% saline solution whilst the controls were given 0.85% saline solution without the extract. The results showed that the flavonoid extract was not hypoglycemic as it had no effect on the plasma glucose levels of non-diabetic mice. Although the extract did not prevent the development of hyperglycemia in the IDDM model after the STZ injection, it did reduce the non-fasting plasma glucose levels significantly, whereas such effect on fasting levels was absent or not significant. In the case of the NIDDM model the extract decreased significantly the non-fasting plasma glucose levels by about [illegible] but there was no effect on the levels of the fasting plasma glucose. The results also showed that all the test animals in both IDDM and NIDDM models had lower NADPH cytochrome P450 reductase activity than their controls. Although both test and controls had comparable liver

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microsomal proteins. The results indicate that, the flavonoids, which are not hypoglycaemic, reduce post prandial plasma glucose levels in diabetic patients. There is also an indication that they reduce the activity of drug metabolising enzymes, probably the ones involving the cytochrome P450DM isozyme. No toxic side-effects on the liver and kidney were observed.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

From time immemorial, man has been discovering a wealth of useful therapeutic agents in the plant and animal kingdoms, with herbal treatment of diseases being a tradition in a large number of different societies. In many of these societies, the knowledge of these medicinal substances and their pharmacological potential are passed on by oral tradition as is typical of traditional healers in this country. Many indispensable drugs of today (eg. quinine, atropine, aspirin, reserpine, codeine and morphine) came into ... through a systematic scientific study of folk remedies.

Of late there has been a shift in consumer preference from chemically-derived medicines to plant derived ones. The United States, United Kingdom, Germany and the Netherlands have doubled their interest in plant as a source of raw material for discovering and developing new pharmaceutical products (Komen, 1991) These developed countries are intensifying their efforts to screen various developing countries plant wealth for new medicinal compounds. As stated in the above publication most of the world's trees and plants that heal are located in the tropics which store about two-thirds of all plant species of approximately 300,000 in the world. Out of this number, about 35,000 is reported to have medicinal value but only 5,000 have been exhaustively

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studied for possible therapeutics.

In Ghana today, the average worker cannot afford to be ill due to the high cost of drugs. It is therefore important to undertake scientific investigations into the therapeutic potentials of our medicinal plants so as to improve and develop them. This will enable our medical doctors use them, thereby reducing dependency on imported drugs.

Traditional methods of healing have successfully served the majority of Ghanaians for years, especially those who form about 70% of the population. Sufficient quantities of these plant-derived drugs must be produced in more acceptable forms to meet the health needs of all the people. Hence developing, improving and promoting these medicinal plants will, in addition to supplementing western-type medicine, be a better approach to meeting the health needs of a majority of the populace who may never have the facilities of a modern hospital, or are unable to afford the high cost of drugs.

For the proper development of herbal medicine, the Ghana government established the Centre for Scientific Research Into Plant Medicine (CSRPM) at Mampong-Akwapim in November 1973, to carry out scientific research into the medicinal properties of several plants used by the country s herbalists. There are other herbal centres complementing the effort of this research centre Among these herbal centres are, the G.K. Noamesi Laboratory at Hohoe in the Volta Region, National Institute of Traditional Medicine at Nsawam, Asthma and Hypertensive Research Centre in Tamale, Apaak Traditional Medicine Manufacturing at Koforidua and Naturo-Herbal Research Clinic,

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Asiadam Herbal Medical Centre, Yadeeya Traditional Science/ Healing Centre and Afladei International Herbal Drugs Manufacturing, all in Accra. Most of these herbal centres have some contact with the country's hospitals, universities and research institutes, and carry out some research into the medicinal plants they use.

A variety of ailments are claimed to be treated by a number of medicinal plant preparations, from stomachache to diarrhoea, skin infections, heart diseases, diabetes, malaria, anaemia and worm infestations. For example, preparations made from the roots of Combretum mucronatum are known to be 98% effective in combating the guinea worm disease (Ayensu, 1978) The Ocimum gratissimum leaf or whole plant is known to be very effective against diarrhoea and the use of Azadirachta indica (neem) to treat malaria has been in existence since time immemorial along the Western, Central and Eastern Africa (Sofowora, 1982) The CSRPM specialises in the treatment of diseases such as asthma, hypertension, anaemia, piles and diabetes.

In spite of the fact that these herbal preparations have been in use since time immemorial, there is still the need for toxicological tests to verify their safety It is an all too acceptable misconception that "all that is natural is good", because a number of highly toxic compounds have been isolated from these plants. For example, many of the tannin-containing plant extracts containing alkaloids have side effects on a number of different organs of the body (Atta-Ur-Rahman and Saman, 1989). Care must therefore be exercised before herbal

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extracts can be fully accepted either in the indigenous or modern systems of medicine. There is therefore the need for scientific investigations not only for efficacy but also for toxicity

There have been several scientific investigations in this department to establish efficacy of herbal preparations. One such investigation has shown that Desmodium adscendens (Papilionaceae) used at CSRPM for the management of asthma, inhibits anaphylactic contractions (Addy, 1989). The plant extract also reduces muscle tone and inhibits agonist induced contractions of airway smooth muscle (Addy and Burka, 1989) D. adscendens was also found to inhibit NADPH-dependent oxygenation of arachidonic acid by kidney cortical microsomes (Addy and Schwartzman, 1992) Studies on Indigofera arrecta, an anti-diabetic plant, indicated that an aqueous extract administered orally, significantly lowered blood glucose levels during the first two weeks of administration (Addy and Nyarko, 1988), prevented the development of hyperglycaemia in the db/db mouse (Addy et. al., 1992), and decreased the plasma glucose levels of fasting normoglycaemic rats The plant, however, did not prevent the rise in plasma glucose after an oral glucose load in these rats (Nyarko et. al., 1993) Piper guineense has been investigated for its insecticidal properties (Gbewonyo and Candy, 1992a and 1992b; Gbewonyo et. al., 1993).

A lot of work has been done in other places to establish the efficacy of herbal preparations. Work at CSRPM on Cryptolepis sanguinolenta has shown that the crude extract is

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very active against malarial parasites. Clinical trials at the centre showed a complete cure of the disease within 3-5 days (Boye and Ampofo, 1983).

There has been intensive research work on *Piper guineense* a plant used locally for many medicinal purposes (Addae-Mensah, 1992). These include the treatment of gonorrhoea, syphilis, rheumatism, the management of cough, bronchitis, intestinal disorders, and treatment for various other viral and bacterial infections. More than 20 different amide alkaloids have been isolated from the plant (Addae-Mensah *et al.*, 1977). Two of these 20 amide alkaloids, namely wisanine and dihydrowisanine show significant tranquillising and sedative activity by causing test mice to sleep in a dose-dependent manner for periods varying from 8-72 hours and also protect mice against metrazole-induced convulsions (Ayitey-Smith and Addae-Mensah, 1977). Other amide alkaloids such as piperidine, wisanine, and trichostachine and their dihydro derivatives also possess some activity against mosquito larvae (Addae-Mensah and Achieng, 1986).

As stated earlier, one of the diseases treated at the CSRPM is diabetes mellitus. Two plant materials used for the treatment are *I. erecta* and *B. ferruginea*. Treatment is done, by orally administering an aqueous extract of the plant material to patients for specific periods. According to personnel at the Centre, aqueous extracts from these two plants have been preserved to last longer

In Nigeria, it has been reported that the crude aqueous and methanolic extracts of the leaves of *B. ferruginea*

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significantly lowered plasma sugar levels of fasting albino rats (Iwu, 1980) However, the extract failed to protect the animals against alloxan-induced diabetes, except pretreating the animals with the drug before the alloxan administration in order to obtain a considerable suppression of hyperglycaemia. Additional work on B. ferruginea has been on its flavonoids as the potential active ingredients (Iwu, 1980; Addae-Mensah and Munenge, 1988). For example, Addae-Mensah and Munenge (1989), investigated Quercetin-3-neophesperidoside (Rutin) and other flavonoids as the active hypoglycaemic agents in B. ferruginea using New Zealand white rabbits artificially made hyper-glycaemic by glucose infusion. They observed that the flavonoids lowered the blood glucose levels of these animals.

Since the animal models were made hyperglycaemic by glucose infusion, it implies that, they had no defect and neither was there impairment in the glucose tolerance Such animal models cannot be said to represent the diabetic state. In order to establish that the flavonoid is really anti-diabetic it is better to use diabetic animals which have glucose homeostasis. The overall aim of this study is to evaluate the efficacy of the flavonoids as anti diabetic materials using two animal models, which represent the two major types of diabetes, that is, the IDDM and the NIDDM, to test for possible toxic side effects of these flavonoids, and how they affect the detoxifying enzyme system of the test animals.

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1.2 DIABETES MELLITUS

Diabetes mellitus is defined as an acute and chronic metabolic disorder characterized principally by hyperglycaemia (raised blood glucose level) resulting from an absolute or relative deficiency of metabolically functional insulin or its receptors (Robbin, 1967; Seifter and Englard, 1983).

Insulin is known to exert multiplicity of acute metabolic effects in the regulation of glucose and other nutrient homeostasis. It is known to inhibit hepatic glucose production and promote glucose uptake by causing an immediate inactivation of phosphorylase, which catalyses the breakdown of glycogen. It also activates glycogen synthase involved in the synthesis of glycogen. Insulin blocks gluconeogenesis by inactivating pyruvate carboxylase thereby ensuring that there is no conversion of non-carbohydrates to glucose. Insulin does not directly regulate hepatic glucose uptake, since glucose enters hepatocytes in proportion to extracellular glucose concentration. However, it does enhance the synthesis of glucokinase, the high Km glucose phosphorylating enzyme which is required to phosphorylate the high concentration of glucose entering the hepatocyte. In contrast to liver, glucose uptake by skeletal muscles and adipose tissues is dependent upon the prevailing concentration of insulin. The insulin increases glycogen synthase activity, thereby stimulating glycogenesis and also increases phosphofructokinase activity, thereby stimulating glucose metabolism via glycolysis in fat and muscle cells (Ellenberg and Rifkin, 1983). In the adipose tissue, much of the glucose

taken up by the adipocytes is diverted to triacylglycerol synthesis (Bailey and Marks, 1989) In the absence of insulin therefore, all the above processes will proceed at very low rates with a concomitant increase in the plasma glucose levels.

The clinical picture of diabetes mellitus is a composite of signs and symptoms arising both from the carbohydrate metabolic defect and from widespread vascular disturbances Fasting plasma glucose level rarely exceeds 120 mg/dl in normal humans, but much higher levels are routinely found in patients with deficient insulin action. At plasma glucose level above 180 mg/dl (10mM) (hyperglycaemia) the urine volume is increased owing to osmotic diuresis and coincident obligatory water loss (polyuria). This in turn leads to dehydration, increased thirst and excessive drinking (polydipsia) as well as sugar excreted in urine (glucosuria) As the disease persists, blood vessels can be damaged, leading to heart diseases, stroke, blindness or kidney failure.

The World Health Organisation (W.H.O) has reported that at least 60 million people in the world suffer from diabetes and whilst its prevalence in most Western communities is estimated at 2-5%, it is now known that in some developing populations it can reach 10% or even 20% (Gyesie, 1992) In Ghana, for instance, the rate is found to be increasing. In [illegible] it was estimated that Ghana, with a population of about [illegible].5 million had about 20,000 diabetic patients, that is, about [illegible] 4% of the population. A prevalence rate of 0.2-1% of the general adult population has been found by various

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investigators (Dodu, 1958; Dodu and De-Heer, 1964; Owusu, 1973; Owusu, 1976; Dodu, 1978) In 1992, it was reported that about two people out of every hundred people in Ghana were diabetic, that is, about 2% of the population (Gyesie, 1992) In Ghana, it has been found that the incidence of the disease is equal in both sexes with the prevalence of the diabetes increasing with age, with a peak frequency of onset in the fourth and sixth decades.

Diabetes remains a serious and increasing problem due to the fact that the actual cause of the disease is now unfolding. Several factors have been implicated; from genetic to environmental. The important influence of heredity supported the notion that diabetes mellitus was an inborn disorder leading to abnormal generation of pancreatic beta cells (Rother and Rimoin, 1981). However, environmental factors resulting in acute viral infections such as mumps have been implicated as another possible cause (Boucher et. al., 1974; Martin et. al., 1981). Of late, it appears likely that the condition known as diabetes mellitus can be produced by nearly all the common recognised pathological processes - infections, toxins, immune reactions, inflammation, stressful factors such as emotional disturbances and rarely neoplastic phenomenon (Rother and Rimoin, 1981; Rolfe, 1991). At present there is no known cure for the disease apart from the reduction of the symptoms (Ross et. al., 1976; West et. al., 1981; Rolfe, 1991)

Diabetes therefore demands attention because of its effect on the health of many Much effort would be needed to

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research into, and find a cure for the disease with special reference to what our environment and situation can best offer. For us in Ghana in particular and Africa in general, these resources include traditional medicaments.

Over hundred years ago, it was recognised that the disease appeared in at least two forms, one affecting mainly younger people, often children, and the other more frequent among obese adults (Murray et. al., 1988) The former are those who are ketosis-prone or insulin-dependent (IDDM) and the latter non-ketotic or non-insulin dependent (NIDDM). Some NIDDM patients may, however, require insulin treatment to control excessive hyperglycaemia, although they do not go into coma when treatment is withdrawn (Rolfe, 1991).

1.2.1 Insulin-dependent diabetic mellitus (IDDM)

IDDM is usually characterized by the abrupt onset of symptoms, insulinopoenia, proneness to ketoacidosis, and dependence on insulin therapy to prevent ketosis and to sustain life. While classically this diabetes type occurs in juveniles, it may appear at any age (Bennet, 1983) Insulin facilitates the transport of the metabolites of digestion into tissues dependent on it from the plasma. In its absence, large amounts of glucose builds up in the plasma and is unavailable to be utilized by the tissues. Gluconeogenic activity in the liver increases to make up for the apparent lack of glucose in these diabetics. It is known that, when insulin is present, the concentration of cell protein is maintained within narrow limits, however, absence or

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deficiency of the hormone causes marked alterations. The expression "melting down of flesh into urine" aptly describes the profound loss of tissue protein and increased nitrogen excretion that occur in the uncontrolled insulin-dependent diabetes (Jefferson et. al., 1983) The defects in impaired protein synthesis are believed, in majority of cases, to account for the high susceptibility of the patient to infection and delayed wound healing (Weringer et. al., 1981) Another feature of IDDM is the uncontrolled rate of lipolysis in the adipose tissue. There is also high acceleration of ketone body production in the liver If the ketone bodies produced are not rapidly used, ketoacidosis may result leading to coma and death.

A common feature of all IDDM cases is the presence of pancreatic beta cell damage. It was believed that the damage was probably due to viral infections, toxins or antibodies, affecting a previously normal pancreas Inflammatory and degenerative lesion resulting from pancreatitis and carcinoma were also usually implicated in the destruction or damage to the pancreas (Dolger and Seeman, 1976) Viral infections are known to be one of the environmental agents that produce selective damage to the beta cells of the pancreas (Rother and Rimoin, 1981; Murray et. al., 1988) Current advances in immunology, have led to the important recognition that patients with juvenile-onset diabetes have islet cell antibodies in their circulation that mediate both humoral and cellular immunity (Atkinson and Maclaren, 1990) The findings are that IDDM is the result of an auto-immune, or self-

directed attack on the pancreas. The attack does not, however, affect the majority of pancreatic cells, which secrete digestive enzymes but instead it restricts itself to the hormone producing cells. It is also now clear that, despite its onset, IDDM actually does not develop suddenly at all. Silently for several years it typically "brews" as the immune system slowly eliminates the beta cells with the symptoms appearing only when at least 80% of these cells are gone; the remainder in due course are eliminated over the next two or three years.

In this study non-diabetic littermates of mutant genetically diabetic mice and some albino mice were induced into the IDDM state using streptozotocin as the diabetogenic agent.

1.2.2 Non-insulin dependent diabetes mellitus (NIDDM)

NIDDM refers to the type of the disease in patients who, unlike the IDDM patients, have normal or above normal levels of circulating insulin. The defect, however, is primarily one of insulin resistance of the peripheral tissues rather than lack of insulin. Insulin resistance is a condition in which there might be a decrease in the number of insulin receptors or the affinity of the receptors for the hormone (Harris and Crabb, 1982). This finding goes to support other observations that diabetes often need not be pancreatic in origin (Stainer, 1981) For example, obesity has been strongly associated with the prevalence of NIDDM (Salans et. al., 1983) Obesity frequently induces abnormalities in carbohydrate metabolism.

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However, most individuals overcome these abnormalities completely after losing weight. Although higher levels of insulin may be found in obese NIDDM, the level is not as high as in the non-diabetic obese patients, suggesting that a form of beta cell failure to satisfy a higher insulin requirement may exist in these NIDDM patients who therefore, may require exogenous insulin to reduce extreme hyperglycaemia (Delvin, 1982)

This type of diabetes is typically 'mild" and shows little tendency to ketosis. The majority of subjects with NIDDM are adults at the time of onset and are obese, but the disease can occur in children and non-obese adults (Bennett, 1983). Surveys have, however, shown that while NIDDM accounts for 80-90% of diagnosed cases of diabetes, as much as 80% of the NIDDM population is obese (National Diabetes Data Group, 1979).

NIDDM, like IDDM, is characterized by hyperglycaemia. However, carbohydrate metabolism is different from that of IDDM because insulin is present. In this type of diabetes hyperlipoproteinaemia is observed with high levels of very low density lipoprotein (VLDL) The high levels of lipoprotein may be due to the rapid rates of *de novo* liver synthesis of fatty acids and triacylglycerol from excess calories stimulated by the high glucose level and high level of insulin rather than increased lipid mobilization from the adipose tissue. Unlike IDDM, no ketoacidosis is observed since uncontrolled lipolysis is not a feature of this diabetic state (Delvin, 1982) Repeated pregnancy, menopause, infection,

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illness and ageing are implicated as diabetogenic stress factors in NIDDM (Dolger and Seeman, 1976) With the advent of modern methods of diabetes control, most NIDDM patients are now able to live healthy, normal lives, and for longer periods too.

In this study, genetically diabetic mice, strain C57BL/KsJ, genotype +db/db+, were used as the NIDDM model.

1.2.3 Liver glycogen levels in diabetics

Glycogen, a very large branched polymer of glucose residues, is a readily mobilised storage form of glucose. Although glycogen occurs in many kinds of cells, its main concentration is in cells of the parenchyma of the liver and of skeletal muscles Its concentration in the liver of a fed human adult is estimated to be about 5-7% or less (Seifter and England, 1983) However the metabolic lability of glycogen makes its quantitative determination difficult. Generally, glycogen synthesis in the liver is favoured by insulin and glucocorticoids; some of the effect of the latter are mediated through insulin since such steroids can stimulate its secretion (Ellenberg and Rifkin, 1983). As mentioned earlier insulin inhibits phosphorylase which catalyses the breakdown of glycogen. It also activates glycogen synthase involved in the synthesis of glycogen. In NIDDM, insulin is present and therefore glycogen synthesis is expected to occur In IDDM, there is a relative or absolute deficiency of insulin and therefore glycogen levels are expected to be low. Work done at this department has revealed no significant difference

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between the glycogen content in the liver of normal diabetic mice and STZ-induced diabetic mice (Sittie, 1985) It is therefore difficult to predict glycogen levels in relation to insulin levels in diabetes, since regulation of glycogen synthesis is so complex. Also the effect of the gluco-corticoids are in fact quite complex in that they can also promote glycogenolysis through permissive effects on glucagon and other catabolic hormones (Ellenberg and Rifkin, 1983)

1.2.4 Cytochrome P450 and diabetes

Drugs are important in helping the body to attain a normal state after a disease. However, after they have done their work, drugs must be excreted. This excretion is the function of the liver which utilizes a two-phase reaction. The first phase of the reaction uses the microsomal NADPH-dependent cytochrome P450 monooxygenase enzyme complex to make the drug more polar by hydroxylation reactions. The second phase involves conjugation of the product of the phase one reaction with acids such as glucuronate and sulphate, and subsequent excretion in the bile.

The monooxygenase complex is an electron carrier system in the endoplasmic reticulum and is made up of, at least, two types of proteins; a flavoprotein, NADPH-cytochrome P450 reductase and a haemprotein, cytochrome P450 (CYP) Oxygen is the final electron acceptor in the system; one atom of the oxygen gets inserted into the substrate (the drug), usually in a hydroxylation reaction, and the other is reduced to water using the electrons in the carrier system donated by the

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NADPH. A whole range of constitutive enzymes of cytochrome P450 exist for the normal metabolism of particular tissues. In addition specific cytochrome P450 proteins for the metabolism of particular drugs are inducible and are synthesized in response to the administration of drugs, thus ensuring their metabolism and subsequent excretion. Because of this induction, the protein content of the endoplasmic reticulum goes up when living things are exposed to drugs.

Changes in the activity of the microsomal mixed function oxygenase system in the liver of rats suffering from diabetes have been detected, and induction of CYP by the disease has been documented (Funae et. al., 1988). For example, CYP with the molecular weight of 52,000 has been induced in genetically diabetic rats, and this diabetes-induced CYP seems to be the same as that induced with acetone, ethanol or fasting (Funae et. al., 1988). Funae et. al., (1988) have also been able to purify and characterized a diabetes-inducible CYP (termed P450DM) to electrophoretical homogeneity (M.wt. 51,000) by high performance liquid chromatography (HPLC) from liver microsomes of diabetic rats induced with streptozotocin (STZ). It would be interesting to find out in this study, whether in addition to the diabetes-induced CYP, the flavonoid extract (drug) also induces another CYP for its own metabolism, or rather inhibits the induction of the diabetes-induced CYP

1.3 TREATMENT OF DIABETES

The method of treatment depends on the type and severity of the disease and whether or not the body of the patient is

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able to produce some amount of its own insulin. Several forms of treatment are available. These involve insulin administration, oral hypoglycaemic drug administration, dietary control measures and treatment with traditional plants or herbal preparations. In all cases, strict dietary control measures are beneficial (Lutherodt, 1977)

1.3.1 Insulin administration

IDDM patients who are deficient in the hormone are put on daily insulin injection. The type and quantity of insulin as well as the times of injection depend on the patient's status and are best decided by the clinician. Problems of antigenicity have, however, become common with the intramuscular injection of the insulin (Dolger and Seeman, 1976; Berhanu and Olefsky, 1981). Fortunately, intensive research is underway to find a more convenient way of administering the insulin by upper gastrointestinal absorption (Nishibata et. al., 1981a and 1981b) Insulin is a protein and therefore when taken by mouth is destroyed by the proteinases of the lower gastrointestinal tract. Other drug manufacturers are performing clinical trials of a new form of insulin that can be taken orally The new form uses genetically engineered human insulin molecules protected by an outer wrapping of fatty cells that are gradually broken down to release the insulin in the small intestines where it is absorbed in the blood (Mennun, 1990) .

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1.3.2 Oral hypoglycaemic drugs

Oral hypoglycaemic agents that are effective in the management of the disease in NIDDM patients have been developed for over half a century Typical amongst these are the sulphonylureas. These drugs stimulate the production and secretion of insulin by the islet cell as well as stimulating deposition of glycogen by its action on hepatic enzymes (Liebovitz and Feinglos, 1983) However, after several months or years of treatment, the pancreatic beta cells may lose their responsiveness to sulphonylureas. These drugs are also limited in use due to their clinical hypoglycaemic side effects. Some of these side effects are found to be fatal, especially the impaired counterregulatory response in NIDDM patients.

The biguanidines are another type of oral hypoglycaemic agents which are believed to increase the utilization of glucose by the tissues while decreasing hepatic glucose output without glycogen storage. The biguanidines are however known to cause lactic acidosis (Lebovitz and Fienglos, 1983; Lewis and Elvin-Lewis, 1977) A synthetic fatty acid analogue known as 2-tetradecylglycidate (TDGA), is involved in long chain fatty acid oxidation with a resultant lowering of blood glucose levels in several animal species (Lee *et. al.* 1982) TDGA acts by irreversibly inhibiting the activity of carnitine palmitoyl transferase.

1.3.3 Dietary control

The diet of an obese diabetic patient must provide

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sufficient calories to keep the patient s weight at the normal non-obese level. Patients are mostly advised to avoid foods containing refined carbohydrates such as sugar, biscuits, sweet fizzy drinks such as coca cola and alcohol. Natural fruits and vegetables should be encouraged (Rolfe, 1991). Food scientists have been concerned with the effect of the fibre content of foods on the process of carbohydrate metabolism, digestion and absorption. In a survey conducted at the Nutrition unit of the Noguchi Memorial Institute For Medical Research, plasma glucose response to five Ghanaian foods were determined in nine male non-insulin dependent diabetics in the form of mixed meal. All these nine subjects controlled their diabetes with diet alone. The observations were that, Ga kenkey, rice and big, green plantains could be ideal for diabetic diets in Ghana (Brakohiapa et. al., 1993) According to the authors, this finding, is similar to that observed in normal non-diabetic subjects (Brakohiapa et. al., 1991). The reasons why these foods produce least post-prandial rise in blood glucose levels is yet to be investigated. However, the high fibre content may be a factor (Brakohiapa-personal communication)

1.3.4 Anti-diabetic herbal preparations

The search for new anti-diabetic agents has extended to the investigation of traditional plant medicines. More than 100 different plants and plant extracts are reported to possess hypoglycaemic properties. Few of these "natural treatment" for diabetes have received adequate medical and

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scientific scrutiny and only a small number of purportedly active compounds have been wholly or partially characterized (Bailey and Marks, 1989). A survey of the literature has shown that a large variety of compounds obtained from several plant families are found to be responsible for the hypoglycaemic action. For instance, glycosides isolated from the families Caesalpinaceae, Compositae, Convolvulaceae, Ericaceae, Moraceae, Myrataceae, Papaveraceae, Ranunculaceae, Rhamnaceae and Scrophulaceae have been shown to possess active ingredients which lower plasma glucose in test animals. Polysaccharides, oils and vitamins from the family Graminae show pharmacological activity by decreasing plasma glucose level in animals (Atta-Ur-Rahman and Zaman, 1989) Beta-sitosteryl-D-glucoside isolated from the bark of Ficus religiosa also shows hypoglycaemic activity (Atta-Ur-Rahman and Zaman, 1989) In Madagascar, South Africa, Philippines, Jamaica, India and Australia, C. roseus has long been used as an infusion in the treatment of diabetes (Sofoworo, 1982)

In Ghana, a number of plants have been documented to have anti-diabetic property. Typical examples are I. arrecta, B. ferruginea, Costus schlechteri (Zingiberaceae), Myrianthus arboreous (Moraceae), Anthocleista kerstingii (Loganiaceae) and Catharanthus roseus (Apocynaceae) (Ayensu, 1978) At the CSRPM, decoctions of I. arrecta and B. ferruginea are dispensed as anti-diabetic preparations. In the case of I. arrecta a concentration of 20g/l extract is prepared by adding 20g of the leaves to one litre of boiling water This is further boiled for 20 minutes, filtered and cooled. This is

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then used as a substitute for water For *B. ferruginea* 5kg of the dried leaves is boiled in 80 litres of water for 1.5 hours. It is cooled and the volume readjusted to take care of evaporation. The patient then takes one teacupful of the decoction 3 times daily The *I. arrecta* extract was found to prevent the development of hyperglycaemia in the genetically diabetic mice (db/db mice) without any adverse effect (Gyasi, 1983; Sittie, 1985; Quaye, 1986; Addy *et. al*., 1992).

On oral administration to normal fasting rabbits and rats, flavonoids of *B. ferruginea* have been shown to be active as hypoglycaemic agents (Addae-Mensah and Munenge 1989) Due to the secrecy surrounding traditional medicaments, many potent medicinal plants are not well known (Ampofo, 1977). *B. ferruginea* is a typical example of such plants whose anti-hyperglycaemic effect is not well known. It is therefore hoped that, the results of this present study will enhance the interest and further research on the plant.

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1.4 BRIDELIA FERRUGINEA

Bridelia ferruginea (wild. corr. spreng.) of the family Euphorbiaceae is a widely used plant in West Africa. For example, in Togo the root bark is used for intestinal, bladder as well as skin diseases. In the Hausaland, the leaf and stem bark decoctions are used to immunise a person against syphilis. The pulped bark is used as enema or beverage for dysentery and diarrhoea, for fevers, headaches, stiffness and rheumatic pains. A decoction from the root is also used against gonorrhoea, whilst the leaves are used against fever, and as a mouthwash (Addae-Mensah, 1992).

B. ferruginea in combination with I. arrecta have been in use at the CSRPM for the treatment of diabetes. This plant was used at the Centre to treat some patients whose fasting plasma glucose levels were above 240mg/100ml of blood (Ampofo, 1977) A gradual fall in the fasting plasma glucose resulted until it became normal after 12 weeks. However, in all cases, the patients returned to the diabetic state when treatment was discontinued.

Since 1983 work on this plant has been centred on its aqueous methanol extract of the leaves, mainly the flavonoids. In Nigeria, these have been reported to significantly lower the fasting blood glucose levels of albino rats, but failed to protect the animals against alloxan-induced diabetes (Iwu, 1980) as already mentioned In its preparation a freshly prepared solution of the flavonoids in 0.85% sterilized saline solution is administered intraperitoneally in a volume of 0.5ml to the test animals. A number of these flavonoids have

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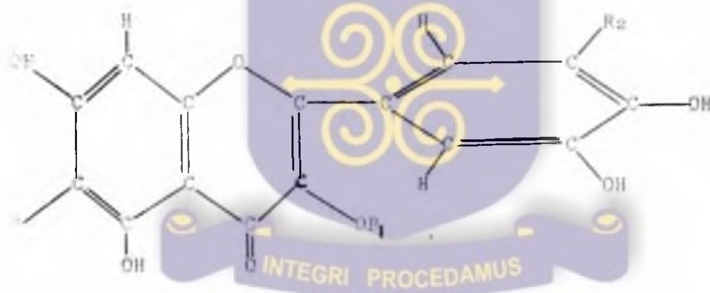
been isolated and among these are quercetin, quercitrin, rutin, myricitrin, myricetin-3-O-β-glucoside and 3-methyl-quercetin (Addae-Mensah 1992). Rutin has been found to be relatively non-toxic since 2.25g daily for 7 days shows no toxic effect in man. It is totally absorbed and rapidly metabolised in humans. Rutin and the other flavonoids are therefore potential anti-hyperglycaemic agents needing further investigations. A typical structure of a flavonoid is as shown below:



(a) $R_1 = R_2 = H$ for Quercetin

(b) R_1 = Glu. R_2 = H for Quercitrin

(c) R_1 = Glu-Rha R_2 = H for Rutin

(d) R_1 = Rha. R_2 = OH for Myricitrin

(e) R_1 = Glu. R_2 = OH for myricetin-3-o-β-glucoside

(f) R_1 = Me R_2 H for 3-Methylquercetin

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1 5 DRUG_TOXICITY_TESTS

Toxicology is defined as the study of poisons A poison may be defined as that which causes sickness or death, when taken in sufficient quantity From the above definitions, one may say that there is no clear-cut difference between a drug and a poison. This is because a drug, administered in doses that alter physiological function in order to produce a desired therapeutic effect, may produce toxic or harmful effects if greater than the therapeutic quantities are administered

In the administration of a drug, the therapeutic index, a measure of the drug s safety becomes important. Thus the concentration of the drug at which 50% of the animals tested will be killed (LD_{50}) can be used as a measure of toxicity Another important index of toxicity involves damage to vital organs such as the liver and kidney

1 5 1 Acute_and chronic_toxicity_tests

Acute toxicity refers to the harmful effect of a drug or an agent that manifests itself in seconds, minutes, hours or days after entering the patient, whilst chronic toxicity entails a long-term harmful effects of an agent weeks, months and years after entering the patient. The number of times the drug is administered can also result in acute or chronic toxicity The results of chronic and acute toxicity experiments together with other information including those derived from pharmacokinetics, teratogenesis and perinatal toxicity form the basis for the evaluation of the possible

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hazardous properties of a drug as well as its safety (Rumke, 1964) The determination of an effective lowest concentration or safe dose level is another important objective in toxicity tests and is needed to determine the margin of safety of a given compound. In the study reported here, short-term effects of the flavonoid extract were related to the acute toxicity whilst long-term administration of the flavonoid extract was related to the chronic toxicity For a measure of the toxicity of the flavonoids, liver and kidney function tests were performed due to the fact that, certain drugs or their metabolites are able to damage these vital organs thereby affecting their function. If the flavonoid is toxic then possible damages to these vital organs would be observed and its administration as a therapeutic agent limited or abandoned.

1.5.2 Liver function test

The liver plays a major role in enzymatic transformation and disposition of drugs. Many drugs will undergo limited or significant transformation resulting in their accumulation and undesirable effects upon the liver The drug's adverse effect on the liver disrupts its function and therefore one can measure liver function test as a test of toxicity of the drug. The liver contains certain specific compounds and enzymes and therefore when it is damaged these compounds and enzymes are released into the blood circulation leading to elevated levels of these compounds and enzymes in the plasma.

A combination of laboratory tests most frequently used in

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confirming liver damage include serum bilirubin, aspartate amino-transferase (AST), alanine aminotransferase (ALT) and gamma glutamyl transferase (GGT). Serum bilirubin gives the most informative biochemical indicators on how severe the liver damage is. Bilirubin itself results from the reduction of biliverdin catalysed by the enzyme bilirubin reductase. The bilirubin then forms a loose association with albumin in the plasma and carried to the liver to be conjugated with glucuronate, the product of which is more soluble for easy excretion into the intestine with the bile. During liver damage, however, the conjugation is not done or reduced to the minimum leading to an elevated concentration of the bilirubin in the serum. Although bilirubin determination was one of the parameters for the liver function test, it could not be performed, due to non availability of the reagents for its determination during the period of the study.

The other most important parameter for the liver function used is gamma glutamyl transferase (GGT). This enzyme is present in serum and in all cells except in muscle. It is predominantly located in the cell membrane and may act to transport amino acids and peptides into the cell in the form of gamma glutamyl peptides. Gamma glutamyl transferase activity is elevated in all forms of liver damage and is the most sensitive serum enzymatic indicator of liver damage (Committee for Clinical Chemistry and Clinical Physiology, 1976). GGT was therefore selected for the liver function tests.

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1.5.3 Renal function test

The functions of the kidney may be characterized as excretory, regulatory and endocrine. The excretory function serves to rid the body of most of the undesirable end-product of metabolism, as well as any excess of inorganic substances ingested in the diet. The regulatory function of the kidney plays a major role in homeostasis (Rock et. al., 1987) Damage to such a vital organ may result in the death of the patient. The adverse effect of the drug or its metabolite may be on the kidney, hence the renal function tests to test for the possible damage to the kidney by the flavonoid extract.

Possible tests for evaluating renal function, involve clearance of various compounds (creatinine in particular), estimation of glomerular filtration rate, assessment of glomerular permeability by establishing the types of proteins appearing in urine, measurement of plasma creatinine, measurement of the concentrating ability of the tubules and the measurement of blood urea nitrogen (BUN) (Heinegard and Tiderstrom, 1973). One disadvantage of the BUN is that, it is elevated during heavy protein meal Due to this demerit of BUN, serum creatinine determination was selected for assaying renal function.

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CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Animals

Genetically diabetic mice, strain C57BL/KsJ m+/+db genotype +db/db+ and their non-diabetic lean littermates were bred locally at the Animal unit of the Noguchi Memorial Institute For Medical Research (NMIMR) from breeding pairs purchased from Jackson laboratory Bar Harbour Main, U.S.A. . Another set of genetically diabetic mice of the same strain and genotype and their lean non-diabetic littermates were purchased from Jackson laboratory to supplement those bred at the NMIMR. In addition, albino mice were purchased from the Animal unit of the NMIMR. The mice purchased from the Jackson laboratory were allowed to stay for at least one week for them to acclimatise before being used. When locally bred, both male and female mice of the diabetic strain with their non-diabetic lean littermates were purchased and used, whereas only female mice were used, when the animals were purchased and used without breeding. Some of the non-diabetic littermates of the mutant diabetic mice and the albino mice from the NMIMR were used for the induction of the Type I diabetes. All the animals were fed ad libitum on pelleted animal feed from the NMIMR. The composition of the feed was 40% wheat bran, 40% maize, 2% fish meal, 17% cod liver oil and 1% sodium chloride.

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3.1.2 Reagents and chemicals

Glycogen, hydrochloric acid, potassium dihydrogen phosphate, sodium chloride, sodium citrate, sodium hydroxide, sucrose and Tris-(hydroxymethyl)-aminomethane were purchased from Fluka Garantie Chemika, Switzerland Amyloglucosidase (EC 3.2.1.3), bovine serum albumin, cytochrome c, reduced nicotinamide adenine dinucleotide phosphate (NADPH) and streptozotocin (STZ) were purchased from Sigma Chemical Company St, Louis, Mo, U.S.A. Citric acid was purchased from Eastman Kodak Company, U.S.A. Potassium chloride was purchased from May and Baker Limited, England. Ethylenediaminetetraacetic acid (EDTA) and Folin-Ciocalteau Reagent were purchased from Hopkin and Williams, England. Creatinine, glucose and gamma glutamyltransferase test kits were supplied by Randox Laboratories limited, Ireland.

3.1.3 Flavonoid extract

The extract made up of Quercetin-3-neohesperidoside (rutin) and other flavonoids extracted from Bridelia ferruginea leaves, was provided by Ivan Addae-Mensah of the Chemistry Department, University of Ghana.

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2.2 METHODS

2.2.1 Induction of diabetes

For the model of insulin-dependent diabetes mellitus, non-diabetic littermates of the mutant strain C57BL/KsJ m+/+db, and albino mice weighing approximately 20-30g were used. A freshly prepared solution of streptozotocin (STZ) in 1mM citrate buffer pH 4.2, was administered intraperitoneally in a volume of 0.2ml for five consecutive days. Each mouse received 40mg per kilogram body weight of the STZ for each of the five days.

2.2.2 Administration of flavonoid extract

A freshly prepared solution of the flavonoid in 0.85% sterilized saline solution was administered intraperitoneally in a volume of 0.5ml to the test animals. The control mice received 0.5ml of the saline solution without the extract. Each test animal was given 10mg of the extract per kilogram body weight. Subsequently some mice were given 20mg of the extract per kilogram body weight.

With respect to the diabetic state there were various treatment groups as follows:

(i) Non-diabetic mice of strain C57BL/KsJ m+/+db or albino mice as one type of control mice without the symptoms of diabetes.

(ii) STZ-induced diabetic mice as the model for the insulin-dependent diabetes mellitus.

(iii) Genetically diabetic mice of strain C57BL/KsJ and

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genotype +db/db+, as the model for the non-insulin dependent diabetes mellitus.

In one type of treatment schedule set up to investigate the short-term effect of the extract, the extract was administered only once to the test animals followed by glucose monitoring at hourly or half-hourly intervals. This was done for all the three different groups of mice with respect to the diabetic states (ie i, ii, iii above)

Another type of treatment schedule was set up to investigate the long-term effect of the extract. A set of mice of the insulin-dependent diabetes mellitus and another set of the non-insulin dependent diabetes mellitus model were given the extract three times a week for at least four weeks. Glucose monitoring in this set up was done weekly

The test mice were given the extract at different times with respect to the induction of diabetes with STZ in the case of the IDDM model, or age of mice in the case of the genetically diabetic NIDDM model. Glucose monitoring here was done weekly

[illegible].3 Plasma glucose determination

Blood samples collected from the tail vein were centrifuged using the Beckman microfuge for one and half minutes to obtain plasma. Samples of plasma were used to measure glucose by a glucose oxidase method, using the Beckman Glucose analyzer II as follows: A precise volume of 10ul sample was manually pipetted into enzyme reagent in a cup containing an electrode that responds to oxygen concentration.

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The rate of oxygen consumption which is directly proportional to the concentration of glucose in the sample was displayed as a direct readout in milligram of glucose per 100 millilitres (mg/dl). This unit of measurement of plasma glucose concentration was used as such and not converted to mmols/l.

Another method used for the determination of glucose was that described in the Sigma glucose kit. This method is a quantitative, enzymatic (Glucose oxidase) determination at 433-475 nm. The procedure is essentially that of Raabo and Terkildsen (1960) with a minor change in the quantity of chromogen to increase sensitivity In this procedure 25 ml of each plasma sample and two standards were carefully pipetted into test tubes and 0.5 ml distilled water added to each tube. Into each tube was added 5.0 ml of combined enzyme-colour reagent solution and the contents mixed thoroughly The enzyme solution contained glucose oxidase from *Aspergillus niger*, peroxidase from horseradish and buffer salts. The colour reagent was made by reconstituting o-dianisidine dihydrochloride provided in the kit with 20 ml distilled water. The combined enzyme-colour reagent solution was prepared by combining 100 ml of the enzyme solution and 1.6 ml colour reagent solution. The test tubes were incubated at 37°C for 30 ± 5 minutes or at room temperature for 45 minutes.

At the end of the incubation period, all the tubes were removed from the incubator and absorbance read at 450 nm using the Shimadzu Double Beam Spectrophotometer, Model UV-190. Concentrations of the glucose in the plasma were obtained using the conversion below:

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Plasma glucose (mg/dl) = Test sample/Standard x 100.

2.2.4 Liver glycogen determination

The procedure used is that of Murat and Serfaty (1974). After each period of study, the mice were anaesthetized, and blood taken by cardiac puncture, centrifuged and the serum set aside for the determinations of GGT and creatinine. The abdomen of each was quickly opened and approximately 0.1- 0.3 g liver tissues removed and immediately placed in pre-weighed vials containing 10 ml of 0.1 M ice-cold citrate buffer, pH 4.2 and sodium fluoride (NaF, 0.25%). The difference in weight gave the wet weight of the liver tissue used. Immediately after the weighing, the tissues were individually homogenized using a Polytron homogenizer

After homogenization, free glucose in the tissue was determined using the Beckman glucose analyzer or the Sigma glucose kit as described above. Ten milligram amyloglucosidase powder was then added to each homogenate and the mixture allowed to stand at room temperature overnight. Total glucose was then determined for each hydrolysed homogenate and the amount of glucose equivalent to the glycogen content of the tissue was obtained by subtracting initial free glucose value from the total glucose value. In determining the glycogen content of the liver samples, a calibration curve for glycogen was prepared for each series of determination using different concentrations of commercial glycogen, hydrolysed alongside the tissue homogenates to ensure that conditions were similar for both. For the glycogen calibration, a 100ml

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stock solution of 5 mg/ml glycogen was prepared by dissolving 0.5 g commercial glycogen in 100ml distilled water. From the stock of 5 mg/ml, serial dilutions were made with concentrations ranging from 0-3 mg/ml. The amount of glycogen in each liver sample was determined using the calibration curve.

2.2.5 Preparation of liver microsomes

Mice in treatment groups for the evaluation of the long-term effect of the extract were killed at the end of the experimental period, the abdomen was opened immediately and about 0.1-0.3 g of the liver excised for liver glycogen determination as described above. The rest of the liver was removed and pooled for each group of mice undergoing the same treatment. These were frozen below -70°C if not used immediately. The frozen tissues were thawed, placed in ice-cold 0.15M KCl and homogenized (4ml/g wet weight) in 10mM Tris-buffer, pH 7.5, containing 0.25M sucrose. The tissue homogenates were centrifuged first at 10,000g for 20mins at 4°C. The supernatant was centrifuged at 100,000g for 60mins at 4°C and the resulting microsomal pellet resuspended in 0.1M potassium phosphate buffer pH 7.5, divided into small aliquots and frozen immediately below -70°C. Protein concentration in each preparation was determined using the Folin-Lowry method described below

2.2.6 Protein concentration

To 50μl microsomal preparation, 5ml of an alkaline copper solution was added, mixed thoroughly and allowed to stand at

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room temperature for at least 10 minutes. Ten millilitres of diluted Folin-Ciocalteau reagent was added rapidly with immediate mixing. After 30 minutes of incubation at room temperature the absorbance at 750 nm against the sample blank was read. A calibration curve was prepared for each series of determination using commercial bovine serum albumin, from a stock solution containing 0.2 mg/ml. From the stock solutions, serial dilutions were made with concentrations ranging from 0-0.2 mg/ml. The amount of microsomal protein was determined using the calibration curve.

2.2.7 Determination of NADPH-dependent cytochrome c reductase (NCCR) activity

In a preliminary assay, NCCR activity was measured in a 3.5ml solution containing 0.3M potassium phosphate buffer pH 7.4, 1mM EDTA, 136uM cytochrome c and 50ul microsomal preparation. Subsequently the volume of microsomal preparation was adjusted to contain 0.3-0.4mg protein. The reaction was initiated by adding 20ul of 10mM NADPH. The increase in absorbance at 550 nm was recorded at 25°C for 3 minutes. The amount of cytochrome c reduced was calculated using the extinction coefficient of 2.77×10^7 cm^2/mole.

2.2.8 Gamma glutamyl transferase (GGT) determination

The procedure used was the one found in the Randox GGT test kit. The assay is based on the use of L-gamma-glutamyl-p-nitroanilide as the donor substrate and glycylglycine as the glutamyl acceptor. The p-nitroaniline produced in the

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reaction is determined by its yellow colour, which is monitored at 405nm. To 0.20ml of the serum prepared as above (section 2.2.3) was added 2.0ml of the reagent (Tris-buffer 100mmol/l pH 8.25, glycylglycine 60mmol/l, L-gamma-glutamyl-p-nitroanilide 4mmol/l, and surfactant) This was mixed and initial absorbance read at time zero. The time was noted and absorbance were read after 1, 2, and 3 minutes against air at 405nm. The concentrations of the GGT in the serum were obtained using the conversion below:

Serum GGT activity (U/I) = 1111 x dA_{405} /min

2.2.9 Creatinine determination

The method used is based on the Jaffe reaction, which occurs between creatine and picrate ion formed in alkaline medium. A red-orange adduct develops and the rate constant of adduct formation is a linear function of the hydroxyl ion concentration over a broad range of picric acid concentrations (Bartler, 1976).

Randox creatinine test kit provided the detailed procedure. A standard was prepared by adding 0.2ml standard solution provided in the kit to 2.0ml of the reagent mixture (picric acid 35mmol/l, surfactant and 0.32mol/l of sodium hydroxide). These were mixed and absorbance of the standard, $A_{standard1}$ and sample (serum), $A_{sample1}$, read after 30 sec. Exactly 2 minutes later absorbance $A_{standard2}$ and $A_{sample2}$ were read. Creatinine concentration in serum, (x). was calculated as follows:

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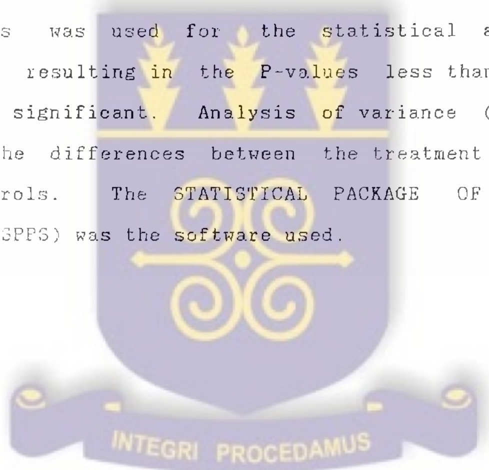
$A_{standard2} - A_{standard1} = A_{standard}$

$A_{sample2} - A_{sample1} = A_{sample}$

$x\ (mg/dl) = A_{sample}/A_{standard} \times 2$

3.2.10 STATISTICAL ANALYSIS

The mean ± standard error of the mean (MEAN ± SEM) was used in expressing the data. The t-test for paired observations was used for the statistical analysis and differences resulting in the P-values less than 0.05 were considered significant. Analysis of variance (one-way) was used for the differences between the treatment groups and their controls. The STATISTICAL PACKAGE OF THE SOCIAL SCIENTIST (SPSS) was the software used.



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CHAPTER 3

RESULTS

3.1 SHORT - TERM EFFECT OF THE EXTRACT ON NON-DIABETIC MICE

Figure 1, shows the short-term effect of the flavonoid extract on plasma glucose levels of non-fasting non-diabetic mice which were lean littermates of the diabetic mutant strain used. Each animal was given 10mg/kg body weight of the extract. In the males, the initial plasma glucose level (130.20 ± 3.51mg/dl) determined before administration of the extract for the test animals was not significantly different from the plasma glucose levels of the control animals of 124.50 ± 3.25mg/dl. No significant difference was found three hours after the flavonoid administration, between the plasma glucose level of the test animals (122.25 ± 6 50mg/dl) and the plasma glucose level of the control mice (130.00 ± 6.31mg/dl)

In the females, the plasma glucose levels of the test animals 135.15 ± 4.58mg/dl and the controls 140.20 ± 2.86 mg/dl were not significantly different before the start of the extract administration. As in the case of the males, no significant difference in the plasma glucose levels of the test mice (139.90 ± 5.85mg/d) and the control mice (138.90 ± 5.00mg/dl) after the three hour period.

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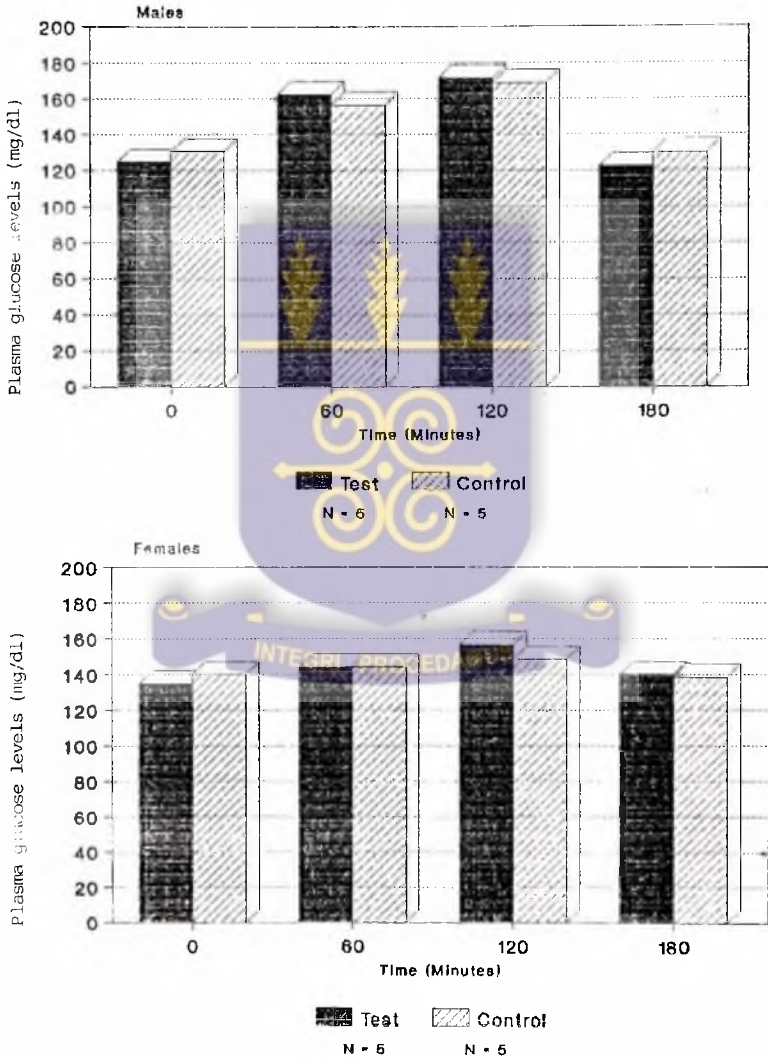
Figure 1: Short-term effect of the flavonoid extract on non-fasting plasma glucose levels of non-diabetic mice over a three hour Period.



N = number of mice used

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3.2 INSULIN - DEPENDENT DIABETES STUDIES

3.2.1 Short-term Effect of the Extract

(a) Plasma glucose levels

Figure 2a, shows the effect of the flavonoid extract on fasting plasma glucose levels of STZ-induced diabetic mice. There were three groups of mice, each group was given the extract at a different time with respect to the induction of the diabetes. The test animals in Group-1, which were given the extract immediately after the STZ administration, had an initial plasma glucose level of 166.79 ± 22.05mg/dl. This was not significantly different from that of control mice which had a glucose level of 181.25 ± 36.38mg/dl before the extract was administered. After a two hour period, the difference in the glucose level of the test mice, 181.25 ± 18.06mg/dl and the control mice with glucose level of 234.75 ± 28.04mg/dl was not statistically significant.

In Group-2, where the test animals were given the extract one week after the STZ injection, initial plasma glucose level of both test and control animals were very similar: 368.30 ± 15.02mg/dl and 365.20 ± 32.35mg/dl respectively. After the two hour period, significant difference (P<0.05) was found between the plasma glucose level of the test mice (365.50 ± [illegible].24mg/dl) and that of the control mice (530.00 ± [illegible]mg/dl). Within the test animals, there was no significant difference between any two mean levels within the two hour period, however, within the control animals, the initial glucose level of 365.20 ± 32.25mg/dl was found to be significantly lower than the level of 530.00 ± 30.43mg/dl

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obtained after the two hour period.

In Group-3, in which the test animals were given the extract two weeks after the STZ, no significant difference was found between the initial plasma glucose level of the test animals (303.75 ± 18.74mg/dl) and the control animals, (282.83 ± 22.44mg/dl) before the extract administration. After the two hour period, again no significant difference was observed between the test mice 388.75 ± 7.43mg/dl and the control mice, 409.67 ± 7.70mg/dl. Within the test group, the initial plasma glucose level of 303.75 ± 18.74mg/dl was significantly not different at the end of the two hour period with plasma glucose level of 388.75 ± 7.43mg/dl. In the case of the control animals, the initial plasma glucose level of 282.83 ± 22.44mg/dl was found to be significantly different from 409.67 ± 7.70 mg/dl glucose at the end of the two hour period.

Due to the high plasma glucose levels of the Group-2 animals, it was suspected that, the animals might not have been fasted overnight. However, because significant difference was found between the test mice and the control mice, the experiment was repeated but instead of fasting, the non-fasting plasma glucose levels were measured

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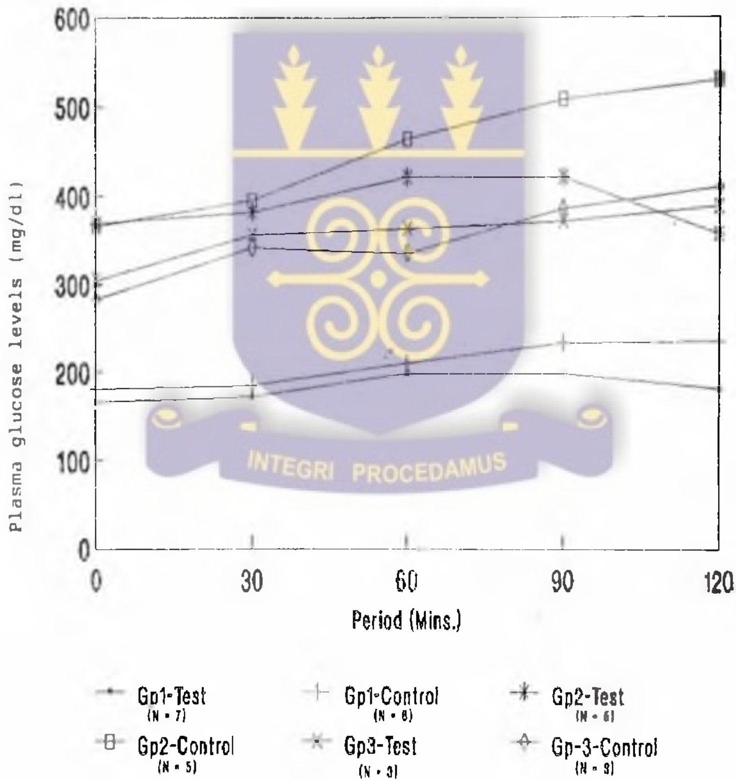
Figure 2a: Short term effects of the flavonoid extract on plasma glucose levels of STZ-induced diabetic mice. Extract administered immediately, one week after and two weeks after STZ injection for group 1, 2 and 3 respectively.



Gp = Group

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Figure 2b represents the non-fasting plasma glucose level in three groups of albino mice. The test animals in Group-1, were given the extract immediately after the STZ administration. The initial plasma glucose level of the test mice, 178.50 ± 18.73mg/dl and the control mice, 171.75 ± 30.00mg/dl were not significantly different before the extract administration. After a three hour period, there was again no significant difference between the plasma glucose level in the test mice (175.10 ± 12.37mg/dl) and the control mice (232.50 ± 31.43mg/dl) Within the group, no two mean plasma glucose levels were found to be significantly different for the test animals, whilst in the controls, the initial plasma glucose level was found to be significantly higher compared to the level after one hour and also after the three hour period.

In Group-2, where the test animals were given the extract one week after the STZ administration, the initial plasma glucose levels of the test mice, 286.80 ± 24.71mg/dl and the control mice, 274.50 ± 57.12mg/dl were not significantly different before the extract was administered. After the three hour period, again no significant difference was observed between the test mice (285.50 ± 35.57mg/dl) and the control mice (319 75 ±73.70mg/dl)

In the Group-3, the test animals were given the extract two weeks after the STZ administration. The initial plasma glucose level of the test mice, 280.42 ± 31.87mg/dl and the control mice 291.63 ± 57.21mg/dl were not significantly different. After the three hour period, the plasma glucose level of the test mice (297.33 ± 32.24mg/dl) was again not

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significantly different from the control mice with 413.13 ± 42.94mg/dl glucose. In the group, no two mean plasma glucose levels were found to be significantly different in both the test animals and the control animals.

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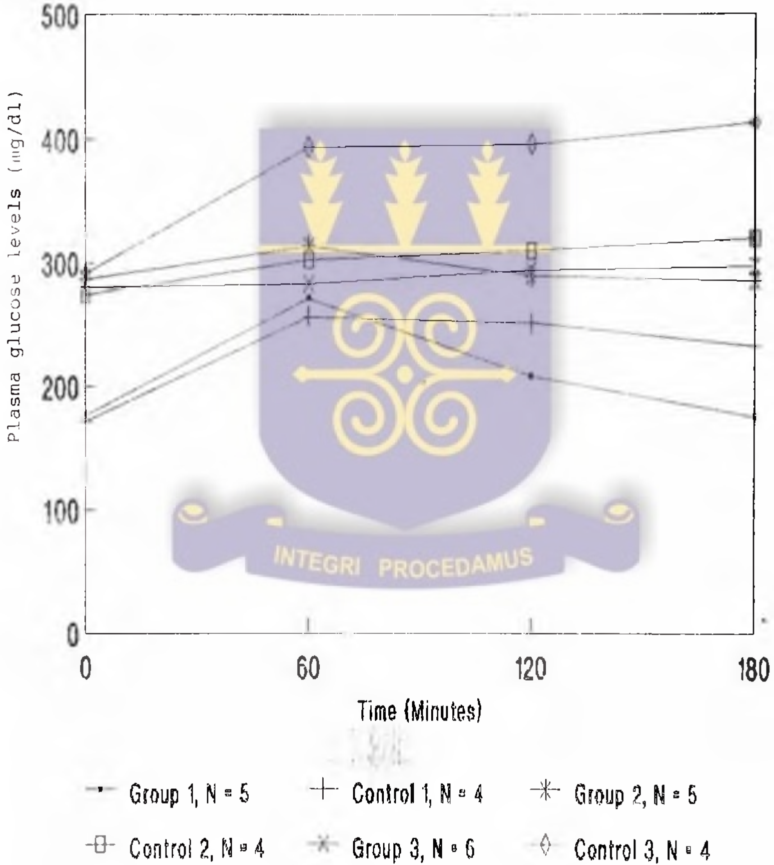
Figure 2b: Effect of the flavonoid extract on non fasting plasma glucose levels of 3 groups of STZ-induced diabetic Mice. Extract administered immediately, one week after and two weeks after STZ injection for group 1, 2 and 3 respectively.



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(b) Liver glycogen Levels:

Figure 2c shows the mean liver glycogen levels (% wet weight tissue) of fasting STZ-induced diabetic mice whose glucose levels are shown in Figure 2a. In Group-1, the liver glycogen level of the test mice was 3.28 ± 0.22% whilst that of the control mice was 3.51 ± 0.29%. These values were not significantly different. In Group-2, the liver glycogen level of the test animals was found to be 3.74 ± 0.51% as against [illegible]4[illegible] ± 0.48% in the control animals. This difference was also not statistically significant. No significant difference in liver glycogen level was found among the Group-3 animals. The liver glycogen level for the test animals was 5.40 ± 0.22% as against 4.70 ± 1.26% for the control mice.

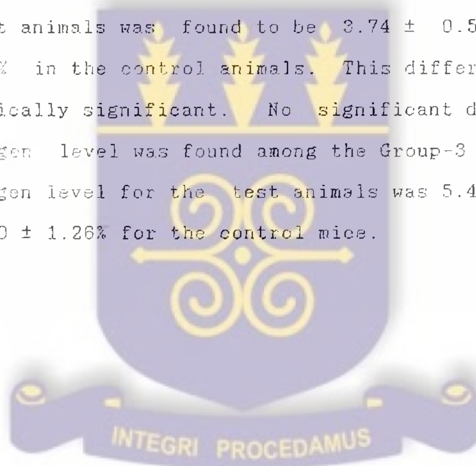

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Figure 2c: Effect of the flavonoid extract on liver glycogen levels of 3 groups of STZ-induced diabetic Mice.

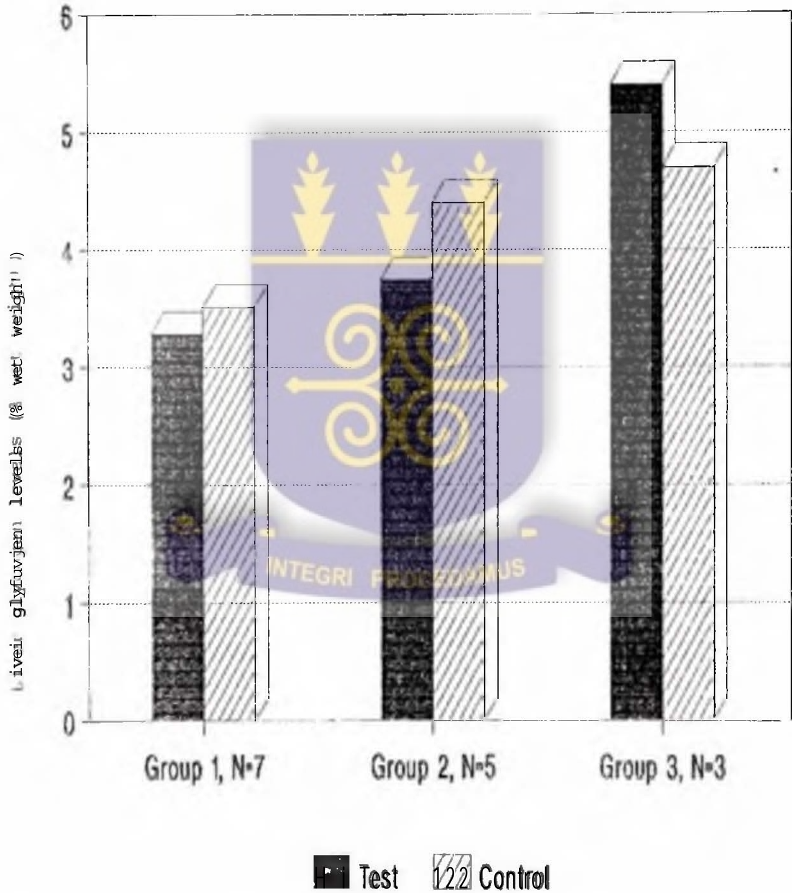

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Figure 2d shows the mean liver glycogen levels of non-fasting STZ-induced diabetic (albino) mice whose glucose levels are shown in Figure 2b. In Group-1, the liver glycogen level of the test animals was found to be 2.33 ± 0.22% whilst that of the control animals was 2.39 ± 0.54%. In Group-2, the liver glycogen level of the test animals was observed to be 2.14 ± 0.36% as against 2.50 ± 0.55% for the control animals. In the Group-3, the liver glycogen level of the test animals was 3.22 ± 0.53% as against 4.73 ± 0.54% in the control animals. The values for the tests animals were not significantly different from those of the control animals.

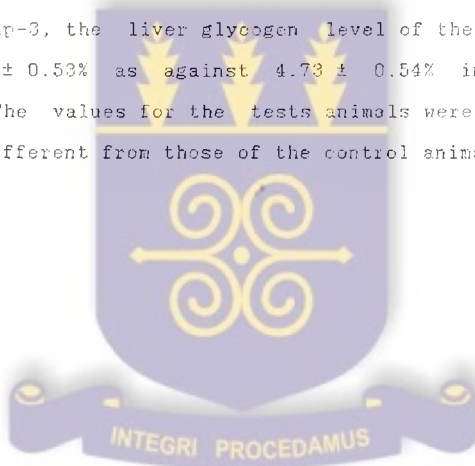

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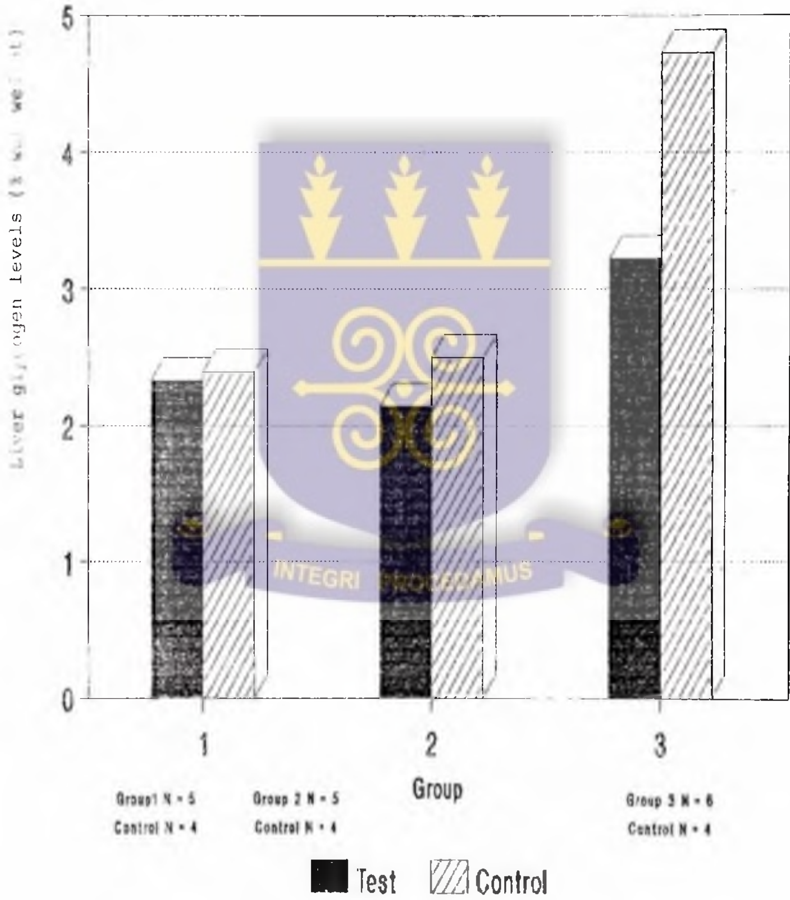
Figure 2d: Liver glycogen Levels of STZ - induced diabetic Mice (Albino) in response to administration of the flavonoid extract.



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3.3.3 Long-Term Effect of the Extract

(a) Plasma Glucose Levels

The results of the non-fasting plasma glucose levels of STZ-induced diabetic mice that were given the flavonoid extract (20mg/kg body weight) three times a week for at least four weeks are shown in Figure 3a. The initial plasma glucose levels of the three treatment groups were determined and shown not to be significantly different. Group-1 in which the animals were given the extract immediately after the STZ, had initial plasma glucose level of 163.30 ± 8.28mg/dl. Group-2 in which the animals were given the extract two weeks after the STZ, had plasma glucose level of 160.50 ± 2.24mg/dl and the controls which were not given the extract had initial glucose of 179.38 ± 14.91mg/dl. Two weeks after the STZ injection, all the animals developed diabetes with plasma glucose levels above 400mg/dl. For instance, the plasma glucose level of Group-1 animals increased from the initial level of 163.30 ± 8.28 to 480.90 ± 73.19mg/dl, that of the Group-2 animals increased from an initial level of 160.50 ± 2.24 to 436.40 ± 129.65mg/dl whilst that of the control animals increased from 179.38 ± 14.91 to 468.50 ± 67.59mg/dl. [illegible] six weeks of the experimental period, plasma glucose levels of the Group-1 mice rose from the initial level of 163.30 ± 8.28 to 408 ± 69.25 mg/dl, that of the Group-2 rose from an initial level of 160.50 ± 2.24 to 472.80 ± 122.69mg/dl whilst the control mice rose from 179.38 ± 14.91 to 780.50 ± 112.2mg/dl. In the seventh week, all the animals in the three treatment groups were fasted overnight and the plasma glucose

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[illegible] measured and compared with the non-fasting levels determined in the sixth week. The results are presented in Figure 3b For the Group-1 mice, the non-fasting glucose level of 408.80 ± 69.25 mg/dl in the sixth week and the fasting level 371.30 ± 49.95 mg/dl in the seventh week were not significantly different from each other The same was true of the Group-2 mice where a non-fasting glucose level of [illegible] ± 122.69mg/dl and a fasting level of 363.10 ± 28.12mg/dl were measured. However, in the control mice, the non-fasting level of 780.50 ± 122.2 mg/dl was found to be significantly different from the fasting level of 406.63 ± 33.14mg/dl ($P<0.05$).

These same animals, including the controls, were given the extract for another three week period. After this period, [illegible]-fasting plasma glucose level of the Group-1 animals was observed to be 499.63 ± 135.66mg/dl, that of Group-2 was 470.99 ± 124.76mg/dl whilst that of the controls rose to 1125.00 ± 166.31mg/dl.

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Figure 3a: Long-term effects of the flavonoid extract on non fasting plasma glucose levels of STZ-induced diabetic mice in three treatment groups (see text for description of treatment groups).

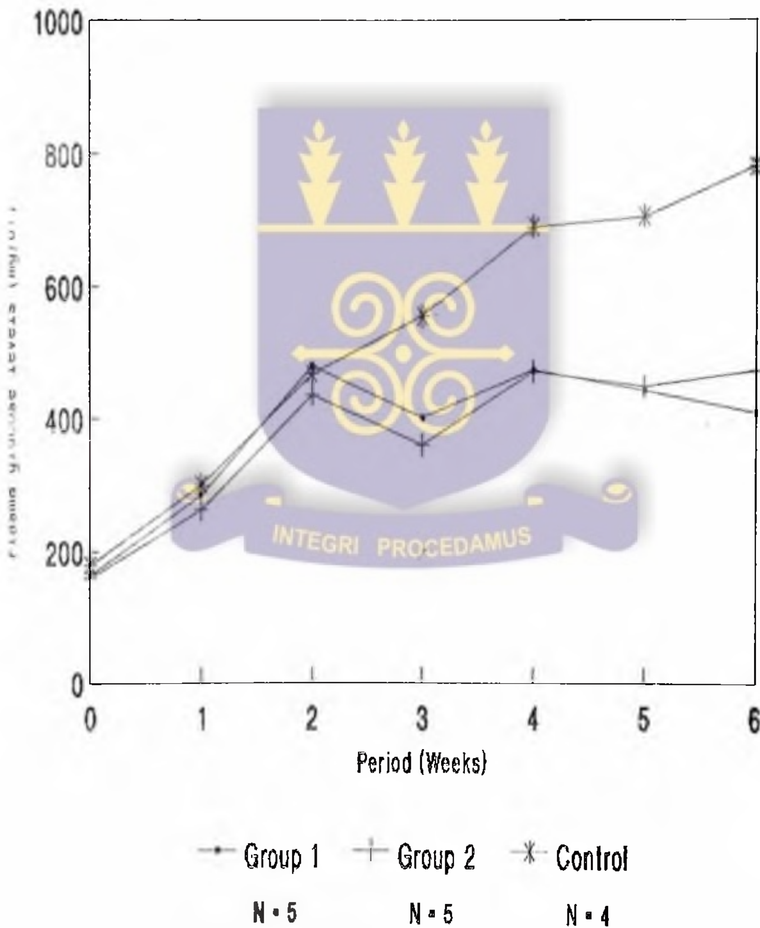

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Figure 3b: Comparison of fasting and non-fasting plasma glucose levels in STZ-induced diabetic mice after flavonoid administration.

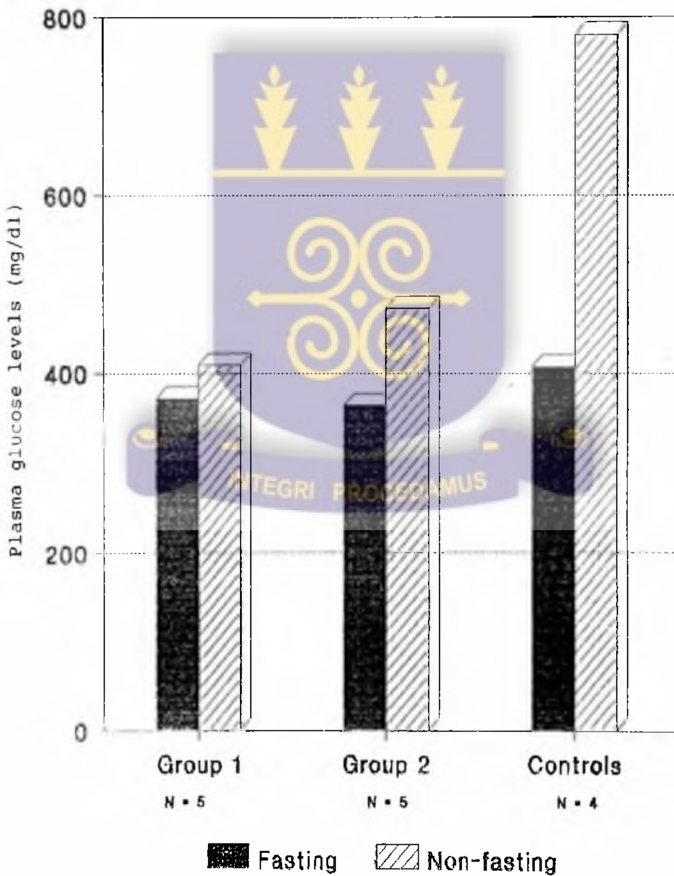

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Figure 3c shows the results obtained from experiments in which the long-term effect of the flavonoid extract on non-fasting plasma glucose levels was measured in STZ-induced diabetic mice that were given the extract three times a week for four weeks. As shown in the figure, all the three treatment groups developed the diabetes after two weeks of the STZ injection with plasma glucose levels above 600mg/dl The initial plasma glucose levels of the three treatment groups were not significantly different before administration of the extract. After two weeks of the STZ injection, plasma glucose levels of Group-1 animals rose from 219.67 ± 23.97mg/dl to 725.83 ± 33.50mg/dl with levels in Group-2 rising from 210.58 ± 23.52mg/dl to 698.33 ± 34.41 mg/dl whilst the value for the controls rose from 219.79 ± 31.69mg/dl to 616.29 ± 27.49 mg/dl. At the end of the experimental period of seven weeks, the glucose level of Group-1 animals had decreased gradually from 725.83 ± 38.50mg/dl to 470.17 ± 30.91mg/dl whilst that of the Group-2 animals also decreased from 698.33 ± 34.41 to 570.50 ± 24.54mg/dl. The glucose level of the control animals increased steadily from 616.29 ± 27 49 mg/dl to 850.00 ± 39.96 mg/dl/. After the seven weeks of investigations, the glucose levels of Group-1 and Group-2 animals were lower than that of the control animals. However, the difference was not statistically significant.

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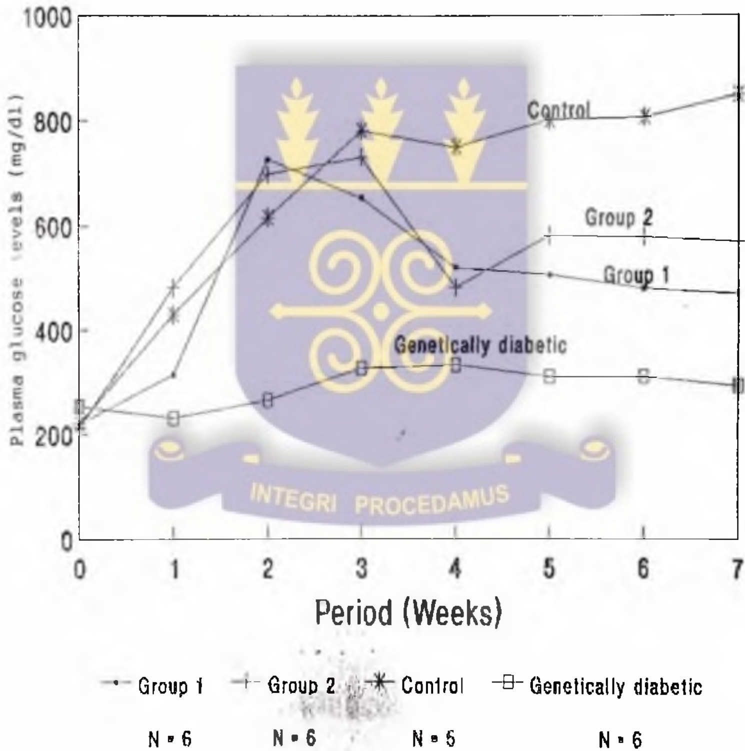
Figure 3c: Long-term effect of the flavonoid extract on non-fasting plasma glucose levels of genetically diabetic mice, and STZ-induced diabetic mice.



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(b) Liver Glycogen Levels

Figure 3d shows the mean liver glycogen levels (% wet weight tissue) of the STZ-induced diabetic mice that were used in the study of the long-term effect of the flavonoid extract on plasma glucose levels (Figure 3a) After ten weeks of the investigations, the liver glycogen level of mice in Group-1 was found to be 2.78 ± 0.38%, those in Group-2, had a glycogen level of 2.33 ± 0.14% whilst the level for the control animals was 3.28 ± 0.50%. These glycogen levels were not significantly different from each other.

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Figure 3d: Effect of the flavonoid extract on liver glycogen levels of STZ-induced diabetic mice over a ten week period.

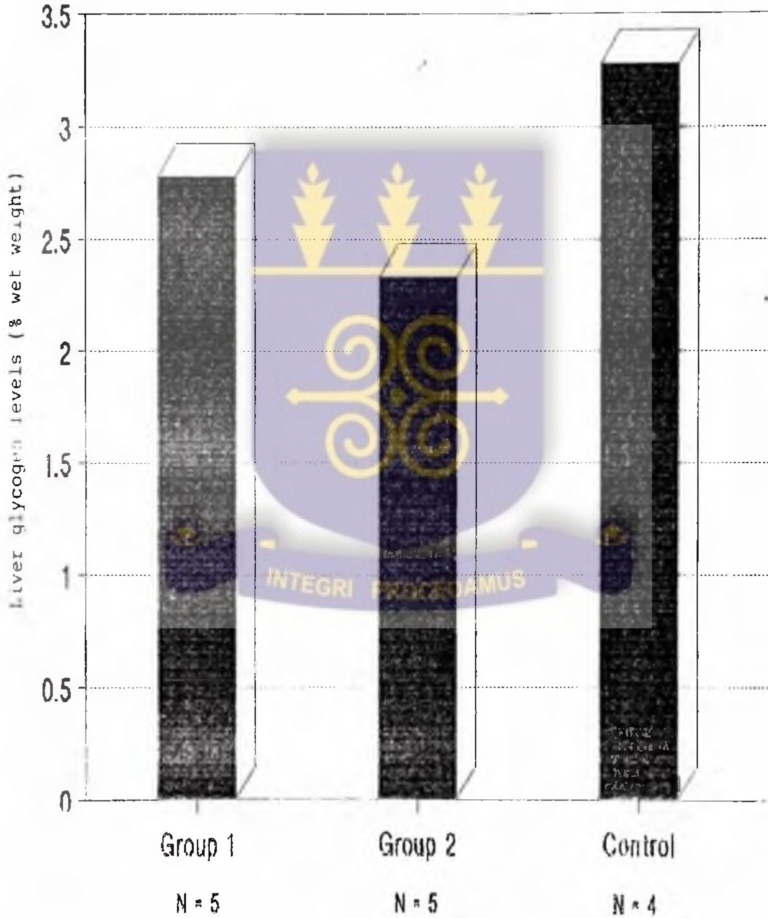

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Figure 3[illegible]. represents the results obtained from experiments in which the long term effect of the flavonoid extract on the test liver glycogen levels of STZ induced diabetic mice were measured. These animals are the animals with plasma glucose levels shown in Figure 3a. After seven weeks of the investigations the liver glycogen levels of Group-1 mice was found to be 6.41 ± 0.05%, Group 2 mice had [illegible]75 ± 0.03% whilst the control mice had 4.65 ± 0.75%. All three values were not significantly different from each other

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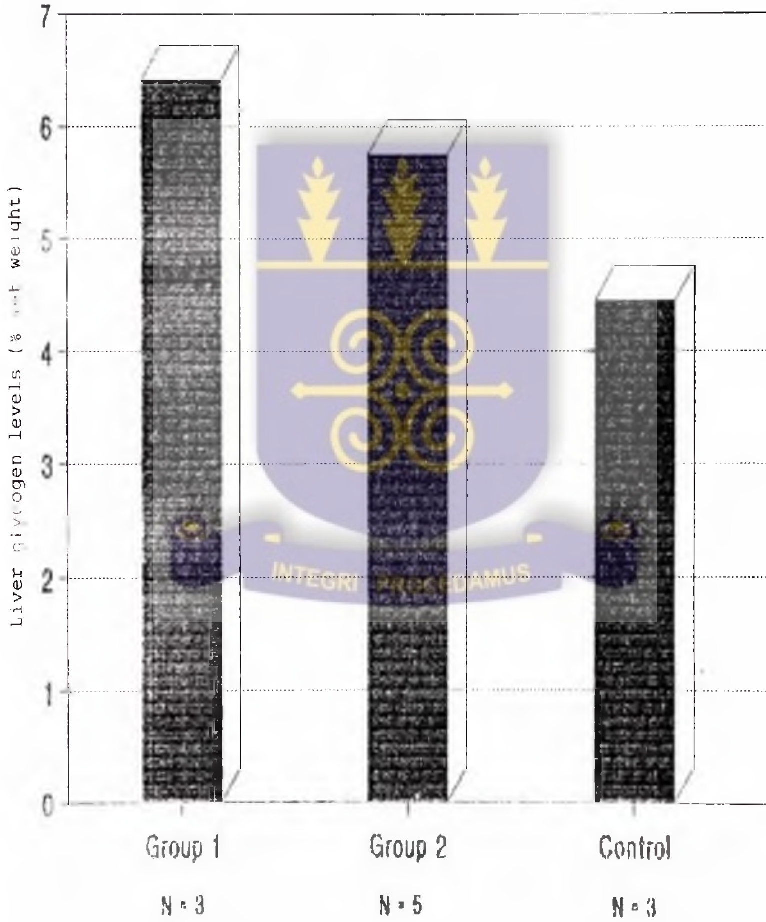
Figure 3e: Effect of the flavonoid extract on liver glycogen levels of STZ-induced diabetic mice over a seven week period.



e) Liver microsomal proteins:

The long-term effect of the flavonoid extract on the liver microsomal proteins (total) of STZ induced diabetic mice are as given below. The microsomes were prepared using livers of animals with plasma glucose shown in Figure 3a. Animals in Group-1 had microsomal protein of 9.[illegible]3mg/g liver (wet weight tissue). The value for the Group-2 animals was found to be 7.40mg/g and that for the control mice was 8.61mg/g (Fig. 3f). Another set of mice (albino) given the same treatment as above, had the following levels of liver microsomal proteins: Group-1, 11.54mg/g, Group-2, 18.37mg/g and the control mice, 11.30mg/g (Fig. 3g). The blood glucose levels of these animals are shown in Figure 3c.

The long-term effect of the flavonoid extract on reductase (NCCR) activity in the microsomal protein of these mice are as follows: At the end of the ten week period animals in Group-1 were found to have an NCCR activity equivalent to 0.1756 x 10^{-7}M of reduced cytochrome c per milligram microsomal protein, Group 2 had 0.[illegible]038 x 10^{-7} M/mg whilst the control mice had 0.8714 x 10^{-7}M/mg (Fig. 3f). With the albino mice, the reductase activities were, Group-1, 0.1264 x 10^{-7}M/mg, Group-2, 0.0722 x 10^{-7}M/mg, and the control mice had 0.2127 x 10^{-7}M/mg (Fig. 3g)

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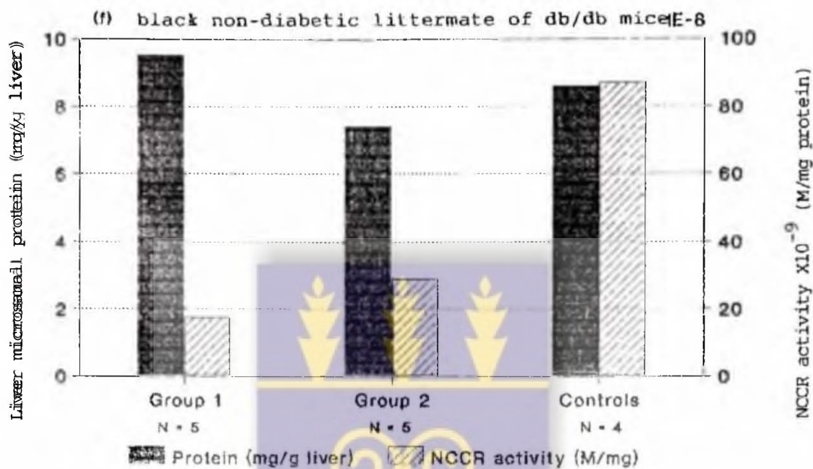
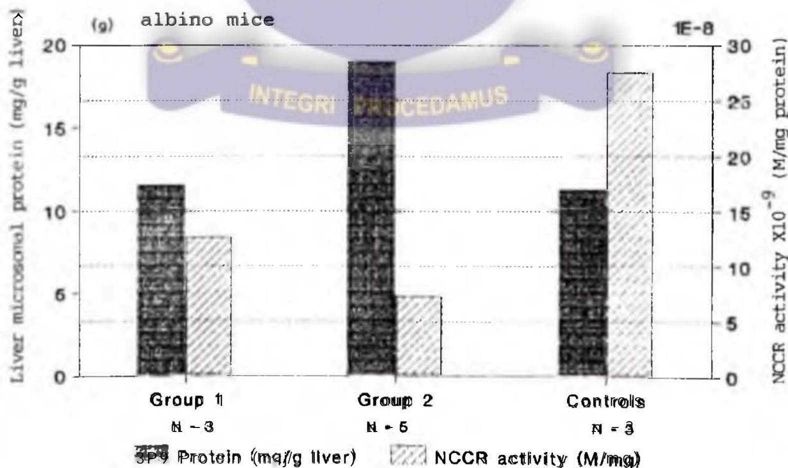
Figure 3 (f, g): Long- term effect of the flavonoid extract on liver microsomal protein and NCCR activity.





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(d) Toxicological evaluation of the flavonoid extract:

The serum GGT level of the STZ-induced diabetic mice was measured after ten weeks of the flavonoid extract administration for possible toxic side effect on the liver All the animals, both the test and the control animals, had GGT level of 11.11 (U/I).

The results obtained for the creatinine levels in the STZ-induced diabetic mice, determined for possible toxic side effect on the kidney after ten weeks of flavonoid administration, were 0.6190mg/dl, Group-2 had 0.9762mg/dl and 1.0714mg/dl, respectively for groups 1,2 and controls.

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3.3 NON-INSULIN DEPENDENT DIABETES STUDIES

3.3.1 Short-term effect of the extract

Figure 4a shows the short-term effect of the flavonoid extract on non-fasting female genetically diabetic mice over a three hour period. Both the test and control animals had mean plasma glucose levels at the start of the study not significantly different from each other. The level was 277.90 ± 20.77mg/dl for test animals and 250.80 ± 37.48mg/dl for the control animals. After an hour of the flavonoid administration, no significant difference was recorded in the plasma glucose concentration of the test animals (230.90 ± 17.37 mg/dl) and that of the control animals (249.80 ± 44.86mg/dl). However, after two hours, the test mice showed a reduction in the plasma glucose levels of about 39% to 168.80 ± 16.32mg/dl from the initial glucose level whilst the level in the control animals was reduced by about 4% to 242.00 ± 23.39mg/dl. The difference between the test and control was not statistically significant. At the end of the three hour period the plasma glucose level of the test animals was reduced by about 48% to 144.80 ± 10.33mg/dl whilst that of the control animals was reduced by about 19% to 204.50 ± 9.04mg/dl. The difference between the blood glucose level for the test and control animals was found to be statistically significant. (P 0.05).

Within the test group, the plasma glucose level after two hours and three hours were found to be significantly different from the mean plasma glucose level at the start of the study. With the control mice no significant difference in plasma glucose level was found within the three hour period.

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Figure 4a: Effect of the flavonoid extract on plasma glucose levels of female genetically diabetic mice over a three hour Period.

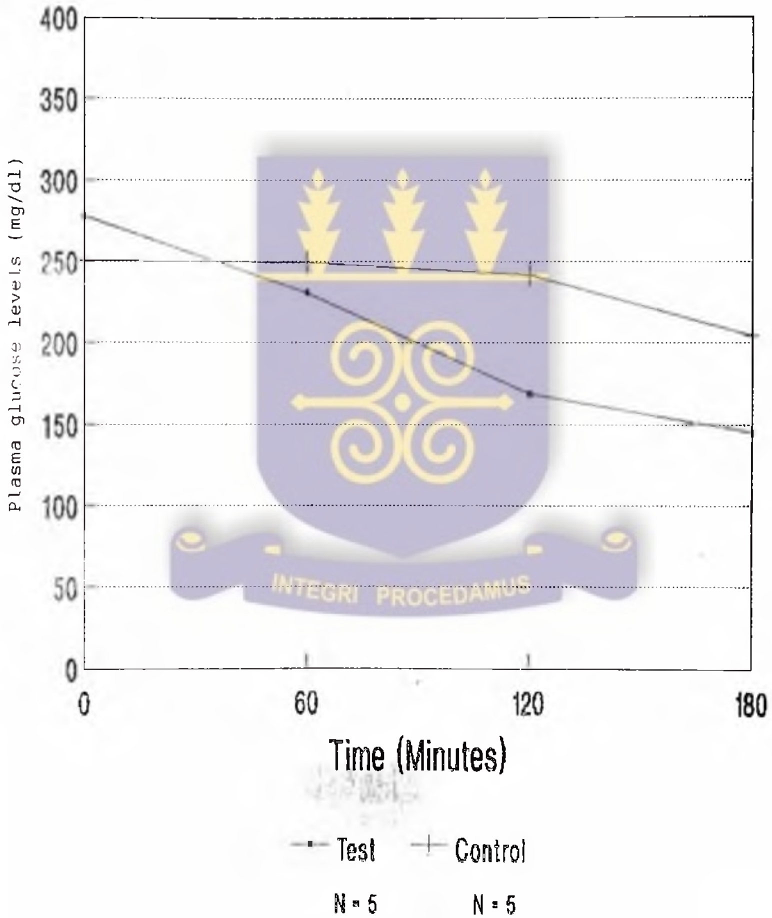

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Figure 4b shows the short-term effect of the flavonoid extract on non-fasting male genetically diabetic mice over a three hour period. Before the flavonoid administration, the test and control animals had no significant difference in their mean plasma glucose levels. One hour after administration of the extract, the plasma glucose level of the test animals was reduced by about 9% to 204.20 ± 24.42mg/dl from the initial level of 223.70 ± 39.85mg/dl whilst the plasma glucose level in the control animals reduced by about 10% to 193.90 ± 26.15mg/dl from the initial level of 215.25 ± 31.02 mg/dl. However, the two mean values were not significantly different. Two hours after administration of the extract, the plasma glucose levels of the test animals reduced by about 40% to 134.00 ± 10.64mg/dl whilst that of the control animals reduced by about 11% to 192.63 ± 27.26 mg/dl. The two mean plasma glucose levels were, however, not significantly different. At the end of the three hour period, the plasma glucose level of the test animals reduced by about 47% to 118.70 ± 7.35mg/dl whilst that of the control animals reduced by 4% to 207.38 ± 31.15mg/dl. The two values were found to be significantly different ($P<0.05$).

Within the test group, the mean plasma glucose levels after two hours and three hours following the administration of the extract were found to be significantly different from the mean glucose level at the start of the study. In the control animals, no two groups were found to be significantly different.

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Figure 4b: Effect of the flavonoid on non-fasting plasma glucose levels of male genetically diabetic mice over a three hour Period.

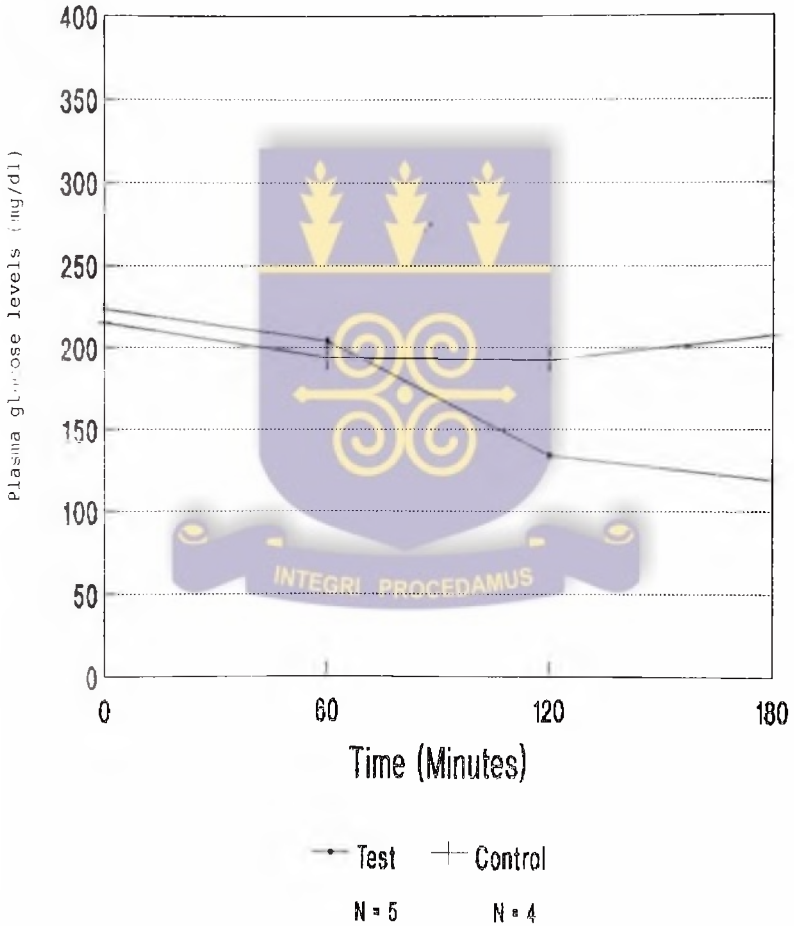

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Figure 5a, shows the short-term effect of the flavonoid extract on non-fasting plasma glucose levels of genetically diabetic mice over a two hour period. In this study, the plasma glucose level of the test animals (191.58 ± 10.58 mg/dl) and the control animals (192.20 ± 16.91mg/dl) were not significantly different, before the flavonoid extract was administered. At the end of the three hour period, no significant difference in plasma glucose levels between the test animals 187.20 ± 5.99mg/dl and the control animals, 231.60 ± 31.24mg/dl was detected.

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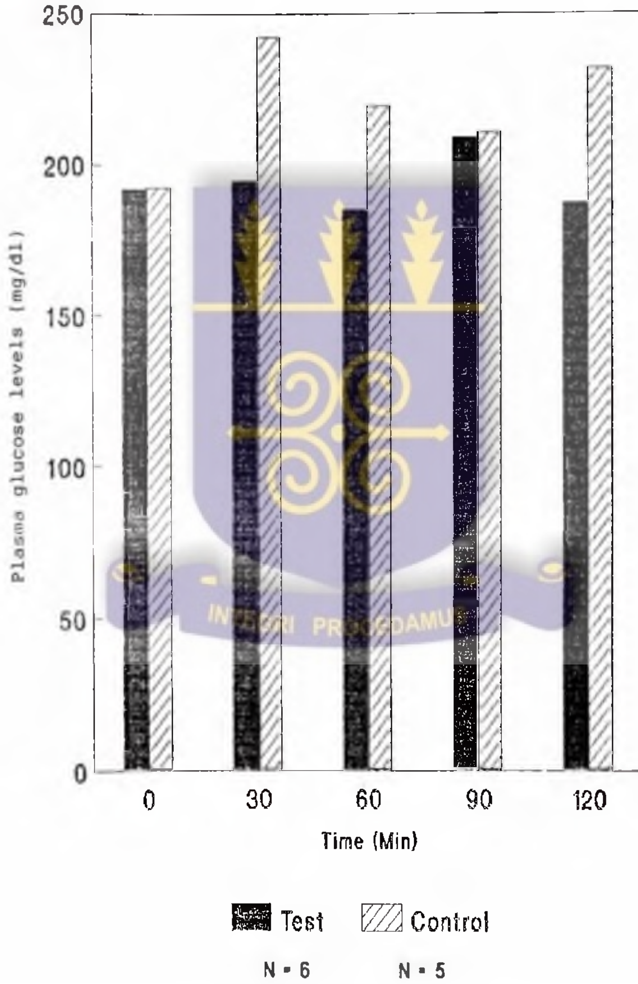
Figure 5a. Effect of the flavonoid extract on non-fasting plasma glucose levels of young genetically diabetic mice over a two hour period.



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The animals with plasma glucose levels in Figure 5a were kept for four weeks and after the fourth week, another short-term effect of the extract was investigated. Figure 5b, shows the effect of the flavonoid extract on these animals. The initial mean plasma glucose levels of both test mice with plasma glucose level, 363.50 ± 36.32 and control mice with plasma glucose level, 390.10 ± 43.99 were not significantly different. After the three hour period, no significant difference was found between the test mice, 216.50 ± 23.58 and the control mice, 206.20 ± 35.91

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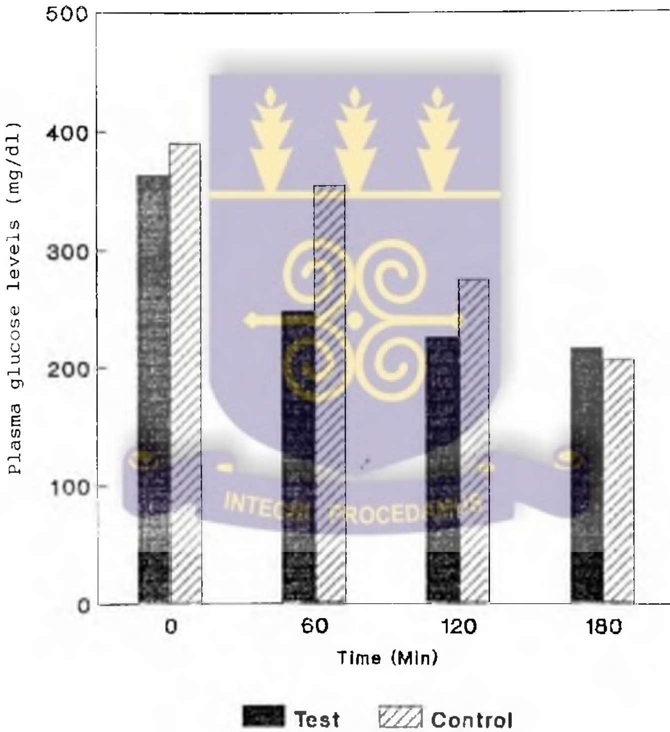
Figure 5b: Effect of the flavonoid on fasting plasma glucose levels of genetically diabetic mice over a three hour period.



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3.3.2. Long-term Effect of the Extract

(a) Non-fasting plasma glucose levels

The long-term effect of the flavonoid extract on non-fasting plasma glucose levels of genetically diabetic mice was studied using the db/db mice. The animals had initial mean plasma glucose level of 252.75 ± 37.55mg/dl before the extract was administered. Four weeks after the extract administration plasma glucose levels of the animals (six in all) was found to be 332.75 ± 47.46mg/dl (see Figure 3c). Half of these animals were killed and the other half kept for another three weeks without the extract. After this period, the plasma glucose level was found to be 292.17 ± 50.15mg/dl. Statistical analysis showed that, the initial and the final mean plasma glucose levels were not significantly different. Also no two mean plasma glucose levels were significantly different during the seven week period of investigations. The plasma glucose levels of these animals were compared with glucose levels of mice of the same strain and age which had been killed earlier. This was due to the unavailability of mice to serve as controls during the period of investigations. These animals had plasma glucose levels above 550 mg/dl.

(b) Liver glycogen levels

Figure 6, shows the liver glycogen levels of non-fasting genetically diabetic mice studied for the long term effect of the flavonoid extract. The animals in Group-1 were given the extract three times a week for four weeks. Those in Group-2 were the animals which were not killed but kept for another

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three weeks without the extract. The liver glycogen level in the Group-1 was found to be 5.75 ± 0.48% liver (wet weight tissue). The glycogen level in the livers of the Group-2 mice was found to be 5.02 ± 0.42%. The liver glycogen levels of these animals were compared with glycogen levels of the same strain and age as described in (a) above. These animals had liver glycogen level of 5.39 ± 0.34%. All the three glycogen levels were not significantly different from each other.

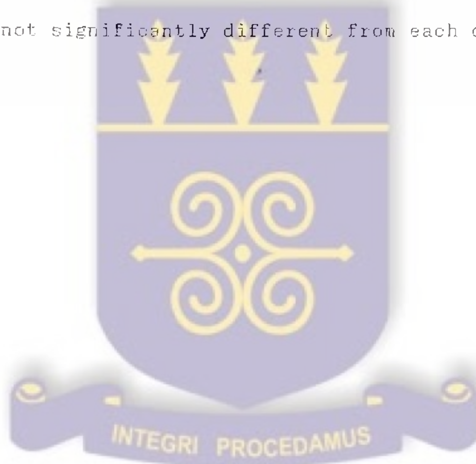

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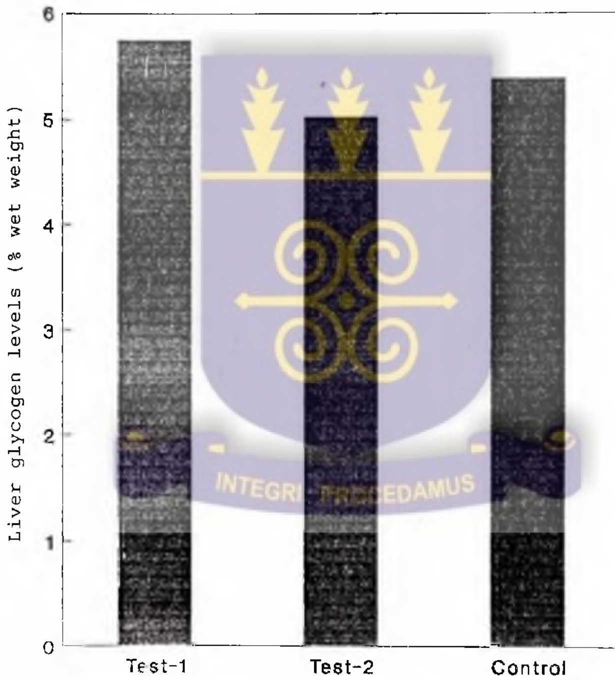
Figure 6: Effect of the flavonoid/on liver glycogen levels of / extra genetically diabetic mice over a seven week period.



N - 3 for Test-1, Test-2 & Control

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(c) Liver microsomal proteins

The values obtained for the liver microsomal protein of the non-fasting genetically diabetic mice which were studied for the evaluation of the long-term effect of the flavonoid extract are shown in Figure 7. At the end of the experimental period, mice in Group-1 which were given the extract three times a week for four weeks had 15.29mg of total protein/g liver (wet weight). The Group-2 animals which were also given the extract three times a week for four weeks, but had the extract administration stopped for another three weeks had a total protein concentration of 9.01mg/g liver. The above values were compared with those of mice of the same strain and age as described in (a) above. These animals had microsomal protein concentration of 15.97mg/g liver.

The reductase activity of the non-fasting genetically diabetic mice is also shown in Figure 7. At the end of the investigations, the activity in Group-1 was 0.4636 x 10^{-7}M/mg microsomal proteins. Mice in Group-2 had activity of 0.0930 x 10^{-7}M/mg. These values were compared with the reductase activity of mice of the same strain and age as described in (a) above. The value for these animals was found to be 0.9767 x 10^{-7}M/mg protein.

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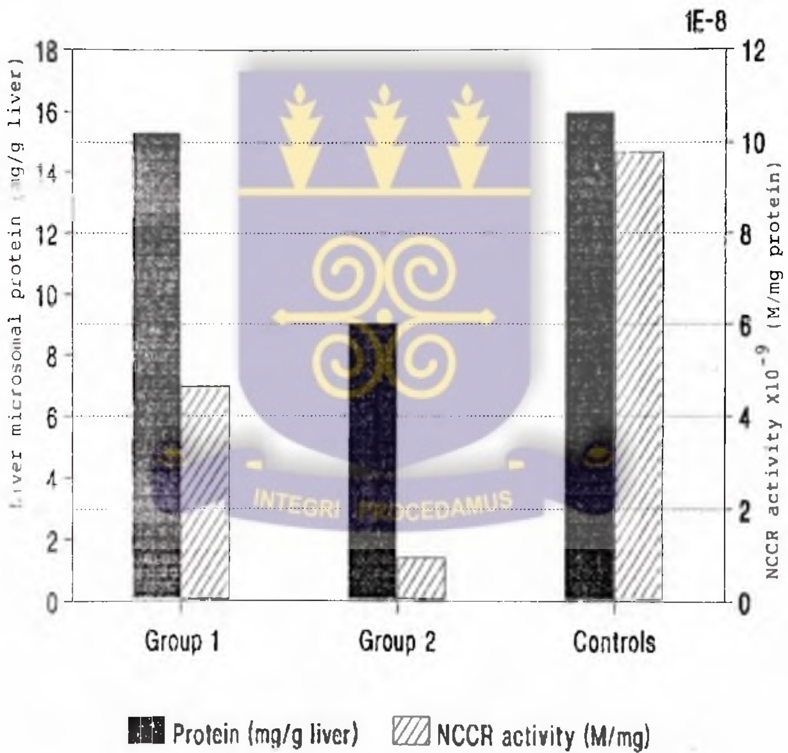
Figure 7: Long-term effect of the flavonoid extract on liver microsomal protein and NCCR activity of genetically diabetic mice (see text for description).



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CHAPTER 4

DISCUSSION AND CONCLUSION

In this study, the possible anti-diabetic therapeutic effects of some flavonoids extracted from B. ferruginea and their possible effect on hepatic and renal functions were investigated using non-diabetic mice, STZ-induced diabetic mice and genetically diabetic mice. The evaluation was done in two parts, one involving a short-term effect, and the other involving a long-term effect of the flavonoid extract.

The short-term effect of the extract on the normal non-diabetic mice showed that the extract is not hypoglycaemic as the plasma glucose levels of these mice were not reduced below 120mg/dl after three hours of receiving the flavonoid extract. This property of the extract is advantageous because the risk of a possible "shock" resulting from a sharp drop in plasma glucose levels will be avoided. In fact, there are many hypoglycaemic plants known but not being used as sources of anti-diabetic drugs because of the sharp drop in plasma glucose levels after their administration, a typical example being hypoglycin.

In the IDDM model studied for the short-term effect of the extract, the test animals were found to have fasting plasma glucose levels that were not significantly different from those of the control animals. However, when these animals were not fasted, marked differences in the glucose levels between the test animals and the control animals were

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observed. For example, when the mice studied initially for the short-term effect of the extract in the IDDM were fasted overnight before blood was taken for the glucose level determinations, it was observed that marked differences in glucose levels between the test mice and control mice occurred [illegible] in the case of the Group-2 mice which were given the extract one week after the STZ injection. The amount of glucose in the Group-2 mice were also found to be higher than those in Group-3, which were given the extract two weeks after the STZ administration. There was therefore the suspicion that, the animals in the Group-2 might not have been fasted well enough hence the higher plasma glucose levels. This prompted a repetition of the experiment. However, due to lack of lean littermates of the diabetic mice, which were used in the previous experiment, albino mice were used, and instead of the fasting plasma glucose levels, the non-fasting levels were determined. As found in Figure 2b, although the normal pattern was observed, that is, the non-fasting plasma glucose level of the Group-1 was lower than that in Group-2 which was also lower than that in Group-3, no significant differences were observed between the test mice and the control mice in all the three groups. The glucose levels in these albino mice were also found to be lower than those of the lean littermates used previously. This may be due to the fact that, the albino mice which belong to a different strain, respond differently to the diabetogenic agent. They are probably less sensitive, and therefore were not quite diabetic, with non-fasting plasma glucose levels below 400mg/dl.

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In the long-term investigations, it was found that, the extract could not prevent the development of the diabetes. Plasma glucose in all three groups increased steadily even for the animals which were given the flavonoid extract immediately after STZ injection. This means that the extract has little, if any, effect on the beta cells of the pancreas. However, after the diabetes had developed, the extract was able to maintain or stabilise the plasma glucose level in the test mice compared to the rising levels in the control mice. For these animals, it was observed that all the three treatment groups developed diabetes during the first two weeks of the treatment with STZ, with plasma glucose levels between 400-500mg/dl at the end of the second week. In subsequent weeks, the test mice still had plasma glucose levels between 400-500mg/dl whilst that of the control mice rose from the 500mg/dl range to over 1000mg/dl. These animals were fasted and glucose levels compared with the non-fasting ones, as shown in Figure 3b. No significant differences were found between the fasting and non-fasting glucose levels in both the Group-1 and Group-2 mice, but the difference in the control mice was found to be statistically significant. The above may suggest that the flavonoid extract works by lowering post-prandial glucose, thereby having little or no effect on the fasting levels of these animals. This might explain why with artificial hyperglycaemia (Addae-Mensah and Munenge, 1989) the flavonoids caused a marked reduction in glucose levels in the animals infused with glucose

From the 6th week onwards the test mice and the control

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were again given the extract three times a week for three weeks but while the glucose levels of the test animals were kept between 400-500mg/dl that of the control mice stayed high above 1000mg/dl as found in the 6th week (Figure 3a). This implies that the flavonoids maintain their effect once shown but earlier and seem to be ineffective with extreme hyperglycaemia.

The above experiment was repeated with the animals having higher glucose levels than the animals in the previous experiment. With this experiment, the difference among the three treatment groups was not statistically significant. A dose-response of the flavonoid extract in these animals is recommended for future work since an increase in the dose in these animals might have been able to reduce the high glucose levels to significant levels. The results could also indicate that when the hyperglycaemia is extreme, the plant extract may not be able to reduce it effectively

In agreement with the literature, these results show that when diabetes becomes very severe its treatment with anti-hyperglycaemic agents becomes a problem, since most anti-hyperglycaemic agents require functional beta cells for activity. Therefore, where the hyperglycaemia is not so severe, indicating that there might still be a certain amount of beta cells not destroyed by the diabetogenic agent, significant reductions in plasma glucose levels were observed in this study. This implies that, the plant extract could be working by enhancing insulin production by these cells.

In the NIDDM model, no significant differences in the

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Fasting plasma glucose levels were observed for the test and control mice three hours after they had been given the extract. However, when the mice were not fasted in this short-term experiment, the glucose levels of the test mice were all found to be significantly different from those of the control mice three hours after they had been given the extract. As shown in Figures 4a and 4b, some of the test animals had about 50% reduction in their plasma glucose levels after the three hour period. These results confirm what was observed in the IDDM models, that is, the flavonoid extract affect non-fasting plasma glucose levels.

In the long-term investigations, (Figs.6&7 and Figs.f&g) the test animals were given the extract three times a week for four weeks. For the genetically diabetic mice (NIDDM model), it was found that, the extract maintained the plasma glucose levels between 200-300mg/dl, even when the animals were over five months old as compared to age-matched control mice with glucose level of over 500mg/dl. For instance, the initial plasma glucose level of 252.75 ± 37.55mg/dl just increased by about 40mg/dl of glucose to 292.17 ± 50.15 during the seven weeks of investigations.

The C57BL/KsJ +db/db+ strain of mouse is characterised by severe hyperglycaemia throughout life (Bray and York, 1979). The pathogenesis of diabetes in this mutant strain is known to occur in phases (Dulin et. al., 1983). Phase-1 involves obese characteristics, with normal pancreatic insulin and hyperinsulinaemia implying that, the beta cells are able to provide the increased insulin demand. In Phase-2, the obesity

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becomes pronounced with extreme hyperinsulinaemia and decreased pancreatic insulin. At this stage, the beta cells are not able to meet the demands of the insulin levels required, therefore leading to the onset of hyperglycaemia. In the third phase, both the pancreatic and plasma insulin decrease, with beta cells losing their function as they degenerate and necrotise.

The sulphonylureas are the main anti-diabetic compounds used in treating NIDDM. They act as stimulants for the release of insulin from beta cells. Drugs with mechanism of action similar to sulphonylureas will therefore not be effective during the third phase of pathogenesis of diabetes in the db/db mouse. The flavonoids were found to maintain plasma glucose levels of the NIDDM models even when they were as old as six months and might have been in the third phase of the pathogenesis. The flavonoid extract somehow maintain the beta cell function. It therefore seems to work with a different mechanism from that of the sulphonylureas although [illegible] both manifest their effect within hours of administration.

Hepatic, rather than peripheral insulin resistance is the major factor responsible for fasting hyperglycaemia in the NIDDM (Campbell et. al., 1988). High levels of glucose in the fasting state is mostly due to lack of hepatic suppression of glucose production, and less glucose uptake by extrahepatic [illegible]. Since the flavonoid extract could not reduce the high fasting plasma glucose levels, a most suitable mechanism of action for the flavonoids might be the stimulation of the

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extrahepatic tissues to take more glucose since marked reductions were observed in the non-fasting plasma glucose levels. Thus the effect of the flavonoids is not to suppress glucose production in the liver but rather on the stimulation of peripheral glucose uptake by the extrahepatic tissues.

In all cases, that is, for both the short-term and the long-term, and for all the treatment groups, that is, normal, IDDM and NIDDM models, the extract could not reduce the fasting plasma glucose levels significantly whilst it manifested itself within hours of administration in the non-fasting models. Unfortunately, however, the unavailability of insulin kit prevented the correlation between the plasma glucose reduction with that of the insulin levels. For instance, in normal animals a correlation exists between plasma glucose levels and the circulating insulin levels such that, an increase in plasma glucose level results in a corresponding rise in plasma insulin levels. However, the extract cannot be said to work by stimulating the release of insulin alone since the old db/db mice with no functional beta cells had marked reductions in non-fasting plasma glucose levels.

Liver glycogen level was investigated to find out the effect of the flavonoid extract on hepatic glycogen deposition For instance, if the levels were to increase in the presence of the extract compared to the control animals then one would be in a position to attribute the reduction in plasma glucose partly to the production or deposition of glycogen in the liver In both the short-term and the long-

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term effects of the flavonoid extract on the IDDM model, no significant differences were observed in the glycogen levels between the test mice and the control mice. One can therefore state that, the extract does not work by promoting the deposition of glycogen in the liver. In the case of the NIDDM model, the glycogen levels were determined for only those studied for the long-term effect of the extract. Results showed that, the glycogen levels in the test mice were not significantly different from the control mice as shown in Figure 6. This confirms what was observed in the IDDM models. However, it was observed that animals with higher levels of plasma glucose always had higher levels of glycogen, whether the animals had been given the extract or not. This is additional evidence to the proposition made above, that the extract has no effect on the metabolism of the liver.

In the evaluation of a possible toxic side-effect of the flavonoid extract, only the models studied for the long-term effect of the extract were used. In these investigations, the plasma GGT levels of the test mice were compared with those of the control mice. The results showed the same GGT levels for all three treatment groups, implying that, there was no possible damage to the liver. To find out whether a defective kit might have been used, human plasma from different subjects were analyzed. The results (not included here) showed very marked differences which showed that the kit was in good order. In the case of the renal function test, the creatinine levels of all three treatment groups were comparable with that of the test mice being slightly less than that of the control

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mice. This suggests that the extract did not affect the kidney adversely, making its administration safe. This confirms earlier reports that, the flavonoids are non-toxic and that they are totally absorbed and rapidly metabolized in humans (Addae-Mensah, 1992).

Drugs which help the body to return to the normal state after a disease must be excreted, and the mechanism for its excretion involves a hydroxylation reaction catalyzed by the NADPH cytochrome P450 monooxygenase system which is inducible. Diabetes is also known to induce its own cytochrome P450 (CYP). Reagents for the determination of the CYP were not available during the period of study. However, since the reductase is part of the monooxygenase complex, measurement of the reductase activity was used as a measure of the effect of the flavonoids on this elimination process.

The total liver microsomal protein and the reductase activity were measured for mice set up to study the long-term effects of the extract. The results showed that in the IDDM models, the test animals which were given the extract had total microsomal protein levels comparable to, or higher than the levels found in the control animals (Figs.3(f&g)). However, the reductase activities in the test animals were found to be 2-4 fold less than those in the control animals. Diabetes-inducible CYP, termed P450DM exists, and changes in the activity of the microsomal mixed function monooxygenase system in rats suffering from diabetes have also been detected (Funae *et. al.*, 1988). Since the flavoprotein reductase is a component of the monooxygenase system one may regard the

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reduction in reductase activities as a reduction (to normal values?) of the increased drug metabolising system in the diabetic. Basal NCCR levels in the non-diabetic mice would have confirmed the above. However, due to the smaller number of experimental animals during the period of study, this level could not be determined. It is therefore recommended that further work be done on the NCCR levels of the non-diabetic mice. This action of the flavonoid may be of some benefits to the diabetics since the high levels of drug metabolizing enzymes may account for the high inactivation of anti-diabetic drugs, thereby rendering them ineffective in treating diabetes.

In the case of the NIDDM model (figs 6&7), whereas the total microsomal protein level of the test animals was comparable to the control animals, the reductase activity of the test animals was 2-10 fold less than in the control animals. This, again, confirms what was obtained in the IDDM studies; that the extract is a potential suppressor of drug metabolizing enzymes involving the cytochrome P450DM isozyme. The flavonoid could therefore, be used synergistically with other anti-diabetic drugs in the treatment of diabetes. The fact that the flavonoids did not induce specific drug metabolizing enzymes for their own metabolism and subsequent elimination from the body implies that they will be able to stay for a considerable length of time for their effect to be felt. It is recommended that a study of the possible interactions of the flavonoids with the cytochrome P450 protein in diabetics be investigated. Also baseline levels of

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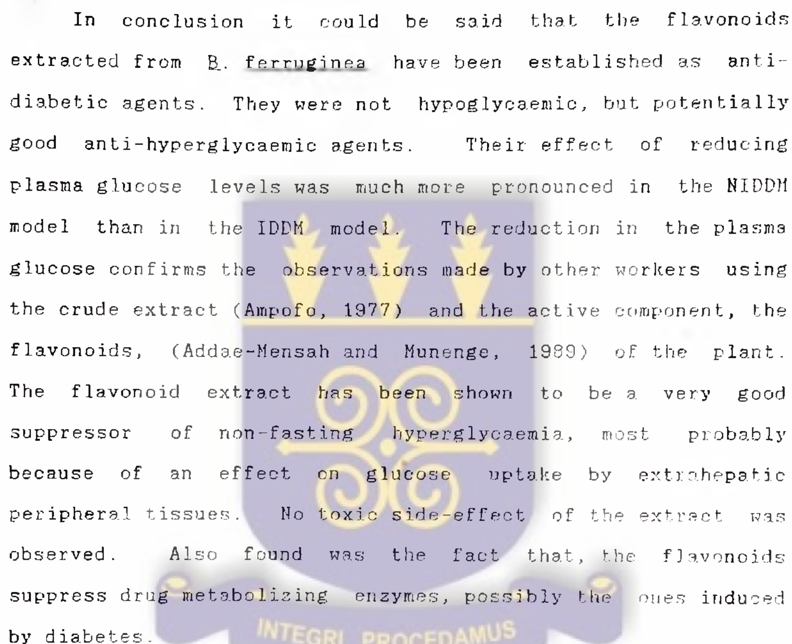
cytochrome P450 in non-diabetic mice, STZ-induced diabetic mice and genetically diabetic mice could be included.

In conclusion it could be said that the flavonoids extracted from B. ferruginea have been established as anti-diabetic agents. They were not hypoglycaemic, but potentially good anti-hyperglycaemic agents. Their effect of reducing plasma glucose levels was much more pronounced in the NIDDM model than in the IDDM model. The reduction in the plasma glucose confirms the observations made by other workers using the crude extract (Ampofo, 1977) and the active component, the flavonoids, (Addae-Mensah and Munenge, 1989) of the plant. The flavonoid extract has been shown to be a very good suppressor of non-fasting hyperglycaemia, most probably because of an effect on glucose uptake by extrahepatic peripheral tissues. No toxic side-effect of the extract was observed. Also found was the fact that, the flavonoids suppress drug metabolizing enzymes, possibly the ones induced by diabetes.



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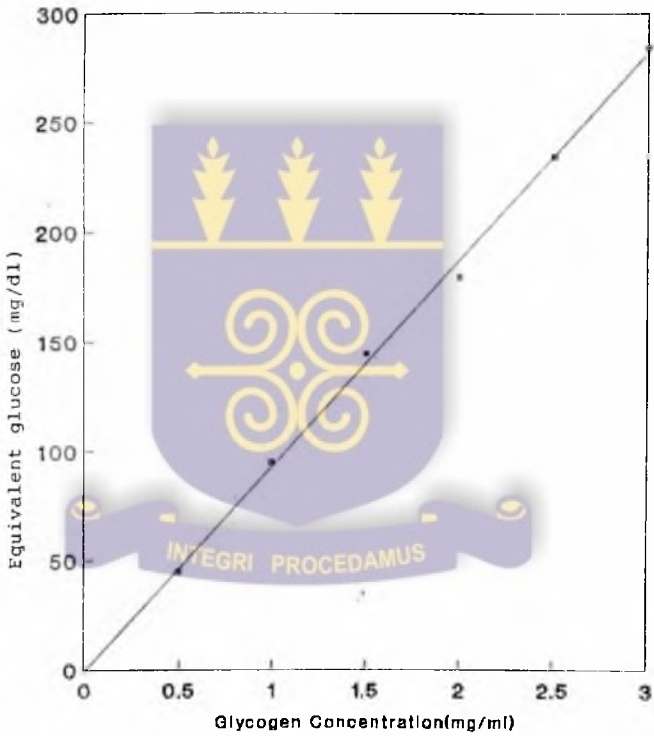
Appendix 1a: Calibration Curve for Liver Glycogen Determination.



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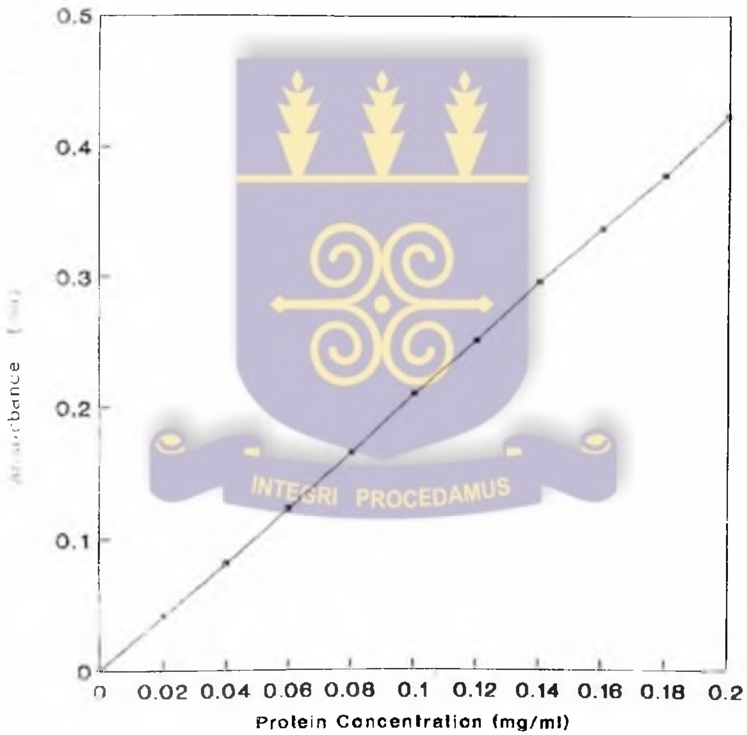
Appendix 1b: Calibration Curve for Liver Microsomal Protein Determination



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Appendix 2

A sample result of the Statistical Analysis used in testing for significant differeces between and within groups.

```
ONEWAY /VARIABLES BGL by Hour (10,17) /RANGES DUNCAN.
```

Page 4 SPSS/PC+ 9/7/93

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Variable BGL Blood Glucose Level
By Variable HOUR

Analysis of Variance

Source	D.F.	Sum of Squares	Mean Squares	F Ratio	F Prob.
Between Groups	7	401056.7438	57293.8205	1.8855	.1049
Within Groups	32	972382.7000	30386.9594		
Total	39	1373439.444			

Page 5 SPSS/PC+ 9/7/93

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Variable BGL Blood Glucose Level
By Variable HOUR

Multiple Range Test

Duncan Procedure
Ranges for the .050 level -

2.88 3.03 3.13 3.19 3.24 3.29 3.32

The ranges above are table ranges.
The value actually compared with Mean(J)-Mean(I) is..
123.2618 * Range * Sqrt(1/N(I) + 1/N(J))