EFFECTS OF ACUTE AND CHRONIC ADMINISTRATION OF ETHANOL EXTRACT OF *Cola nitida* SCHOTT AND ENDLICHER (KOLANUT) AND CAFFEINE ON GLUCOSE METABOLISM IN RATS

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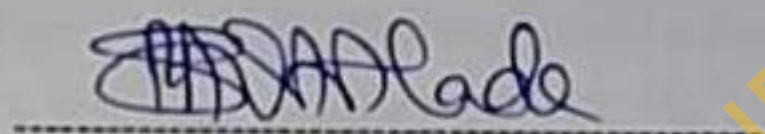
DEDICATION

This dissertation is dedicated to all scientists striving to discover the mysteries of God who created us in His image for just that purpose

CERTIFICATION

I certify that this work titled "EFFECTS OF ACUTE AND CHRONIC ADMINISTRATION OF ETHANOL EXTRACT OF *Cola nitida* SCHOTT AND ENDLICHER (KOLANUT) AND CAFFEINE ON GLUCOSE METABOLISM IN RATS" was carried out by Oladele Ayobami AFOLABI under my supervision in the Department of Physiology, University of Ibadan, Nigeria.



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ABSTRACT

Cola nitida is known for its high caffeine content. Caffeine administration has been shown to cause glucose intolerance and insulin resistance. However, there are few studies on the effect of kolanut and decaffeinated kolanut on glucose metabolism. This study was designed to investigate the effect of acute and chronic administration of Ethanol Extract of Kolanut (EEK) and Decaffeinated Ethanol Extract of Kolanut (DEEK) on glucose metabolism in Wistar rats.

Kolanut was obtained from Ilobu, Osun State and authenticated at Department of Applied Biology, LAUTECH, Ogbomoso with herbarium number LHO 442. The seeds (500 g) were air- dried, powdered and macerated in cold ethanol. Decaffeination was achieved by soaking equal amount of the seeds in distilled water and adding dichloromethane. Caffeine content of EEK and DEEK was analysed using Gas Chromatography-Mass Spectrometry (GC-MS). In acute studies, 24 Wistar rats (235-300 g) divided into 4 groups were orally administered single dose of caffeine (6 mg/Kg), EEK (6 mg/Kg), DEEK (6 mg/Kg), and 0.3 mL distilled water (control). Sample collection commenced 30 minutes after administration. In chronic studies, the same doses were orally administered daily for eight weeks to another set of 24 rats (240-310 g). Samples were collected a day after the final dose was given. In both phases, oral glucose tolerance test was carried out using standard procedure and the Areas Under Curve (AUC) calculated. Blood samples (0.2 mL) and liver biopsies were taken from each animal to determine insulin levels, Liver Glycogen Synthase (LGS), and phosphorylase activities using ELISA. Plasma glucose was determined using glucose oxidase method. Glycogen level was determined spectophotometrically by anthrone method. Data were subjected to descriptive statistics and analysed using ANOVA and Student t-test at α 005.

The GC-MS revealed caffeine content of 17.5% and 3.3% in the EEK and DEEK, respectively. In the acute study, $AUC_{glucose}$ increased by 19.0% and 11.8% for caffeine and EEK, respectively while DEEK had no effect on $AUC_{glucose}$. The $AUC_{insulin}$ increased by 66.6%, 21.5% and 10.6% for caffeine, EEK, and DEEK, respectively. The liver glycogen content significantly decreased from 2.4±0.1 (control) to 1.1±0.1 and 1.3±0.2 g/100g for caffeine and EEK, respectively while DEEK showed no effect. Acute administration of caffeine and EEK significantly decreased the activities of LGS and phosphorylase while DEEK had no effect. Chronic caffeine treatment increased

$AUC_{glucose}$ by 9.2% while EEK reduced $AUC_{glucose}$ by 3.2% and DEEK had no effect. Caffeine increased $AUC_{insulin}$ from 131.4±5.2 to 157.1±6.9 ng.min/mL, while EEK reduced $AUC_{insulin}$ to 114.5±5.6 ng.min/mL, DEEK had no effect on $AUC_{insulin}$. Caffeine and DEEK had no significant effects on LGS and phosphorylase activities. On the contrary, EEK increased liver glycogen content from 1352.0±253.1 to 1987.8±304.9 mg/100 g, and LGS activities from1270.0±154.4 to 2470.0±229.9 ng/mg protein while the phosphorylase activities reduced from 21.3±1.1 to 17.6±0.9 ng/mg protein.

Chronic administration of ethanol extract of kolanut enhanced glucose tolerance and insulin sensitivity while its acute administration resulted in glucose intolerance and insulin resistance. Decaffeination had no effect on insulin sensitivity and glucose tolerance.

Keywords: Caffeine, Decaffeinated Kolanut, Glucose tolerance, Insulin sensitivity.

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LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
a-pv)	Arterial-portal vein
AR	Adenosine receptors
ATP	Adenosine triphosphate
AUC	Area under the curve
CAF	Caffeine
cAMP	3',5'-cyclic adenosine monophosphate
CHO	Carbohydrate
CNS	Central Nervous System
CoA	Coenzyme A
DMS	Dichloromethane sodium
DEK	Decaffeinated Kolnnut
EDs	energy drinks
EEG	Electroencephalorgram
EEK	Ethanol extract of Kolanut
EGP	Endogenous glucose production
FFA)	Free fatty acids
G-6-Pase	Glucose 6-phosphatase
GC-MS	Gas chromatography and mass spectrophotometry
GDM	Gestational diabetes mellitus
GLUT	Glucose transporters
GPCRs	G-protein coupled receptors
GR	Glucose-responsive
GTT	Glucose tolerance test
HCl	Hydrochloric acid
HFD	High-fat diet
HGP	Hepatic glucose production
HOMA	Homeostatic model assessment
HOMA-%B	Homeostatic model assessment β-cell function

HOMA-IR	The homeostatic model assessment for insulin resistance
HSu	High sucrose
ICV	Intracerebral ventricle
IDDM	Insulin dependent diabetes mellitus
IP	Intraperitoneal
IPGTT	Intraperitoneal glucose tolerance tests
IRS	Insulin Receptor Substrate
ISI	Insulin sensitivity index
ISI_{Clamp}	insulin sensitivity index glucose clamp
ISI_{OGTT}	Insulin sensitivity index OGTT
KOH	Potassium Hydroxide
LH	Lateral hypothalamus
LIRKO	Liver-specific insulin receptor knockout mice
L-PIA	L-N6-phenylisopropyladenosine
NADH	Nicotinamide adenine dinucleotide
ND	Normal diet
NECA	adenosine-5'-N-ethylcarboxamide,
NHGU	Net hepatic glucose uptake
NO	Nitric oxide
NTS	Nucleus tractus solitarics
OGTT	Oral glucose tolerance test
PDK	Pyruvate dehydrogenase kinase
PI3K	Phosphatidylinositol 3-kinase
POD	` Peroxidase conjugate
PPAR:	Peroxisome proliferator-activated receptor
T1DM	type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TMB	3,3',5, 5' tetramethylbenzidine substrate solution
VMH	Ventromedial hypothalamus

CHAPTER ONE

1.0 INTRODUCTION

Cola nitida (Vent.) Schott Endl. (Kolanut), a member of the tropical family sterculiaceae, is indigenous to West African (Russel, 1955). Its fruits contain seeds known as kolanuts which are consumed by humans in different parts of the world particularly in Nigeria and other West African countries because of its stimulatory properties (Jayeola, 2001). It is a major source of caffeine and can therefore be described as the "coffee" of West Africans. Kolanut is the seed of the Cola plant which is indigenous to the tropical rain forests of West Africa, West Indies, Brazil and Java (Rusell, 1955). *Cola nitida* and *Cola acuminata* are the most common Cola species of commercial value in Nigeria. The kolanut is a source of essential flavouring in the confectionery industries and was part of the original formulation of the popular soft drink Coca-Cola (Beattie, 1970). Kolanuts are however widely used as pycho-stimulants and have been found to contain caffeine up to 51% in *Cola* nitida extract (Salahdeen *et. al.*, 2014) in recent analysis using gas chromatography and mass spectrophotometry (GC-MS). Apart from caffeine, kolanut also contains small amounts of the related methylxanthine theobromine, flavonoids, anthrocyanins, protein, carbohydrate, fat and ash (Jayeola, 2001; Odebunmi *et. al.*, 2008). Studies on the effects of kolanut are scanty and mostly epidemiological (Lawoyin *et. al.*, 2005; Morakinyo and Odejide 2003). Small quantities of kolanut have been found to empirically increase mental activity (Lawoyin *et. al.*, 2005), reduce the need for sleep and to dispel hunger and thirst (Abdulkarim *et. al.*, 2005). These properties make it useful for students and long distance drivers who wish to remain active and alert for long periods of time. However, despite this widespread consumption of kolanut, investigations on the physiological or clinical effects of kolanut have not received much attention. Experimental studies on the effects of kolanut have mostly investigated the psychostimulatory effects of the extract (Scotto *et. al.*, 1987). Ajarem (1990) found that intraperitoneally (i.p.) injected kolanut extract significantly increased the locomotor activity of mice at a dose of 5 mg/kg while a low dose of 2.5 mg/kg had no effect, a high dose of 10 mg/kg depressed effects on locomotor activity. The results suggest that the Kola extract induces biphasic changes in the locomotor activity of mice depending on the dose and the treatment duration. Osim *et. al.*, (1991) found that Kolanut extract greatly stimulated gastric acid secretion in cats. However, an equivalent amount of caffeine contained in the Kolanuts had 42% of the potency of the kolanut extract in inducing acid secretion. The results showed that consumption of kolanut

caused an increase in gastric acid secretion which was greater than that produced by an equivalent dose of caffeine thereby suggesting that constituents other than caffeine may be responsible for the stimulation of gastric acid production. In a similar study, (Ibu *et. al.,* 1986) also found that extracts of both *Cola nitida* and *accuminata* stimulated gastric acid secretion. However, there are relatively few studies on the effect of kolanut on carbohydrate metabolism which have yielded conflicting results. While the study of Onyeanusi and Ikpe, (2004) showed that kolanut supplementation of rat diet for six weeks significantly elevated blood glucose and glycosylated haemoglobin above those of controls, the study of Salahdeen and Alada, (2009) showed that intravenous infusion of the ethanol extract of kolanut increased canine hind limb glucose uptake mainly by increased glucose extraction in a manner comparable to that of caffeine.

Caffeine, a methylxanthine derivative is arguably the most widely consumed drug in the world (Fredholm, 1999) mainly due to its ubiquitous nature being a major component of many beverages such as coffee, tea and soft drinks (Beattie, 1970). Furthermore, besides being present in all cocoa based confectionaries, it is a common additive in energy drinks (EDs) and pain relieving drugs (Barone and Roberts, 1996; Tanda and Goldberg, 2000). In the USA and many western countries caffeine intake could be as high as 151–300 mg/day (Martin and Bracken, 1987) mostly due to consumption of coffee, tea and chocolate. However, here in Nigeria and in much of the West African subcontinent large amounts of caffeine are consumed in the form of kolanut which has recently been shown to contain up to 51% caffeine (Salahdeen *et. al.*, 2014). In comparison, the caffeine content of coffee, tea and some soft drinks averaged 1.-2% and 1-4%.

Caffeine is known to be a powerful stimulant of the nervous system and is in fact so widely consumed because of its psychotropic actions (Fredholm, 1999). However, though it is taken for its psychotropic actions, it affects virtually all the organs and tissues of the body such as the muscles. kidneys and other organs. It readily crosses the blood brain barrier (Olendorf *et. al.*, 1971, Van Handle *et. al.*, 1983) and acts directly on vagal, medullary and vasomotor centers (Syed, 1976). Small amounts of caffeine have been reported to affect blood pressure, heart rate, respiratory rate and metabolic rate (Lombardo, 1986), while large doses may produce wakefulness, nervousness, irritability, anxiety, and insomnia (Goldstein, 1964).

The effects of caffeine on carbohydrate metabolism has been extensively investigated in humans (Graham and Sprict, 1995; Greer *et. al.*, 2001; Keijzers *et. al.*, 2002; Thong *et. al.*, 2002; Lee *et. al.*, 2005), dogs (Pencek *et. al* 2004; Salahdeen and Alada, 2009) and rats (Budohoski *et. al.*,

1984; Steinfelder and Petho-Schramm, 1990; Vergauwen *et. al.*, 1993) among many other animal models. A large number of the human studies in healthy, obese and type 2 diabetic men and pregnant women have documented insulin resistance and reduced glucose tolerance on administration of caffeine. Graham *et. al.*, (2001) showed that caffeine ingestion resulted in an exaggerated response in blood glucose and insulin during an oral glucose tolerance test (OGTT) when eighteen healthy adult males received caffeine (5 mg/kg) or placebo and 1 h later ingested 75 g of dextrose and underwent OGTT. In the caffeine trial the serum insulin and C peptide concentrations were significantly greater ($P \leq 0.001$) than for placebo but did not result in a lower blood glucose level suggesting that caffeine ingestion may have resulted in insulin resistance. Petrie *et. al.*, (2004) examined the effects of caffeine ingestion on insulin and glucose homeostasis in obese men before and after a nutrition and exercise intervention. Sedentary, obese men, were given 5 mg/kg caffeine or placebo 1 h before each OGTT after a 12-wk nutrition and exercise intervention, the OGTTs were repeated. While the nutrition and exercise intervention resulted in decreases in body weight, percentage body fat, and fasting glucose, insulin, and proinsulin concentrations and increases in the insulin sensitivity index (ISI) for the placebo OGTT, caffeine caused a greater OGTT insulin response and a lower ISI both before and after weight loss. The proinsulin-insulin ratio indicated that neither weight loss nor caffeine affected the nature of the β cell secretion of insulin. Moosa and Akther, (2010) Investigated the effects of caffeine on fasting glucose & insulin levels and on glucose & insulin response to a mixed-meal tolerance test were studied in T2DM habitual coffee drinkers. While caffeine did not affect the fasting levels of plasma glucose or insulin when compared with placebo, the AUC 2h values demonstrated significant caffeine effects for both plasma glucose and plasma insulin ($P < 0.05$) responses to the mixed-meal tolerance test. They concluded that acute administration of caffeine and carbohydrate impaired post-prandial glucose metabolism and insulin responses. Such effects could have implication for the management of type 2 diabetic patients. Robinson *et. al.*, (2009) assessed the effect of acute caffeine ingestion on glucose tolerance in women with or without gestational diabetes mellitus (GDM). The diabetic and non diabetic women completed two trials one week apart in a double-blind randomized crossover study. In the control group, caffeine did not significantly affect blood glucose, insulin, or C-peptide. In the GDM group, glucose area under the curve (AUC) was greater C-peptide AUC was greater and insulin sensitivity index was lower

after caffeine than after placebo. They therefore reached the conclusion that caffeine impaired insulin sensitivity in women with GDM.

In dogs which are known to be very sensitive to caffeine, experimental studies haveparadoxically shown caffeine to stimulate glucose uptake in the liver (Pencek *et. al*., 2004) and skeletal muscles of the hind limb (Salahdeen and Alada, 2009a). Pencek *et. al*., 2004, infused caffeine via the intraportal route at rates (1.5 µmol/kg · min) designed to create concentrations similar to that seen with normal dietary intake. Although arterial insulin, glucagon, norepinephrine, and glucose did not differbetween groups, in dogs infused with caffeine, net hepatic glucose uptake (NHGU) was significantly higher than in controls even as net hepatic lactate output was found to have increased compared with controls indicating that physiologic circulating levels of caffeine can enhance NHGU during a glucose load, and that added glucose consumed by the liver is in part converted to lactate. Salahdeen and Alada (2009a) showed that caffeine increased glucose uptake in the canine hind limb at rest and during contraction and that the increased uptake was due to increased glucose extraction. A similar study determined that the increased glucose uptake at rest but not during contraction was modulated by adrenergic receptors (Salahdeen and Alada, 2009b).

In rats Kolnes *et. al*., 2010 incubated rat epitrochlearis muscles and soleus strips with insulin and different concentrations of caffeine and theophylline in order to measure glucose uptake, force development and PKB phosphorylation. They found that caffeine and theophylline completely blocked insulin-stimulated glucose uptake in both soleus and epitrochlearis muscles at 10 mM. Caffeine reduced and theophylline blocked insulin-stimulated glycogen synthase activation. Caffeine and theophylline reduced contraction-stimulated glucose uptake by about 50%, whereas contraction-stimulated glycogen breakdown was normal. They hypothesized that caffeine and theophylline also inhibit glucose uptake in skeletal muscles via an additional and hitherto unknown molecule involved in GLUT4 translocation. However, there have been recent reports of caffeine administration improving insulin sensitivity and glucose tolerance in the rat. Coelho, (2014) in a PhD Thesis used prediabetic Wistar rats which were given to 35% of sucrose (high sucrose- Hsu model) in drinking water over a period of 28 days, 0.5, 0.75 and 1g/l of caffeine given to control rats and in HSu model over a period of 12 weeks. Insulin sensitivity, basal glycemia, glucose tolerance, adipose tissue mass, Glut4 transporters and nitric oxide (NO) content in skeletal muscle and in the liver were determined. They found that caffeine restored insulin sensitivity and glucose tolerance in HSu rats. Caffeine (1g/l) restored Glut 4 expression levels in skeletal muscle in HSu

animals. Based on these results they suggested that caffeine could be used as a therapeutic tool for the treatment of prediabetes and prevention of T2DM. Similarly, Yeh *et. al.*, (2014) found that chronic caffeine consumption reduced superoxide generation and enhanced insulin signaling in the nucleus tractus solitaries (NTS) leading to a reduction in blood pressure in rats with fructose-induced hypertension. Furthermore, the treatment reduced serum fasting glucose, insulin, homeostatic model assessment-insulin resistance, and triglyceride levels and increased the serum direct high-density lipoprotein level in fructose-fed rats. Their results suggested that caffeine may enhance insulin receptor substrate 1-phosphatidylinositol 3-kinase-Akt-neuronal nitric oxide synthase signaling to decrease blood pressure by abolishing superoxide production in the NTS.

However, caffeine is rarely if ever taken in its pure form outside experimental conditions but consumed as a major constituent of coffee, tea, chocolate, and energy drinks. Coffee being the most common form in which caffeine is consumed has been widely studied. An overwhelming number of studies mostly epidemiological have indicated that coffee consumption is associated with lower risk of diabetes and metabolic disorders (van Dam and Feskens, 2002; Isogawa *et. al.*, 2003; Salazar-Martinez *et. al.*, 2004; Santorelli *et. al.*, 2010). In Europe and North America, coffee is the most common form in which caffeine is consumed particularly in the adult population. While acute caffeine ingestion has consistently been shown to decrease glucose tolerance, epidemiological studies have equally consistently demonstrated that chronic ingestion of coffee decreases the risk of developing type 2 diabetes mellitus (T2DM) (Salazah-Martinez *et. al.*, 2004; van Dam *et. al.*, 2004; Yamaji *et. al.*, 2004). Most published articles epidemiological whether retrospective or prospective investigating the association between coffee and T2DM, although strong in their methodology, have relied heavily on a self-reported diagnosis of diabetes, which may be more prone to misclassification or underreporting. Experimental studies in which coffee was administered to humans and laboratory animals have sometimes corroborated (Rustenbek *et. al.*, 2014, Momkinyo *et. al.*, 2014) the epidemiological findings and at other times refuted them (van Dam *et. al.*, 2004). Rustenbeck *et. al.*, (2014) studied male mice in which diabetes and obesity was induced by high-fat diet (55%lipids, HFD) were given regular coffee or water compared with mice feeding on a defined normal diet (9% lipids ND) found that weight gain in HFD mice was dose-dependently retarded, the moderate weight gain in ND mice was abolished by coffee consumption. Intraperitoneal glucose tolerance tests (IPGTT) showed a dose-dependent faster decline of elevated glucose levels in coffee-consuming HFD mice, but not in ND mice. They also

observed a decrease in non-fasting glycaemia after week 21 in all treatment groups. IPGTT showed diminished peak of glucose levels in coffee-consuming HFD mice. Untreated HFD mice were hyperinsulinaemic and had significantly enlarged islets. Coffee consumption did not affect islet size or parameters of beta-cell apoptosis, proliferation and insulin granule content. Coffee consumption retarded weight gain and improved glucose tolerance in a mouse model of type 2 diabetes and corresponding controls. Morakinyo *et. al.*, (2014) showed that Coffee consumption retarded weight gain and improved glucose tolerance in a rat model of type 2 diabetes. These varying findings with regards to the effect of caffeine and coffee have raised many questions concerning its safety to human health not only in healthy individuals but also in T2DM patients (Lane *et al.*, 2008; Lane, 2011). The disparity in the findings can be said to be due to the differing animal models used, the experimental method whether epidemiological or experimental or whether the caffeine is administered acute or chronic. Moreover there is a question as to whether coffee consumption can be taken to be the same as caffeine consumption. Experimental studies addressing this question added the experimental dose of caffeine to decaffeinated coffee or coffee itself and found similar results to those that presented pure caffeine in capsules, with both kinds of treatments leading to insulin resistance or impaired glucose tolerance (Battram *et. al.*, 2006; Lane *et. al.*, 2007; Louie *et. al.*, 2008; Greenberg *et. al.*, 2010), suggesting that coffee has effects similar to pure caffeine. Furthermore, the duration of consumption of caffeine whether acute or chronic has produced differing results which tend to add to the confusion as to whether it is harmful or beneficial to health (Conde *et. al.*, 2011; Sacramento, 2015). There has however been a lot of debate in recent times regarding the acute and chronic effects of caffeine in coffee regarding the possibility of tolerance to caffeinebeing behind the observed difference. While some investigators (Robertson *et. al.*, 1981; Denaro *et. al.*, 1991) believe that habitual consumption of coffee or other caffeinated beverages leads to the development of tolerance to caffeine, and therefore disappearance of its adverse acute effects over the long term, others (Lane, 2007; Dekker *et. al.*, 2007) have shown that with chronic caffeine intake, insulin resistance and disrupted glucose tolerance persisted after 2 weeks of daily consumption. Studies on the effect of caffeine and coffee have therefore produced varied and conflicting results. Given the widespread consumption of caffeine in its various forms and the documented adverse and beneficial effects with regards to carbohydrate metabolism, there is a need to reconcile the disparity in the findings. While the disparity in the results of acute treatment and chronic treatment may be due to tolerance, other

factors such as route of administration of the caffeine oral or parenteral may account for the differences in the results. Moreover, the large numbers of chemical components of coffee could account for the differences observed in the actions of caffeine and that of coffee as some workers have identified substances that may modulate the adverse effects of caffeine (Shearer *et. al.*, 2003). The lack of consistency in the effect of caffeine on glucose tolerance has warranted further study on the effect of caffeine on glucose tolerance under chronic condition. Although there are reports which showed that Kolanut seed extract significantly increased both blood glucose and hind limb glucose uptake in dogs (Salahdeen and Alada, 2009), there is no information on the effect of Kolanut on glucose tolerance in any animal model. Given the widespread consumption of Kolanut in the West African subcontinent and recent reports of high caffeine content (31%) in kolanut extracts, studies implicating caffeine in insulin resistance in healthy (Greer *et. al.*, 2001; Keijzers *et. al.*, 2002; Battram *et. al.*, 2006) and type 2 diabetic subjects (Lane *et. al.*, 2004; Robinson *et. al.*, 2004; Lane *et. al.*, 2007; Robinson *et. al.*, 2009), and the contradictory experimental and epidemiological findings that caffeine (Conde 2012; Yeh, 2014; Sacramento 2015), and coffee (whose major active component is caffeine) reduced insulin resistance and prevent the development of type 2 diabetes, there is the need to investigate the effects of kolanut on carbohydrate metabolism in the rat. Moreover, many studies on biological effects of kolanut have attributed the effects of kolanut to the caffeine contained therein (Salahdeen and Alada 2009; Salahdeen *et. al.*, 2014; Ibu *et. al.*, 1986) even though none of these studies have investigated the effect of decaffeinated kolanut. The study of Osim *et. al.*, (1991) found that the effect of kolanut in increasing gastric acid secretion was greater than that caused by an equivalent amount of caffeine suggesting that components of kolanut other than caffeine were responsible for observed effect of kolanut on gastric acid secretion. The use of decaffeinated kolanut would have given an indirect indication of the significance of caffeine content in the actions of kolanut.

This study was designed to investigate further the effects of caffeine and Kolanut on glucose tolerance in the rat. Specifically, the following issues were addressed:

1. The effect of caffeine on glucose tolerance, insulin response and liver glycogen deposition in the rat.
2. The effect of ethanol extract of kolanut on glucose tolerance, insulin response and liver glycogen deposition in the rat.

3. The effect of decaffeinated extract of kolanut on glucose tolerance, insulin response and liver glycogen deposition in the rat.
4. The effect of acute and chronic administration of caffeine on glucose tolerance, insulin response and liver glycogen deposition in the rat
5. The effect of acute and chronic administration of ethanol extract of kolanut on glucose tolerance, insulin response and liver glycogen deposition in the rat
6. The effect of acute and chronic administration of decaffeinated ethanol extract of kolanut on glucose tolerance, insulin response and liver glycogen deposition in the rat

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 CARBOHYDRATE METABOLISM

Carbohydrate metabolism is the series of biochemical processes responsible for the formation, breakdown and interconversion of carbohydrates in living organisms.

In the immediate postprandial or fed state, under the influence of insulin, the liver, muscles and adipose tissues become important sites of glucose disposal. Under the influence of prevailing high concentrations of glucose, insulin is secreted by the beta cells of the pancreas. Insulin stimulates the transfer of glucose into the cells, especially in the liver and muscles, although other organs are also able to metabolize glucose. In the liver and muscles, most of the glucose is converted into glycogen by the process of glycogenesis (Shulman *et. al.*, 1995). Glycogen synthase is the enzyme responsible for adding UDP-glucose to a growing chain of glycogen. There are two forms of this enzyme. The inactive form which carries a phosphate group is called glycogen synthase b and it is dephosphorylated to the active form which is called glycogen synthase a, which does not carry a phosphate group. The activity of glycogen synthase is controlled by covalent modification through phosphorlation of the enzyme by kinases (Haystead *et. al.*, 1989; Dent *et. al.*, 1990), cyclic AMP-dependent protein kinase A the activity of which is regulated by the messenger molecule cyclic AMP (cAMP). Cyclic AMP is made from ATP by the enzyme adenylyl cyclase and it is degraded by the action of phosphodiesterase.

While the glycogen stored in the muscle is used for the provision of energy, the glycogen in the liver is stored until needed at some later time when glucose levels are low particularly in the remote post absorptive state. Insulin directly or indirectly influences the uptake of glucose by the liver. It acts directly by binding to hepatic insulin receptors and thereby activating insulin signaling pathways in the liver. These effects have been demonstrated in various models. In isolated rat hepatocytes, insulin inhibits glucose production through inhibition of gluconeogenesis (Claus and Pilkis, 1976) and glycogenolysis (Marks and Botelho, 1986). In the dog, an acute selective increase (Sindelar *et. al.*, 1996) or decrease (Sindelar *et. al.*, 1998) in hepatic insulin level (so that the arterial insulin level was kept constant) resulted in very rapid suppression or stimulation, respectively, of hepatic glucose production (HGP). In addition, liver-specific insulin receptor knockout (LIRKO) mice, which lack hepatic insulin receptors from birth, have been shown to demonstrate severe hepatic insulin resistance (Fisher and Kahn, 2003).

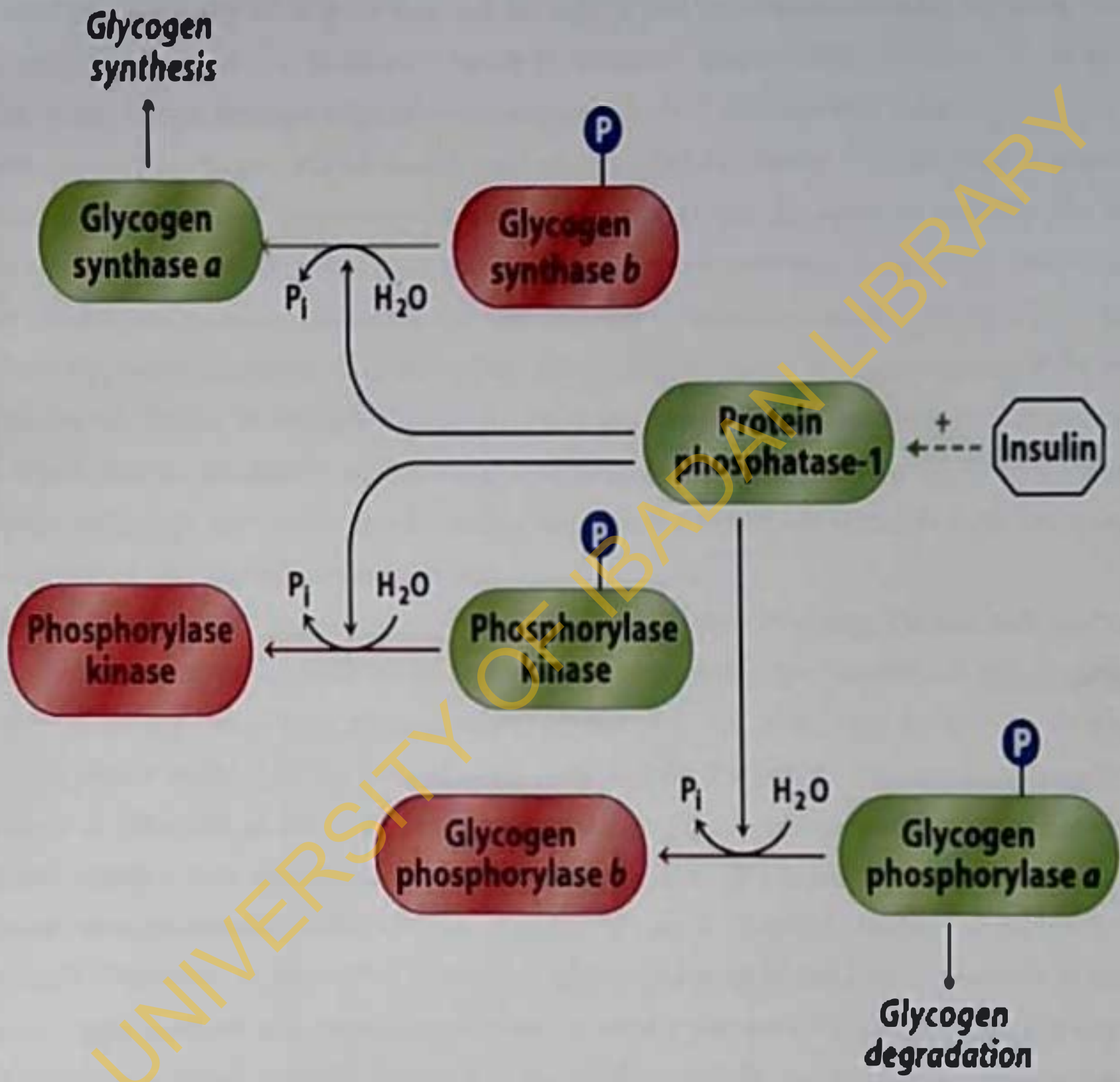

Figure 1 Schematic diagram of steps in activation of glycogen synthase and glycogen phosphorylase. (Principles of Biochemistry 4/e ©2006 Pearson Prentice Hall)

Insulin's indirect effects include reduction of glucagon secretion at the pancreas (Ito *et. al.*, 1995), inhibition of lipolysis in fat (which reduces circulating lipids and glycerol availability for gluconeogenesis) (Sindelar *et. al.*, 1997), and decreased protein catabolism in muscle (protein sparing effect) further reducing gluconeogenic substrate availability (Wolfe and Volpi, 2001), and in addition, recent studies in the mouse and rat suggest that hypothalamic insulin signaling may also play an important role in insulin's ability to indirectly regulate HGP (Obici *et. al.*, 2002a; Obici *et. al.* 2002b). Insulin's indirect hepatic effects were well demonstrated in the dog, where an acute selective increase in arterial insulin level while the hepatic insulin level was kept constant, resulted in inhibition of HGP (Sindelar *et. al.*, 1996). This was shown to be partially due to insulin's effect on lipolysis, since when plasma FFA levels were prevented from falling, HGP only decreased by half as much (Sindelar *et. al.*, 1997). In rats, intracerebral ventricle (ICV) infusion of insulin suppressed glucose production, and this effect was independent of circulating insulin levels (Obici *et. al.*, 2002a). In addition, in mice, HGP was suppressed by insulin despite 95% reduction of hepatic insulin receptors by antisense oligonucleotide treatment (Buettner *et. al.*, 2005). These studies, and others, demonstrate that in addition to insulin's direct effects on the liver, the hormone also regulates HGP through indirect means.

The amount of glucose taken up by the liver is regulated by circulating glucose and insulin concentrations and by the arterial-portal vein (a-pv) glucose gradient (Pagliassotti and Cherrington, 1992). It has previously been demonstrated (Pagliassotti *et. al.*, 1996) that the increase in net hepatic glucose uptake (NHGU) induced by the portal signal is a result of the stimulation of hepatic glucose uptake processes and not a suppression of hepatic glucose production. The rise in hepatic glucose uptake results in increased intracellular concentrations of glucose 6-phosphate and other hexose monophosphates, which in turn stimulate glycogen synthase, leading to significant glycogen deposition. In fact, ~75% of the extra glucose taken up by the liver in response to the portal signal is stored as glycogen, whereas the remainder primarily leaves the liver as lactate. Other metabolic pathways (oxidation, pentose phosphate cycle, conversion to lipids) appear to play minor roles in the intrahepatic fate of glucose.

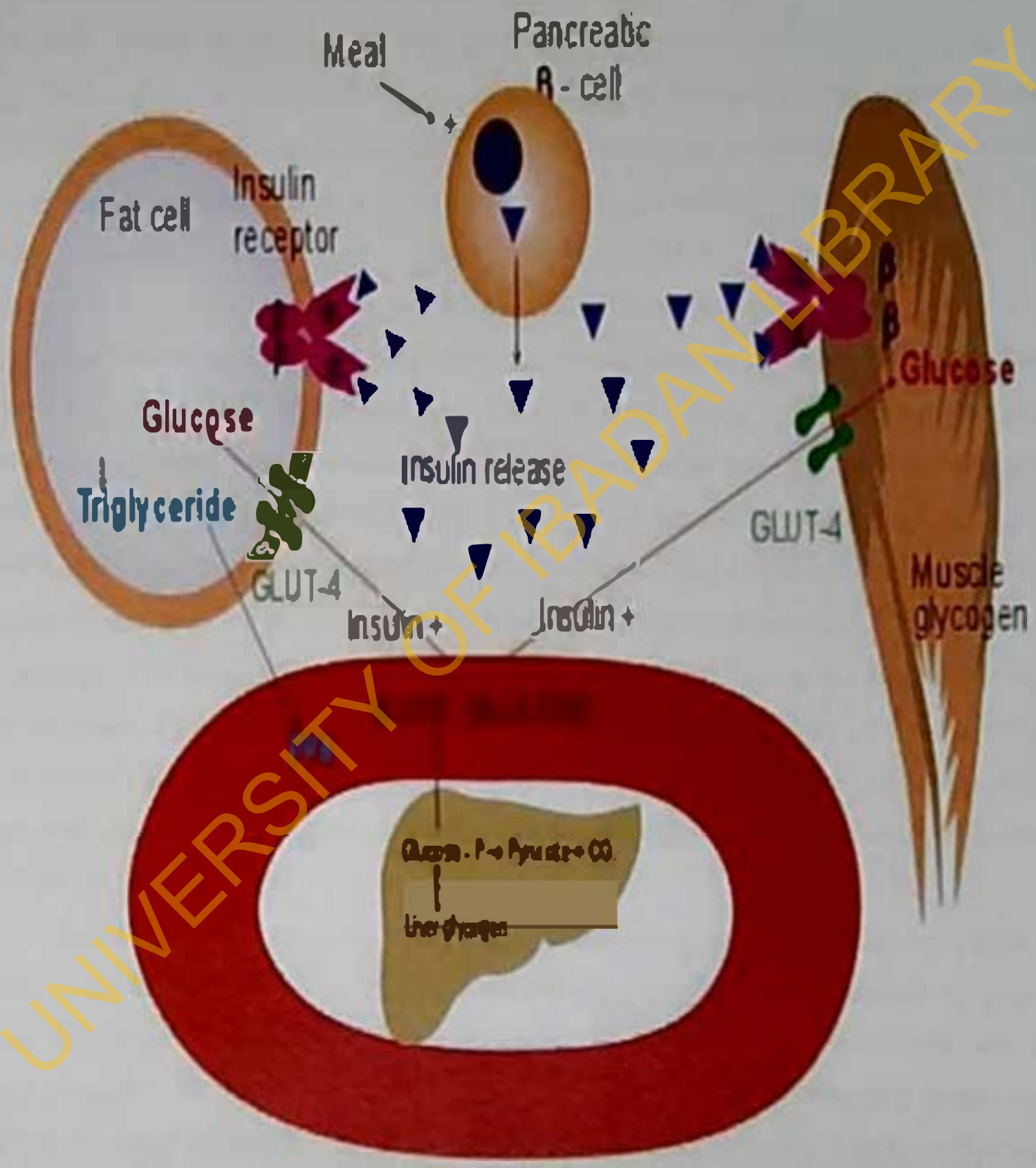

Figure 2 Tissue targets of insulin action (http://www.zuniv.net/physiology/book/chapter)

In the late postabsorptive state as occur in an overnight fast. Glucagon is secreted by the cells of the pancreas in response to low blood glucose concentration. Glucagon restores the blood glucose concentration to a steady-state level by stimulating glycogen degradation and gluconeogenesis. The liver cells almost exclusively possess glucagon receptors (Gerich, 1993), so glucagon is extremely selective in its target. The effect of glucagon is opposite to that of insulin in that it triggers cAMP synthesis leading to the shutting down of glycogen synthesis while at the same time stimulating glycogen degradation to make more glucose available for utilisation by glucose dependent tissues such as the brain (Biorn and Graves, 2001). Glucagon activates the glycogen degradation enzyme glycogen phosphorylase by phosphorylating it from glycogen phospborylase b in its unphosphorylated form to glycogen phosphorylase a the active form (Hendrickx and Willems, 1996). There is a reciprocal relationship of the glycogen synthase and glycogen phosphorylase enzymes. When both are phosphorylated, glycogen degradation is active and glycogen synthesis is not. When both are dephosphorylated, glycogen synthesis is activeand glycogen degradation is blocked suggesting a similar but mutuallyexclusive mechanism of regulatioo for the two enzymes (Aiston *et al.*, 2003). Epinephrine has similar effects on glycogen but is released by the adrenal glands in response to neural signals that trigger the fight-or-flight response. Epinephrine stimulates the breakdown of glycogen to glucose 1-phosphate, which is converted to glucose 6-phosphate. The increase in intracellular glucose 6-phospbate increases both the rate of glycolysis in muscle and the amount of glucose released into the blood stream from the liver.

While epinephrine triggers a response to a sudden energy requirement; glucagon and insulin work in tandem over longer periods to maintain a relatively constant cooconcentration of glucose in theblood. Epinephrine binds to β-adrenergic receptors of liver and muscle cells and to α1-adrenergic receptors of liver cells. The binding of epinephrine to β-adrenergic receptors or of glucagon to its receptors activates the adenylyl cyclase signaling pathway. The second messenger, cyclic AMP (cAMP), then activates protein kinase A which in turn activates glycogen phosphorylase by adding a phosphate group to it (figure 3)

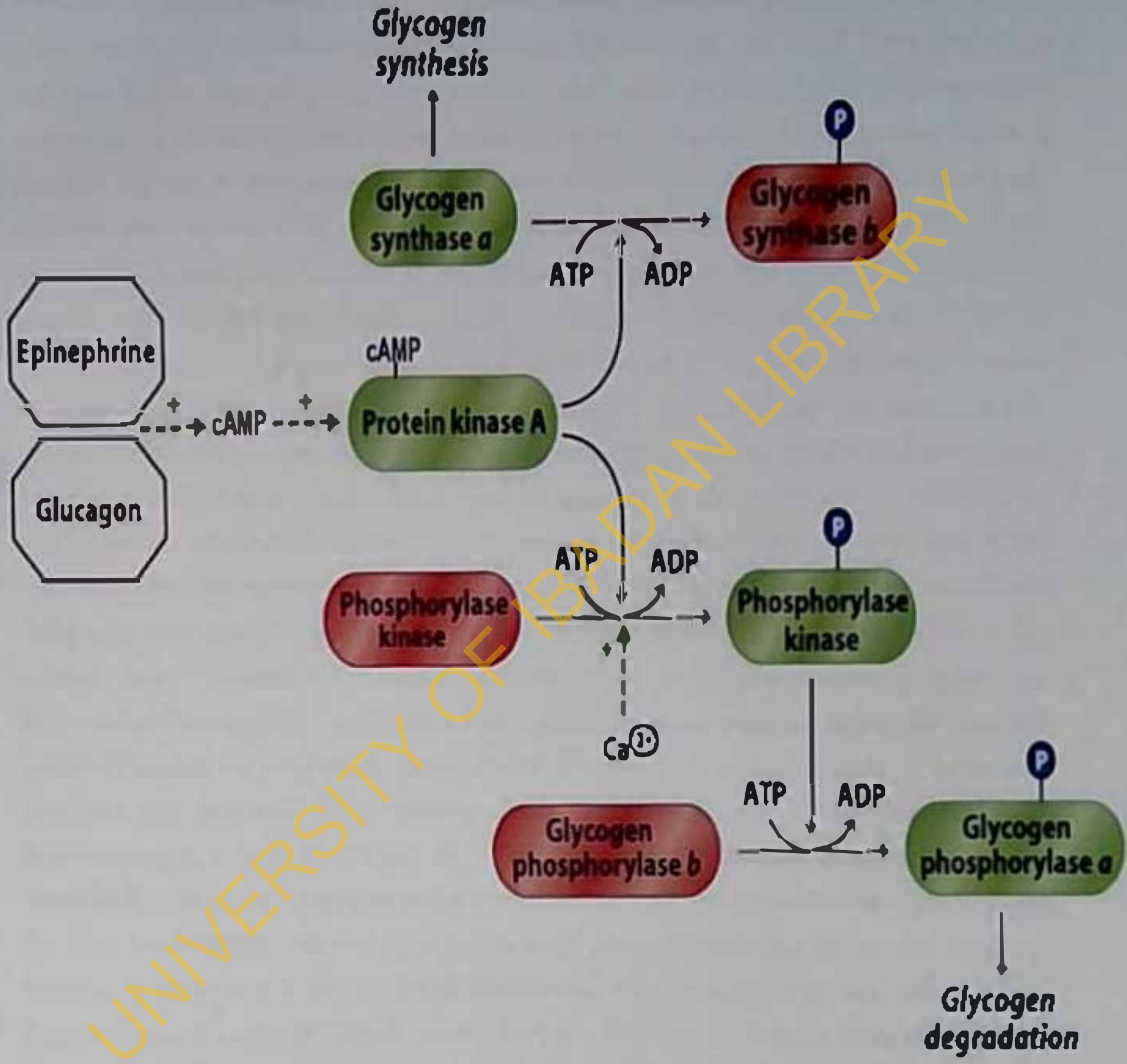

Figure 3 Schematic diagram of hormonal action on liver enzyme in the late post absorptive state (overnight fast). Principles of Biochemistry 4/e ©2006 Pearson Prentice Hall

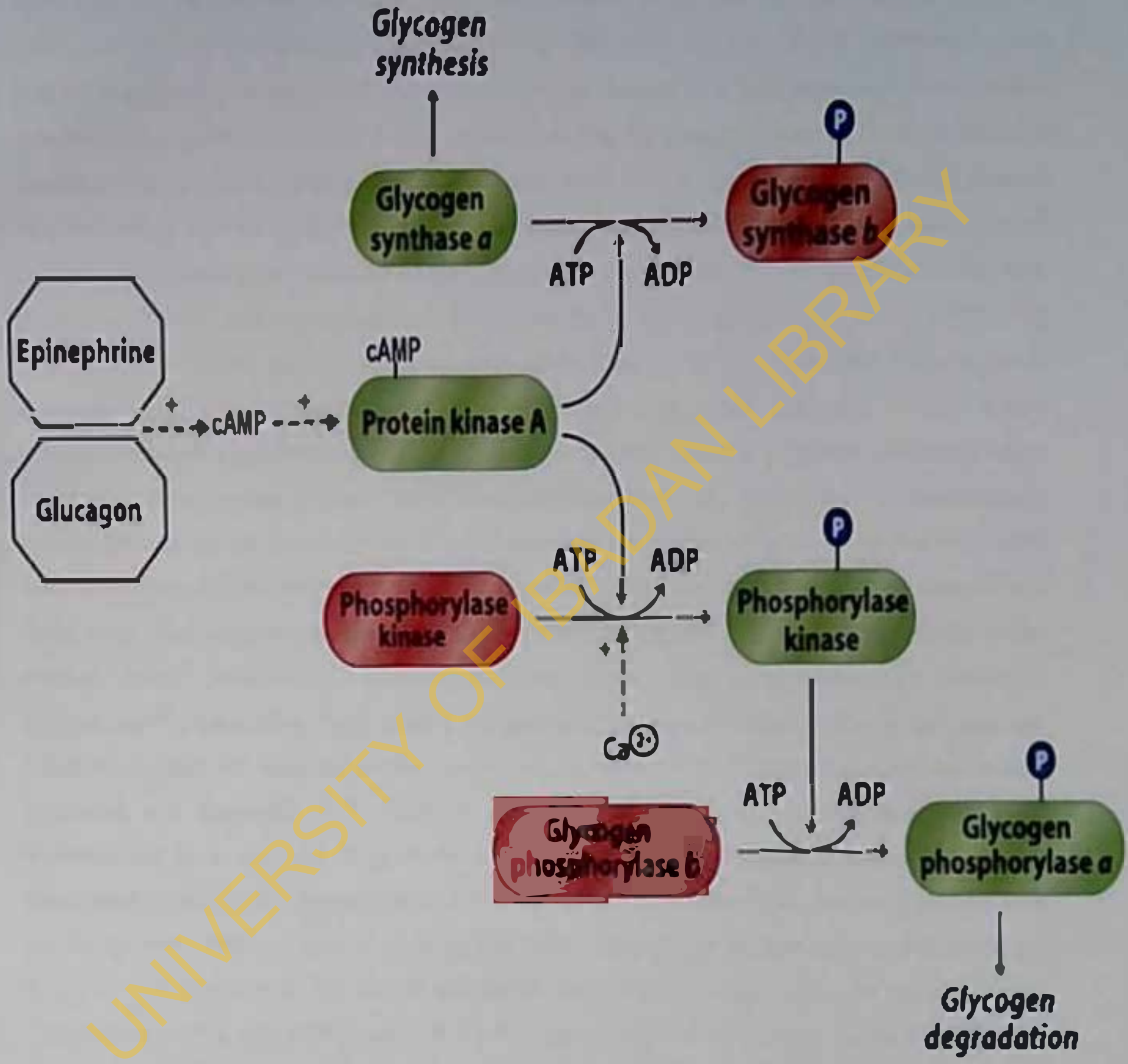

Figure 3 Schematic diagram of hormonal action on liver enzyme in the late post absorptive state (overnight fast). Principles of Biochemistry 4/e ©2006 Pearson Prentice Hall

Apart from glycogenolysis for hepatic glucose production the process of gluconeogenesis is also stimulated by glucagon and to a lesser extent epinephrine, cortisol and growth hormone which are collectively known as counterregulatory hormones. The brain has been found to consume about half of all glucose produced in the body (Eisenberg and Seltzer, 1962), even though it can neither synthesize nor store more than a few minutes' supply of glucose. Normal cerebral function therefore requires a continuous supply of glucose from the circulation. As the plasma glucose levels reaches a low threshold, it is sensed by the brain even while the drop in blood glucose is still asymptomatic causing the secretion of counterregulatory hormones that act in concert to increase glucose production and decrease glucose utilization (Bolli *et. al.*, 1984; Amiel *et. al.*, 1987). The brain monitors plasma glucose levels by both direct (Anand et. al., 1964) and indirect means (Hevener *et. al.*, 1997). Oomura et al. (1964) and Anand et al. (1964) identified neurons within areas of the lateral hypothalamus (LH) and ventromedial hypothalamus (VMH) that altered their firing rates when plasma glucose levels changed. Oomura *et. al.*, (1969) showed that directly applied glucose altered the firing rate of select neurons. They defined "glucose-responsive" (GR) neurons as those that increased and "glucose-sensitive" (GS) neurons as those that decreased their firing rates when ambient glucose levels rose. In areas such as the LH, VMH, and nucleus of the solitary tract, 20-40% of neurons sampled show such glucosesensing properties (Mizuno and Oomura 1984). Studies have demonstrated that neural pathways linking the brain and endocrine organs are involved in the control of secretion of the counterregulatory hormones (Frohman and Bernardis, 1971; Niijima, 1975; Katafuchi *et. al.*, 1985). This was aptly demonstrated in a study by Biggers *et. al.*, (1989), in which peripheral hypoglycemia was maintained while cerebral hypoglycemia was selectively eliminated by the infusion of glucose into the carotid and vertebral arteries of conscious dogs. They observed that the counterregulatory response to hypoglycemia was almost eliminated when cerebral euglycemia was maintained. In further support of a glucose-sensing role for the brain, Borg and co-workers (Borg *et al.*, 1994; Borg *et. al.*, 1995) conducted several studies in the conscious rat. They first observed that bilateral lesions of the ventromedial hypothalamus (VMH) abolished the counterregulatory response to hypoglycemia (Borg *et. al.*, 1994). They then created localized glucopenia within the VMH and observed the initiation of a counterregulatory response in the absence of peripheral hypoglycemia (Borg *et. al.*, 1995). Taken together, these studies provide evidence to support the hypothesis that

the counterregulatory hormone response to hypoglycemia is initiated in, or at least involves, the brain.

2.1.1 Gluconeogenesis

Gluconeogenesis is the formation of 'new glucose' by tissues in the body (liver, kidney and GIT) The liver, kidneys and GIT are able to release glucose into the circulation because they exclusively possess glucose 6-phosphatase (Mayes, 1993 Mithieux *et. al.*, 2004). Glucose 6-phosphatase (G-6-Pase) is a crucial enzyme in the control of glucose homeostasis. It catalyzes the last biochemical reaction of gluconeogenesis and glycogenolysis, *i.e.* the hydrolysis of glucose 6-phosphate (G-6-P) into glucose and Phosphate. Glc-6-Pase is therefore unique in that it confers upon the tissues in which it is expressed the capacity to release glucose into the blood. Glucose-6-phosphatase catalyzes the hydrolysis of glucose-6-phosphate to produce glucose and phosphate. The enzyme, is expressed mainly in the liver, kidney and GIT is critical in providing glucose to other organs during prolonged fast or starvation. More recently, the enzyme has been found in the small intestine (Rajas *et. al.*, 1999; Croset *et. al.*, 2001; Mithieux *et. al.*, 2004) which is also now known to contribute to glucose homeostasis particularly in the fasting state. This enzyme is absent in muscle and other tissues, which therefore cannot release glucose to the blood stream. Activity is inhibited by both insulin and glucose, which become elevated after feeding, thereby reducing endogenous glucose production in the fed state. Levels are increased by glucagon and glucocorticoids (Christ *et. al.*, 1986; Lange *et. al.*, 1994) which are secreted in the fasting state. The enzyme is membrane-bounded, associated with the endoplasmic reticulum. The enzyme glucose-6-phosphatase translocate acts to transport G-6-P from the cytoplasm to the lumen of the endoplasmic reticulum

Release of glucose into the circulation occurs through two main processes: the de novo synthesis of glucose from non-glucose precursors (gluconeogenesis) and the breakdown of glycogen (glycogenolysis) (Gerich, 1993). In normal humans in the overnight fasting state gluconeogenesis and glycogenolysis each contribute approximately 50% of the glucose delivered into the systemic circulation (Landau *et. al.*, 1966). *In vitro* studies have shown that on a gram for gram tissue basis the gluconeogenic capacity of the kidney exceeds that of the liver (Krebs 1963). Gluconeogenesis provides glucose to the tissues of the body in the fasted state when dietary

carbohydrates are not available, by formation of glucose or glycogen from noncarbohydrate source. In addition, the gluconeogenic process clears metabolic products, such as lactate produced by muscle and erythrocytes and glycerol produced by adipose tissue, from the circulation. The regulation of endogenous glucose production is central to the control of blood glucose concentrations, and the liver and kidney are the principal organs responsible for gluconeogenesis. Many of the enzymes of glycolysis and gluconeogenesis are shared, including those from phosphoenolpyruvate to fructose 1,6-diphosphate. In liver, glucose-6-phosphatase catalyzes the rate-limiting step of gluconeogenesis. However, for gluconeogenesis to occur, the enzymes pyruvate carboxylase and phosphoenol pyruvate carboxylase must be present and can limit flux through the gluconeogenic pathway (Barbel and Schmoll, 2003).

2.1.2 Glucose phosphorylation and glycolytic pathways

A balance between hepatic gluconeogenesis and peripheral glycolysis is an important homeostatic function, especially during a prolonged fast. Glycolysis occurs in virtually all living cells with the oxidation of glucose to pyruvate and lactate. A continuous supply of glucose is necessary as a source of energy, especially for the nervous system whose cells have minimal storage capabilities and for erythrocytes, which are unable to store glucose or use other substrates as fuel.

a. Pyruvate formation.

G-6-P is phosphorylated byphosphofructokinase to form fructose 1,6-diphosphate. The reaction is subject to allosteric control by cellular levels of ATP, AMP, and phosphate.

b. Krebs cycle/aerobic glycolysis.

Under aerobic conditions, there can be complete oxidation of carbohydrates, fatty acids, and proteins to carbon dioxide and water, although glucose that enters the cycle can be released as lactate, pyruvate, and alanine particularly during conditions of a prolonged fast. Aerobic glycolysis yields the net production of 38 molecules of ATP per molecule of glucose consumed. Pyruvate crosses the mitochondrial membrane to supply fuel for the Krebs cycle or for glucoocogenesis. The pyruvate dehydrogenase complex determines the transformation of pyruvate to acetyl-CoA (coenzyme A). This enzyme complex is inactivated by ATP when cellular energy stores are high and by pyruvate dehydrogenase kinase (PDK) (Randle *et al.*, 1994). PDK1-4 can regulate the pyruvate dehydrogenase complex by inhibitory phosphorylation of the complex. The enzymes PDK2 and PDK4 are expressed in most tissues, whereas PDK1 and PDK3 distribution is more

limited. Levels of PDK4 are up-regulated during starvation, thereby inhibiting the complex when glucose conservation is necessary (Sugden *et. al.*, 2001, Wu *et. al.*, 2000).

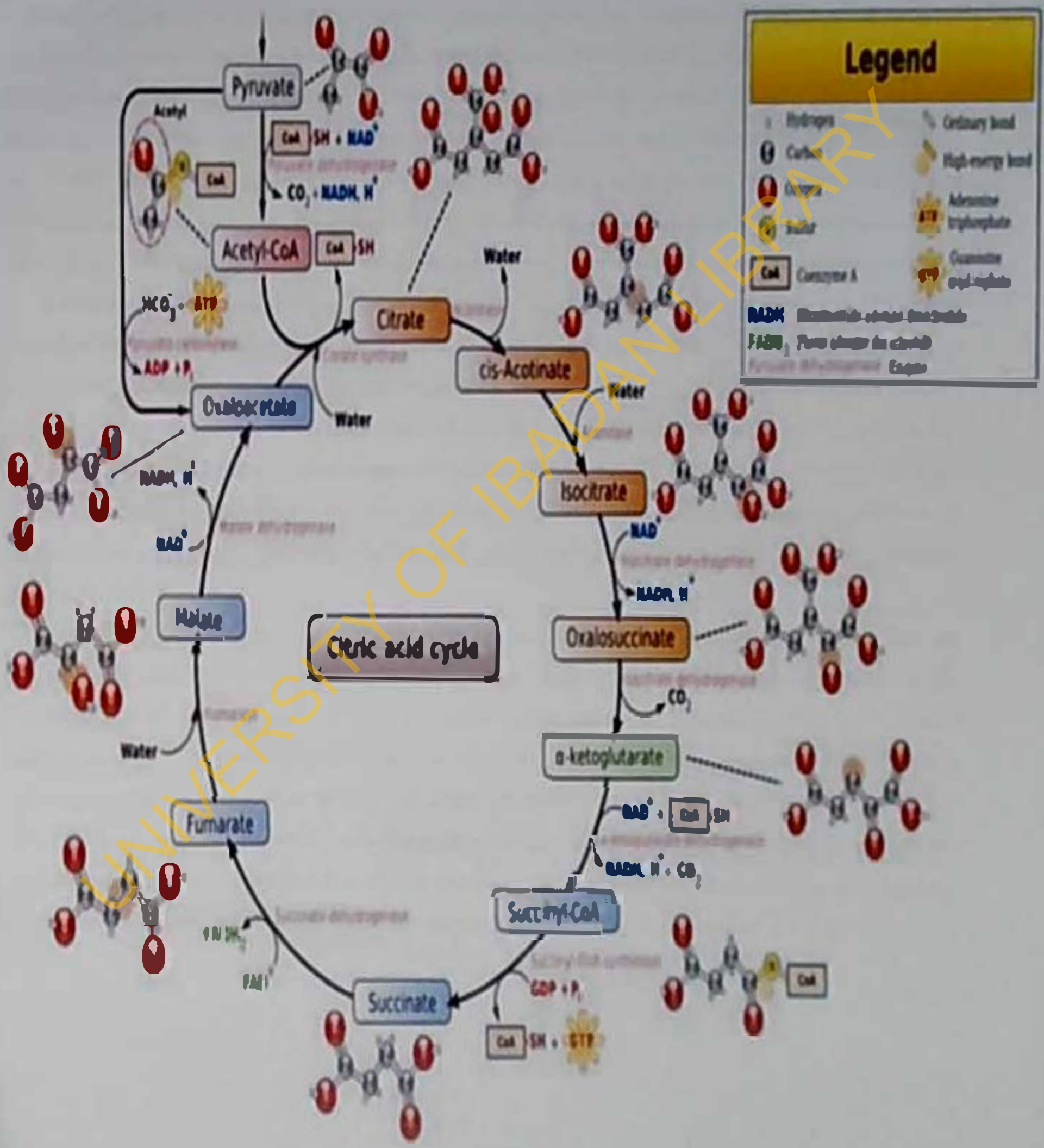

Figure 4 Kreb's (Citric acid) cycle (from Wikipedia Image citricacidcycle bell.svg)

Activity of the pyruvate dehydrogenase complex is a major determinant of the glucose oxidation rate. Glucose oxidation can then proceed though the Krebs cycle, a sequence of reactions in which acetyl-CoA is metabolized to CO_2 and hydrogen atoms. In brief, acetyl-CoA is first condensed with oxaloacetate to form citrate. In a series of seven subsequent reactions, two CO_2 molecules are split off, regenerating oxaloacetate (Coleman and Kuzava, 1991). Exercise can increase the activity of pyruvate dehydrogenase (Nakai *et al*., 2002), and pyruvate dehydrogenase activity is less responsive to insulin stimulation both in patients with diabetes and in their offspring (Mostert *et al*., 1999). Because phosphorylated compounds are charged, most do not cross membranes, and pyruvatedehydrogenaseactivity remains within the mitochondria. Likewise, nicotinamideadenine dinucleotide (NADH) is not diffusible across membranes, so the reduction equivalents produced by Krebs cycle oxidation must be transferred to the cytoplasm by complex alternate reduction-reoxidation cycles involving a membrane diffusible substrate such as malate. The inner mitochondrial membrane contains the respiratory chain proteins, which consist of a series of electron acceptors that are reversibly reduced and then reoxidized as they receive electrons and form ATP. Mitochondria are often located near subcellularstructures that require energy or provide a substrate source (Mostert *et. al*., 1999). Moreover, mitochondrial processes are coupled to peripheral glucose uptake, phosphorylation, and glycolysis by the spatial proximity to hexokinases (Wilson, 1995).

The Randle cycle provides an important link between glucose and fatty acid metabolism (Randle *et. al*., 1963) whereby fatty acid or ketone oxidation leads to elevation of mitochondrial acetyl-CoA and NADH, leading to increases in cytosolic citrate. Increased cytosoliccitrate could inhibit glycolysis at the level of phosphofructokinase, thereby decreasing the use of glucose as a fuel while increasing glucose incorporation into glycogen (Randle *et al*., 1994, Maizels *et. al*., 1977). Malonyl-CoA, which is involved in the regulation of the transfer of long-chain fatty acids into the mitochondria has been proposed to play a central role in this process mediating fuel sensing, glucosemetabolism, and insulin action (Ruderman *et. al*., 1999; Ruderman *et. al*., 2003).

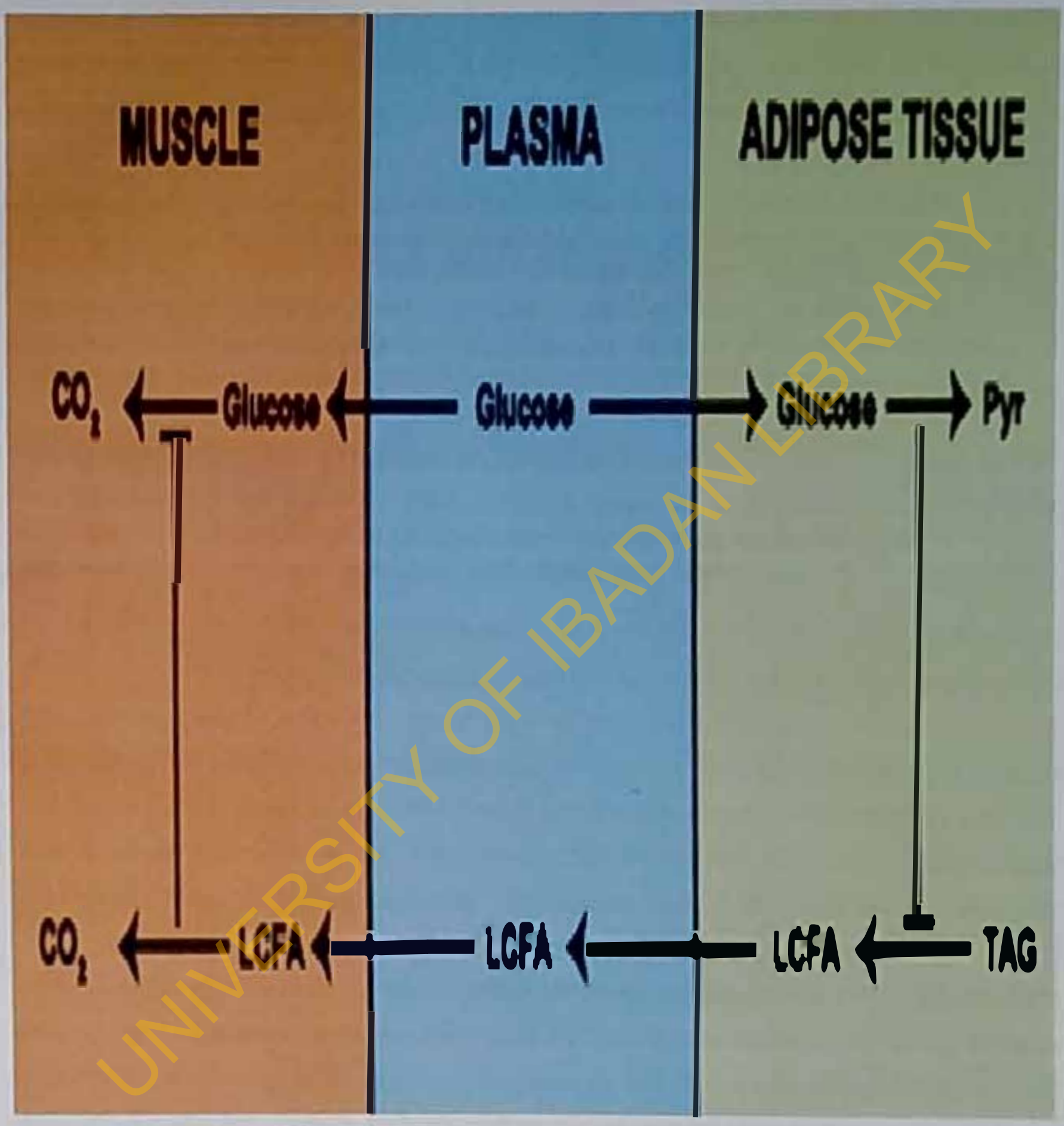

Figure 5 The Randle cycle (Adapted from Randle *et al.*, 1963)

LCFA Long chain fatty acids

TAG Tri acyl glycerol

Mitochondria and glycolytic processes are also coupled with insulin secretion. As a result of glycolysis, rising ATP levels lead to the closure of ATP-dependent potassium channels and opening of calcium channels, which triggers insulin secretion. The role of mitochondria in insulin secretion is highlighted by the finding of defective insulin secretion followed by β-cell loss in transgenic rodents with pancreatic β-cell-specific disruption of mitochondrial transcription factor A (Silva *et al.*, 2000).

Krebs cycle activity is not regulated solely by the mitochondrial acetyl-CoA concentration. Pathway activity varies over a wide range depending on the substrate source; for example, acetyl-CoA levels may be 10-fold lower with glucose compared with fatty acid as a substrate source for the same cycle flux (Williamson and Cooper, 1980). Citrate synthase, isocitrate, and α-ketoglutarate dehydrogenases are generally considered to be important regulatory enzymes controlling flux through the entire Krebs cycle (Rustin *et al.*, 1997). The pyridine nucleotide redox potential ($\frac{NADH}{NAD^+}$ ratio), the matrix phosphorylation potential ($\frac{P_i \cdot ADP}{ATP}$ ratio), and the Ca^{2+} concentration acts key regulatory factors at several steps of the cycle (Rustin et al 1997). Krebs cycle activity is further influenced by thyroid hormone, adrenergic compounds, and glucocorticoids (Williamson and Cooper, 1980; Rustin *et al.*, 1997).

Few cases with primary disorders of enzymes of the Krebs cycle have been reported in humans. However, increased pyruvate dehydrogenase kinase (PDK4) activity seen in insulin resistance and type 2 diabetes, may be a direct or indirect target of Peroxisome proliferator-activated receptors-α (PPARα), and could represent an additional drug target for these medical conditions (Sugden and Holness, 2002; Huang *et al.*, 2002). Deficiency of α-ketoglutarate dehydrogenase, succinate dehydrogenase, and fumarase has been reported in rare patients and leads to neurological impairment with or without muscular involvement. Germline mutations of succinate dehydrogenase cause hereditary paraganglioma and pheochromocytoma (Astuti *et al.*, 2001). More recently, mutations of fumarate hydratase have been associated with uterine fibroids, skin leiomata, and, to a lesser extent, papillary renal cell cancer (Tomlinson *et al.*, 2002). Because neural cells are most dependent on glucose oxidation for fuel, it is not surprising that disorders of these key enzymes are clinically manifest in these tissues, without abnormalities in blood glucose or insulin levels

c. Anaerobic glycolysis: the Embden-Meyerhof-Parnas pathway.

In erythrocytes, the glycolytic pathway always terminates in the formation of lactate, because these cells lack enzymes of the Krebs cycle. However, in other tissues under anaerobic conditions, glucose is used to generate high-energy ATP as fuel, with formation of lactate as a by-product. The reaction is catalyzed by lactate dehydrogenase, and in contrast to the Krebs cycle, which occurs in both cytosolic and mitochondrial compartments, all the enzymes of the Embden-Meyerhof-Parnas pathway are found in the cytosol.

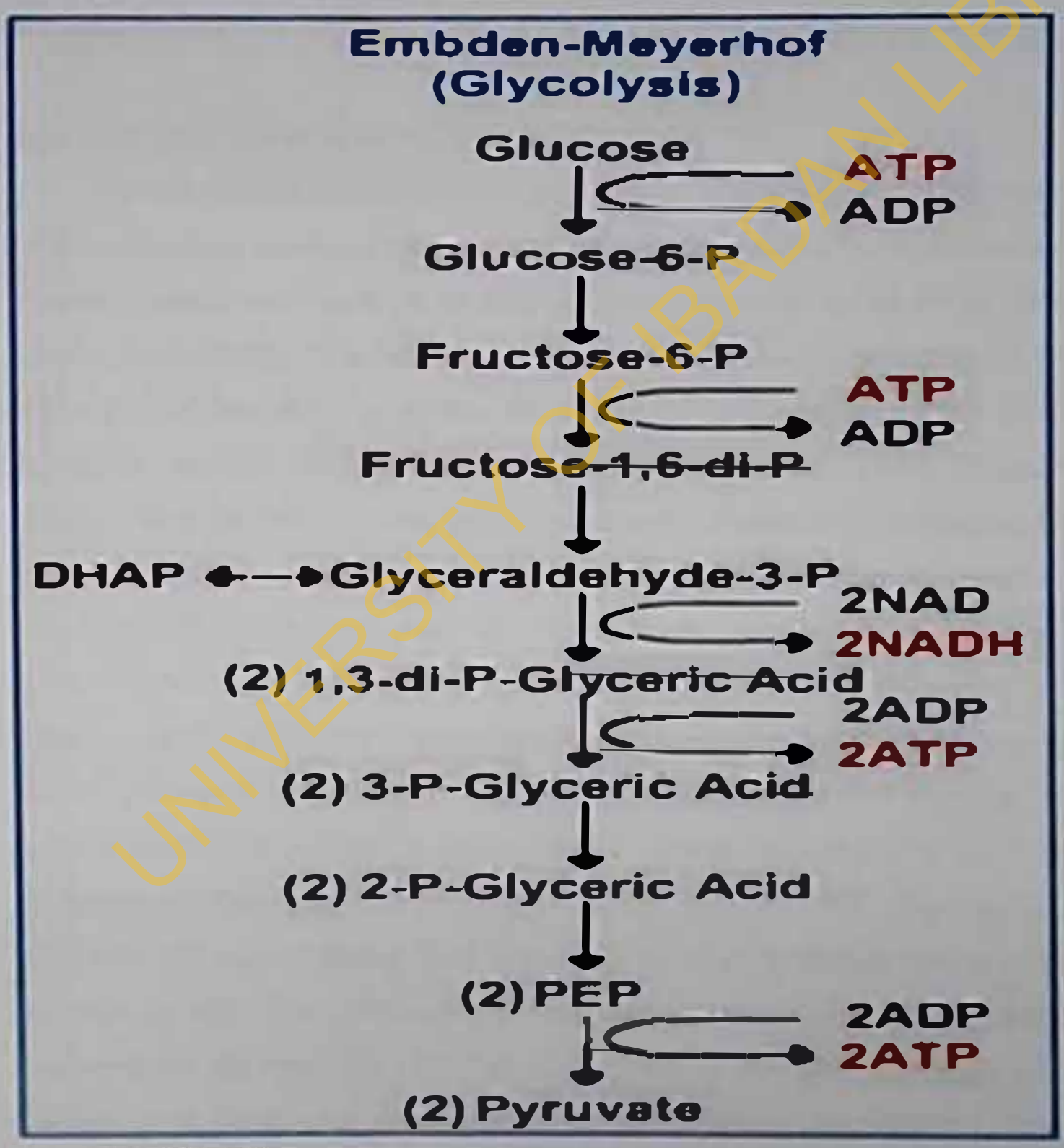

Figure 6 Embden-Meyerhof-Parnas pathway (Source- classes.midlandstech.edu)

Under anaerobic conditions, glucose is the only fuel source that can be used by skeletal muscle, and during muscle contraction a continuous supply of ATP is necessary. Anaerobic glycolysis yields the net production of only two molecules of ATP and only two molecules of reduced NADH per molecule of glucose consumed. Thus, anaerobic metabolism is inefficient and cannot be sustained for long intervals of time. In addition to erythrocytes and skeletal muscle, other tissues that produce lactate include brain, gastrointestinal tract, renal medulla, adipose tissue, and skin. Lactate can be converted back into glucose by the gluconeogenesis pathway, requiring ATP (Duke, 1999), or can be used in muscle to help restore glycogen after intense exercise (McLane and Holloszy, 1979). The energy potential of lactic acid can only be recovered in the presence of oxygen with conversion back to pyruvic acid. In turn, pyruvate can then be metabolized in the citric acid cycle.

2.2 GLUCOSE HOMEOSTASIS

Blood glucose concentration is one of the most finely regulated physiological parameters of the mammalian organism. Glucose homeostasis is achieved by a physiological balance between glucose production and intake in the form of carbohydrates and utilization by the peripheral tissues (Seelay *et. al.*, 1998). Whereas most tissues can readily utilize free fatty acids or other substrates when glucose becomes unavailable, the red blood cells and the brain utilize glucose almost exclusively as their sole energy source (Cryer, 1981; Zierler, 1999). Consequently, sustained hypoglycemia can lead to coma, and if not corrected immediately permanent brain damage and eventually death (Andres *et. al.*, 1956). Abnormal elevation of plasma glucose levels (hyperglycemia) does not pose an urgent threat as does hypoglycemia, yet prolonged hyperglycemia is also ultimately life threatening. Therefore blood glucose has to be kept within a narrow range for the proper functioning of the body in general and these two tissues in particular. Blood glucose homeostasis is achieved by the interaction between insulin and the counterregulatory hormones (glucagon, cortisol, epinephrine and growth hormone) during periods of feeding and fasting (Clarke *et. al.*, 1979; Mitrakou *et. al.*, 1991; Zierler *et. al.*, 1999; Sacca *et al.*, 1979). The normal fasting level of plasma glucose in peripheral venous blood is 80-90 mg/dl (Guyton and Hall, 2000). Following a meal, such as breakfast, glucose is rapidly elevated 30-50 percent above this level; but within two hours glucose levels has been restored to normal, and it remains at 80 mg/dl until the next meal and the pattern is repeated (Scheinberg *et. al.*, 1949,

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Nordlie *et. al.*, 1999). After each meal and in the immediate post absorptive state, insulin stimulates the conversion of glucose to glycogen (in skeletal muscles and the liver) and fats and reduces or prevents hepatic gluconeogenesis (Prager *et. al.*, 1987; Ader and Bergman 1990; Vikaini *et. al.*, 1999). Insulin has been shown to be the only hormone which tends to reduce blood glucose concentration while the counterregulatory hormones have the reverse effect by increasing blood glucose concentration (Woll *et. al.*, 1957; Soskin and Levine, 1987).

High or low blood glucose can cause diabetes or hypoglycemia respectively. When glucose is not being ingested, it is the role of the liver to produce this nutrient, either from its stores (Glycogenolysis) or de novo from three carbon precursors (gluconeogenesis). This maintenance is achieved through a balance of several factors, including the rate of consumption and intestinal absorption of dietary carbohydrate, the rate of utilization of glucose by peripheral tissues and the loss of glucose through the kidney tubule and the rate of removal and release of glucose by the liver (Zieler, 1999).

Sources and Sinks of plasma glucose

2.2.1 Sources of plasma glucose

The great majority of glucose in the plasma comes from two sources: intestinal absorption of dietary glucose and its precursors and the release of glucose from the liver.

Sources of plasma glucose:

Glucose per se (a minor source)

Glucose containing disaccharides

Sucrose (fructosyl glucose) commonly called table sugar

Lactose (galactosyl glucose) called milk sugar

Maltose (glucosyl glucose)

Glucose-containing polysaccharides (a major source): starch from plants and glycogen from animal tissues

Sugars readily converted to glucose: Fructose and galactose

Gluconeogenic amino acids

Glycerol moiety of triglycerides

2.2.2 Sinks of plasma glucose

All body tissues can and do use plasma glucose, but some such as the nervous system which requires about 120-150g of glucose daily under most conditions and the red cells are obligatory

users. In prolonged starvation. studies show that the brain undergoes an interesting metabolic switch that allows it to utilize ketone bodies (β-hydroxybutyrate and acetoacetate) in place of over 50% of its usual glucose requirement (Owens *et. al.*, 1967). Fatty acids are continually mobilized from the huge stores of adipose tissue triglycerides and a portion of them are continually converted to ketone bodies in the liver. This adaptation in metabolism of the brain ensures that it can survive without requiring drastic depletion of muscle protein to provide substrates for gluconeogenesis (West, 1998).The red blood cells, the intestinal mucosa (inner epithelium) and the renal medulla use glucose largely exclusively via anaerobic glycolysis. Most of the body tissues however, are facultative users of glucose. During fasting, these tissues can and do switch to use free fatty acids (FFA) as their primary metabolic fuel (Zieler, 1999). The liver is both a source and a sink of glucose and both uptake and release are occurring at all times with the net balance being under hormonal control (Zieler, 1999). Under normal circumstances, no glucose is lost in the urine through the kidney, so, the kidney is not normally a sink. Although glucose is freely filtered by the kidney, it is reabsorbed into the blood stream unless the capacity for glucose reabsorption by the kidney is exceeded. If plasma levels of glucose are greatly elevated above the renal threshold for glucose (180mg/100ml), then that amount of glucose that cannot be reabsorbed is lost in the urine (Zieler, 1999).

2.2.3 The Role of the Liver in Glucose Homeostasis

The liver plays a unique role in the regulation of carbohydrate metabolism because it is able both to take up and release glucose, thus helping to dampen variations in blood glucose (Soskin et al., 1938). In the postabsorptive state (when all nutrients from the last meal have been absorbed from the intestine), the liver is a net producer of glucose. If adequate amounts of glucose are administered at this point, the liver switches from net glucose production to net glucose uptake. Hyperglycemia, hyperinsulinemia, and a negative arterial-portal venous (a-pv) glucose gradient ("portal signal") are known to positively affect the rate of net hepatic glucose uptake (NHGU). These three factors when present simultaneously result in peak rates of NHGU (Myers *et. al.*, 1991, Pagliassotti *et. al.*, 1996). Circulating insulin increased NHGU in a dose-dependent manner in the presence of a fixed hyperglycemia at all insulin levels tested (Myers *et. al.*, 1991). NHGU was greater in the presence than in the absence of the portal signal. If arterial insulin was maintained constant, both at basal (Adkins-Marshall *et. al.*, 1992, Pagliassotti *et. al.*, 1996) and at fourfold

basal levels (Myers *et. al.*, 1991), NHGU increased as the glucose levels were progressively increased, and again NHGU was greater in the presence of the portal signal than in its absence (Myers *et. al.*, 1991).

The modulation of net glucose uptake by the portal signal also extends to extrahepatic tissues. A decrease in net glucose uptake by nonhepatic tissues in the presence of the portal signal was indirectly demonstrated in several previous studies (Adkins-Marshall *et. al.*, 1992; Pagliassotti *et. al.*, 1996). This reduction was of the same magnitude as the increase in NHGU, thus suggesting that the portal signal controlled the whole body distribution of glucose. Galassetti *et. al.*, (1998) recently demonstrated that the reduction in extrahepatic glucose uptake caused by the portal signal was primarily the result of a decrease in glucose uptake by skeletal muscle.

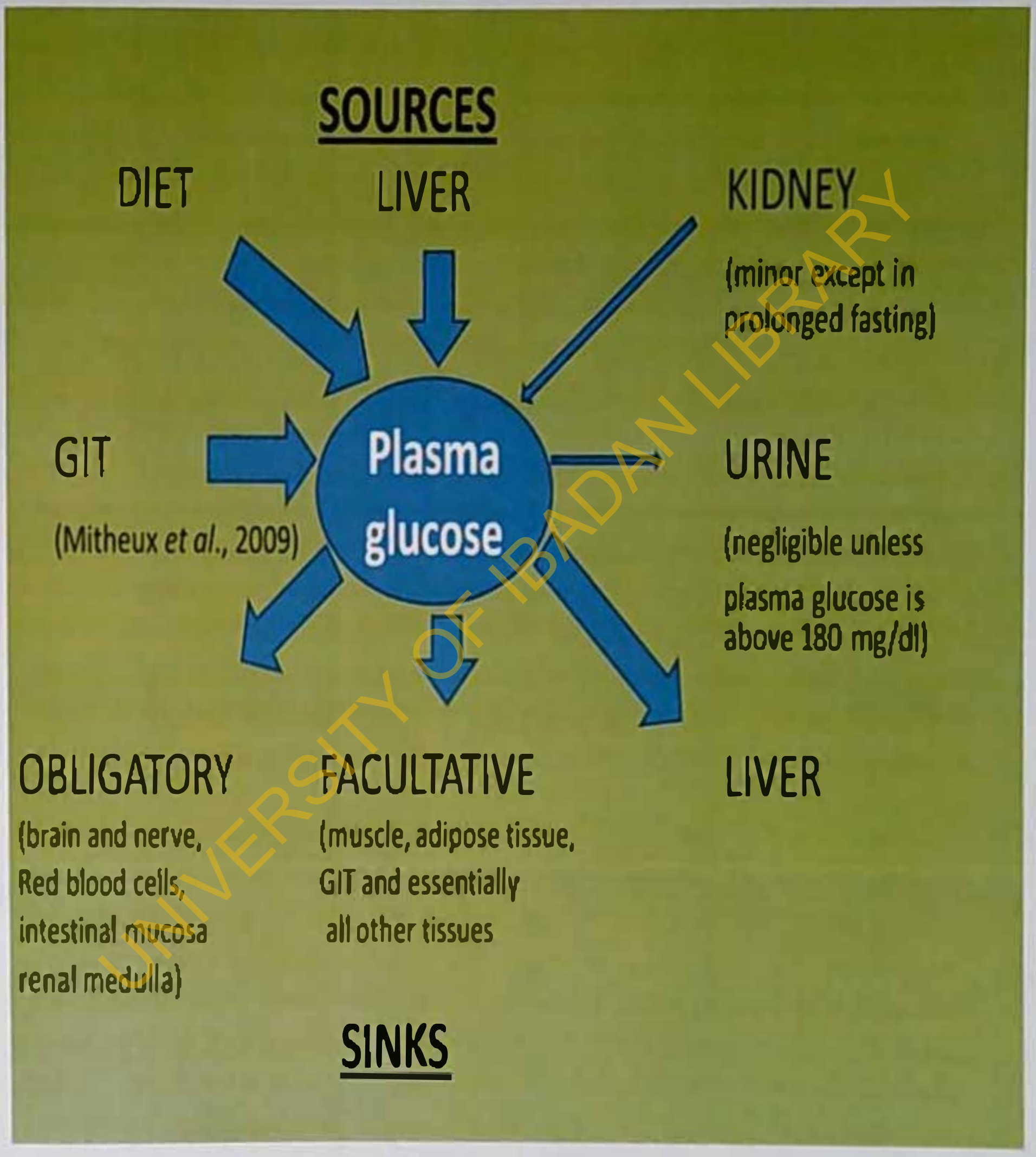


Fig. 7 Sources and sinks of plasma glucose (Steinberg, 1985 with modifications)

Although all previous studies investigating the portal signal were conducted under hyperglycemic conditions, indirect evidence indicates that its effects may be present even under euglycemic conditions. Several studies (Donovan *et. al.*, 1994; Hamilton-Wessler *et. al.*, 1994) showed that the sympathoadrenal response to hypoglycemia was blunted when systemic hypoglycemia was allowed to occur, but the hepatoportal region was kept euglycemic by portal glucose infusion. In the process of maintaining hepatic euglycemia, the authors created a negative a-pv glucose gradient. Therefore, the decrease in the sympathoadrenal response may well have been due to the effects of the portal signal. If the portal signal can be activated under euglycemic conditions, it should increase NHGU in response to insulin and possibly exert its inhibitory effect on glucose uptake by nonhepatic tissues even in the absence of hyperglycemia.

In the postprandial state, the liver becomes an important site of glucose removal. The amount of glucose taken up by the liver is regulated by circulating glucose and insulin concentrations and by the a-pv glucose gradient (Pagliassotti and Cherrington, 1992). It has been previously demonstrated (Pagliassotti *et. al.*, 1996) that the increase in net hepatic glucose uptake (NHGU) induced by the portal signal is a result of the stimulation of hepatic glucose uptake processes and not a suppression of hepatic glucose production. The rise in hepatic glucose uptake results in increased intracellular concentrations of glucose 6-phosphate and other hexose monophosphates, which in turn stimulate glycogen synthase, leading to significant glycogen deposition. In fact, ~75% of the extra glucose taken up by the liver in response to the portal signal is stored as glycogen, whereas the remainder primarily leaves the liver as lactate. Other metabolic pathways (oxidation, pentose phosphate cycle, conversion to lipids) appear to play minor roles in the intrahepatic fate of glucose.

2.2.4 Control of Hepatic Glucose Production

Insulin is known to reduce hepatic glucose production (HGP) by acting both directly and indirectly on the liver (Cherrington *et. al.*, 1998). Insulin acts directly by binding to hepatic insulin receptors and thereby activating insulin signalling pathways in the liver. These effects have been demonstrated in various models. In isolated rat hepatocytes, insulin inhibits glucose production through inhibition of gluconeogenesis (Claus and Pilkis, 1976) and glycogenolysis (Marks and Botelho, 1986). In the dog, an acute selective increase (Sindelar *et. al.*, 1996) or decrease (Sinderlar *et. al.*, 1998) in hepatic insulin level (such that arterial insulin level remained constant) resulted in very rapid suppression or stimulation, respectively, of HGP. Furthermore, liver-specific insulin

receptor knockout (LIRKO) mice, which lack hepatic insulin receptors from birth, demonstrate severe hepatic insulin resistance (Fisher and Kahn, 2003). These studies, and others, demonstrate that insulin acts directly on the liver to regulate HGP.

Insulin's indirect effects include reduction of glucagon secretion at the pancreas (Ito *et al.*, 1995), inhibition of lipolysis in fat thereby reducing circulating lipids and glycerol availability for gluconeogenesis (Sindelar *et. al.*, 1997), and decreased protein catabolism in muscle which further reduces gluconeogenic precursor availability (Wolfe and Volpi, 2001). Studies in the mouse and rat suggest that hypothalamic insulin signalling may also play an important role in insulin's ability to indirectly regulate HGP (Obici *et. al.*, 2002,). Insulin's indirect hepatic effects were well demonstrated in the dog, where an acute selective increase in arterial insulin level (so that the hepatic insulin level was kept constant) resulted in inhibition of HGP (Sindelar *et. al.*, 1996). This was thought to be partially due to insulin's effect on lipolysis, since when plasma FFA levels were prevented from falling, HGP only decreased by half as much (Sindelar *et. al.*, 1997). In mice, HGP was suppressed by insulin despite 95% reduction of hepatic insulin receptors by antisense oligonucleotide treatment (Buettner *et. al.*, 2005). These studies, and others, demonstrate that in addition to insulin's direct effects on the liver, the hormone also regulates HGP through indirect means.

Despite evidence demonstrating both direct and indirect effects of insulin on the liver, it has been hypothesized that control of the liver is primarily indirect (Bergman, 2000). To address the relative importance of insulin's direct versus indirect effects, an experiment was designed to determine which effect dominates control of basal HGP. Experiments were carried out using the overnight-fasted conscious dog, in which, during a pancreatic clamp, insulin infusion was switched from the hepatic portal vein to a peripheral vein. This resulted in a doubling of the arterial insulin level and, at the same time, a 50% decrease in the insulin level within the hepatic sinusoids. It was hypothesized that if HGP is primarily regulated by insulin's indirect effects, then arterial hyperinsulinemia should cause a decrease in HGP, despite a reduction of insulin's direct effect at the liver. On the other hand, if insulin's direct hepatic effects are dominant, increased HGP should occur. Despite mild hyperglycemia and peripheral hyperinsulinemia, HGP rapidly increased more than 2-fold and remained elevated throughout the 3-hour experimental period thereby demonstrating that insulin's direct effect on the liver was dominant and was the primary controller of basal HGP in the nondiabetic dog.

In an effort to determine which mode of insulin action is most important in the regulation of HGP, Buettner *et al.* (2005) recently showed that 95% ablation of hepatic insulin receptors by treatment with insulin receptor antisense oligodeoxynucleotide over 1 week did not impair the ability of insulin to inhibit glucose production during a hyperinsulinaemic clamp. The conclusion drawn from this study was that hepatic insulin receptors are likely to play an important role in the long-term regulation of HGP, mainly via changes in gene transcription, but rapid control can still occur in the presence of a severe impairment in liver insulin signalling. Thus, as previously demonstrated (Sindelar *et. al.*, 1996), in the mouse, HGP can be selectively inhibited by insulin's indirect effects, but these findings do not directly address whether insulin's direct or indirect effects dominate the acute control of HGP. Gupta *et al.*, (2002) found that the prevailing glycemic level is a determinant of the balance between insulin's direct and indirect effects on HGP in individuals with type 1diabetes. They suggested that when glycogenolysis is already maximally suppressed by hyperglycemia, hepatic insulin may not further suppress HGP, whereas, with correction of hyperglycemia, glycogenolysis is restored, allowing for the direct effect of insulin on glycogenolysis to become manifest. In other studies, obese patients with type 2 diabetes were found to have defects in both the direct and the indirect effects of insulin on the liver (Staehr *et. al.*, 2002). When the estimated hepatic sinusoidal insulin levels were increased by 11 μU/ml, in those studies, the impairment in the direct effect of insulin was overcome, but the defective indirect response persisted as a result of impaired suppression of FFA and glucagon levels. Thus, while the direct effect of insulin on HGP is dominant in the normal individual, in the diabetic state, insulin resistance of extrahepatic tissues and the prevailing glucose levels become factors.

In studies in the rat, Obici *et. al.*, (2002) showed that ICV infusion of insulin resulted in increased hepatic insulin sensitivity (so that glucose production fell during a euinsulinemic pancreatic clamp during ICV insulin infusion). In addition, when insulin receptor antisense oligonucleotide, insulin antibodies, or PI3K inhibitors were infused into the third ventricle, glucose production was not suppressed by a rise in arterial insulin as much as in a control group (Obici *et. al.*, 2002). These findings led the authors to conclude that hypothalamic insulin signalling is required for normal inhibition of glucose production by insulin (Obici *et. al.*, 2002).

The blood-brain barrier is permeable to insulin (Woods *et. al.*, 2003), and previous studies in the dog have demonstrated that infusion into the carotid and vertebral arteries resulted in even distribution throughout the blood flowing through the head (Biggers *et. al.*, 2003). In addition, a

rapid change in sympathetic outflow from the brain was previously demonstrated in response to head insulin infusion in the dog (Davis *et. al.*, 1995). Previous studies in the dog demonstrated that cerebrospinal fluid insulin levels increase (after a 30-minute lag) during a hyperinsulinemic clamp (Schwartz *et. al.*, 1990). In that study, when the plasma insulin level was clamped at approximately 90 μU/ml, cerebrospinal fluid insulin was approximately 2 μU/ml after 180 minutes. Okamoto *et al.*, (2005) recently demonstrated hepatic insulin resistance in insulin receptor knockout mice with restored liver insulin signalling, but reduced signalling in the hypothalamus. This suggests that hypothalamic signalling plays an important role in insulin's ability to regulate HGP in the mouse. Thus, background hypothalamic insulin signalling may determine the acute ability of the liver to directly respond to insulin, but not be involved in the minute-to-minute regulation of HGP.

2.2.5 Autoregulation of hepatic glucose production

A major factor affecting blood glucose is the ability of the liver to make glucose available in situations of hyperglycemia even in the absence of direct or indirect insulin action. In response to hyperglycemia, net hepatic glucose output can be decreased by as much as 60±9% by nonhormonal mechanisms. Under conditions in which hepatic glycogen stores are high (i.e. the overnight-fasted state), a decrease in the glycogenolytic rate and an increase in the rate of glucose cycling within the liver appear to be the explanation for the decrease in hepatic glucose output seen in response to hyperglycemia. During more prolonged fasting, when glycogen levels are reduced, a decrease in gluconeogenesis may occur as a part of the nonhormonal response to hyperglycemia. A substantial role for hepatic autoregulation in the response to insulin-induced hypoglycemia is most clearly evident in severe hypoglycemia (<2.8 mmol/l). The nonhormonal response to hypoglycemia is thought to involve enhancement of both gluconeogenesis and glycogenolysis and may supply enough glucose to meet at least half of the requirement of the brain. The nonhormonal response can include neural signaling, as well as autoregulation. However, even in the absence of the ability to secrete counterregulatory hormones (glucocorticoids, catecholamines, and glucagon), dogs with denervated livers (to interrupt neural pathways between the liver and brain) were able to respond to hypoglycemia with increases in net hepatic glucose output. Thus, even though the endocrine system provides the primary response to changes in glycemia, autoregulation plays an important adjunctive role (Moore *et al.*, 1995).

2.2.6 Counter-regulatory Hormones

The counterregulatory hormones are a group of hormones that tend to act on blood glucose levels in a reciprocal manner to insulin. While insulin tends to lower the blood glucose concentration, the counterregulatory hormones which include glucagon, cortisol, epinephrine and growth hormone tend to elevate it. The brain is dependent on a continuous supply of glucose from the circulation (McCall, 1993), depending almost exclusively on glucose for its energy production under physiological conditions. Glucose oxidation normally accounts for almost all of the oxygen consumed by the brain (Sokoloff *et al.*, 1989), and the brain respiratory quotient approaches 1.0 (McCall, 1993). The brain cannot synthesize glucose and it can store only a few minutes' supply as glycogen (McCall, 1993). Therefore, it depends on the circulation for its minute to minute supply of metabolic fuel. Furthermore, the brain cannot quickly increase its extraction of glucose. Normally, the rate of carrier-mediated (GLUT1) facilitated glucose transport across the blood brain barrier down a concentration gradient exceeds the rate of brain glucose metabolism, therefore, transport is not rate limiting. However, if the plasma glucose concentration falls below a critical level (or if brain glucose metabolism increases substantially) glucose transport from blood to brain becomes rate-limiting to brain glucose metabolism and, thus, brain function and survival (McCall, 1993). Hypoglycemia, sensed in the brain itself (Biggers *et. al.*, 1989) and in peripheral structures such as the liver (Donovan *et. al.*, 1991), triggers a series of central nervous system (CNS) mediated changes (Schwartz *et. al.*, 1987; Mitrakou 1991; Cryer, 1993). These include, but are not limited to, changes in hormone secretion, symptoms, cognitive dysfunction, coma and, ultimately, death. Given the survival value of maintenance of the plasma glucose concentration, it is hardly surprising that physiological mechanisms that very effectively prevent or correct hypoglycemia (Cryer, 1993) have evolved. The counterregulatory hormones are vital defence components that prevent life threatening hypoglycaemia which paradoxically occurs as a side effect or complication of insulin replacement therapy for insulin dependent diabetes mellitus (IDDM). Decrements in plasma glucose within the physiological range decrease insulin secretion (Schwartz *et. al.*, 1987, Cryer, 1993). Glucose decrements just below the physiological range increase the secretion of glucose counterregulatory hormones (Schwartz *et. al.*, 1987; Mitrakou, 1991). Further glucose decrements elicit symptoms of hypoglycemia (Schwartz *et. al.*, 1987; Mitrakou, 1991), while even further decrements cause cognitive dysfunction (Mitrakou, 1991). However, these glycemic thresholds are dynamic rather than static.

Plasma levels of glucagon (Gerich *et. al.*, 1974), catecholamines (Vendsalau, 1960), growth hormone (Roth *et. al.*, 1963), and cortisol (Greenwood *et. al.*, 1966) increase in response to hypoglycemia in man. Although each of these counterregulatory hormones has metabolic actions which may potentially reverse hypoglycemia, their individual contribution to the restoration of normoglycemia is unequal. For instance, Garber *et. al.*, (1976) using a sensitive isotope derivative method, found that early increments in plasma catecholamine concentrations during insulin-induced hypoglycemia in human subjects preceded the major compensatory changes in glucose fluxes raising the possibility that adrenergic mechanisms may be important in initiating glucose counterregulation. However, apparently normal glucose counterregulation has been observed in catecholamine- deficient patients with spinal cord transections (Brodows *et. al.*, 1976; Palmer *et. al.*, 1976), epinephrine-deficient adrenalectomized patients (Ensinck *et. al.*, 1976; Gerich *et. al.*, 1979), and normal subjects during the infusion of α or β-adrenergic blocking agents (Walter *et. al.*, 1974; Clarke *et. al.*, 1979). It has therefore been suggested that factors other than catecholamines must be capable of restoring normoglycemia. It is commonly thought that glucagon, growth hormone, and cortisol also play some role in acute glucose counterregulation. The administration of somatostatin, an inhibitor of glucagon and growth hormone secretion, impairs but does not prevent recovery of plasma glucose from hypoglycemia in baboons and normal man (Gerich *et. al.*, 1979; Christensen *et. al.*, 1975). However, somatostatin infusion did prevent recovery of plasma glucose from hypoglycemia in dexamethasone-treated adrenalectomized patients (Gerich *et. al.*, 1979). Although chronic growth hormone and cortisol excess may cause resistance to the actions of insulin (Luft *et. al.*, 1967; Cahill *et. al.*, 1971), the importance of acute changes in growth hormone and cortisol secretion in counteracting the acute hypoglycemic action of insulin can be questioned because normal plasma glucose recovery from insulin-induced hypoglycemia has been observed under conditions in which acute release of these hormones was not possible (Ensinck *et. al.*, 1976; Gerich *et. al.*, 1979; Feldman *et. al.*, 1975). It has since been shown that it is glucagon that plays the primary role in counterregulation and that adrenergic mechanisms, through adrenomedullary epinephrine, play a secondary role by partially compensating for glucagon lack (Gerich *et al.*, 1979). This was demonstrated by Rizza *et. al.* (1979) when they showed restoration of normoglycemia after insulin-induced hypoglycemia is primarily due to a compensatory increase in glucose production where there was intact glucagon secretion, but not growth hormone secretion and that adrenergic mechanisms did not normally play

an essential role in restoring normoglycemia but only became critical to recovery from hypoglycemia when glucagon secretion was impaired. Therefore, glucose homeostasis is maintained by a hormonal network in which insulin and glucagon are the main agents. Synthesis and secretion of insulin is stimulated by increased glucose levels, particularly after feeding. Insulin release allows the quick removal of glucose from circulation by stimulating the entry of glucose into peripheral tissues, mainly in muscle and adipose tissue cells. In parallel, insulin increases energy storage by inducing glycogen synthesis in liver and muscle, and fatty acid synthesis in liver and adipose tissue (Desvergne *et. al.*, 2006). Post-prandial surges and inter-prandial declines of insulin levels tightlycontrol glucose levels such that both hypoglycemia and hyperglycemia, which are intolerable for most tissues, are prevented. One key to glucose control is the ability of pancreatic β-cells to react quickly to changes in blood glucose by altering insulin secretion. A primary effect of insulin on adipocand muscle cell function is its stimulation of glucose transport activity (Levine and Goldstein, 1955; Park *et. al.*, 1959; Crofford and Renold, 1965). Glucose is transported across the cell's plasma membrane by a facilitated diffusion process in both the basal and insulin-stimulated states (Jeanrenaud, 1968). When insulin levels are low, between meals or upon fasting, the hormone glucagon increases the hepatic production and release of glucose by increasing glycogenolysis and stimulating gluconeogenesis. The pancreas is the chief organ of these dual regulations, as it senses glucose levels and produces insulin and glucagon accordingly (Desvergne *et. al.*, 2006).

2.3 PHARMACOLOGY OF CAFFEINE

2.3.0 Introduction

CAFFEINE (1,3,7-trimethylxanthine) is the most ubiquitous member of a group of plant alkaloids found in coffee, tea, chocolate, soft drinks, energy drinks (EDs) and many over-the-counter medications. A large percentage of the human population consume caffeine chronically as a regular part of their diet (Fredholm *et. al.*, 1999). While the estimated mean consumption of caffeine in American adults is 3.0 mg/kg/day with two-thirds of it coming from coffee, in children 0.5 to 1.8 mg/kg/day of caffeine is consumed mainly in form of soft drinks 55% and chocolate products 35–40% (Fredholm *et. al.*, 1999). More recently energy drinks (EDs) such as Red Bull (80 mg of caffeine per can), have been gaining prominence as means of caffeine consumption (Finnegan, 2003). Other means by which caffeine is consumed (sometimes unknowingly), include proprietary analgesics, cold and 'flu remedies, diet pills and diuretics. For example, Anadin Extra,

a well known brand of analgesic contains 90 mg of caffeine per dose. In persons who are habitual coffee drinkers (2–3 cups/day), enough caffeine is consumed to positively affect human psychomotor and cognitive performance (James, 1997). However, high doses of caffeine (>300 mg/kg/day) are known to produce negative effects such as nervousness, anxiety, and sleep disturbance (Benowitz, 1990).

Caffeine being similar in structure to several endogenous metabolites, crosses the blood-brain barrier and the placenta, and is distributed in intracellular fluid (Arnaud, 1987). These properties allow caffeine to affect many human tissues, including the central nervous system, cardiovascular system, and smooth as well as skeletal muscle (Arnaud, 1987, Hardman *et al.*, 1996).

2.3.1 Chemistry

Caffeine, Theophylline and Theobromine are methylated xanthines which are dioxypurines structurally related to Uric acid. Caffeine is 1,3,7-trimethylxanthine; Theophylline, 1,3-dimethylxanthine and Theobromine,3,7-dimethylxanthine. The solubility of the methylxanythines is low and is much enhanced by the formation of complexes with a wide variety of compounds (Goodman and Gilman, 1996). A large number of derivatives of the methylxanthines have been prepared and examined for their ability to inhibit cyclic nucleotide Phosphodiesterases (Beavo and Reifsnyder, 1990) and to antagonize receptor-mediated actions of Adenosine (Daly, 1982; Linden, 1991).The structural formula of caffeine is shown below

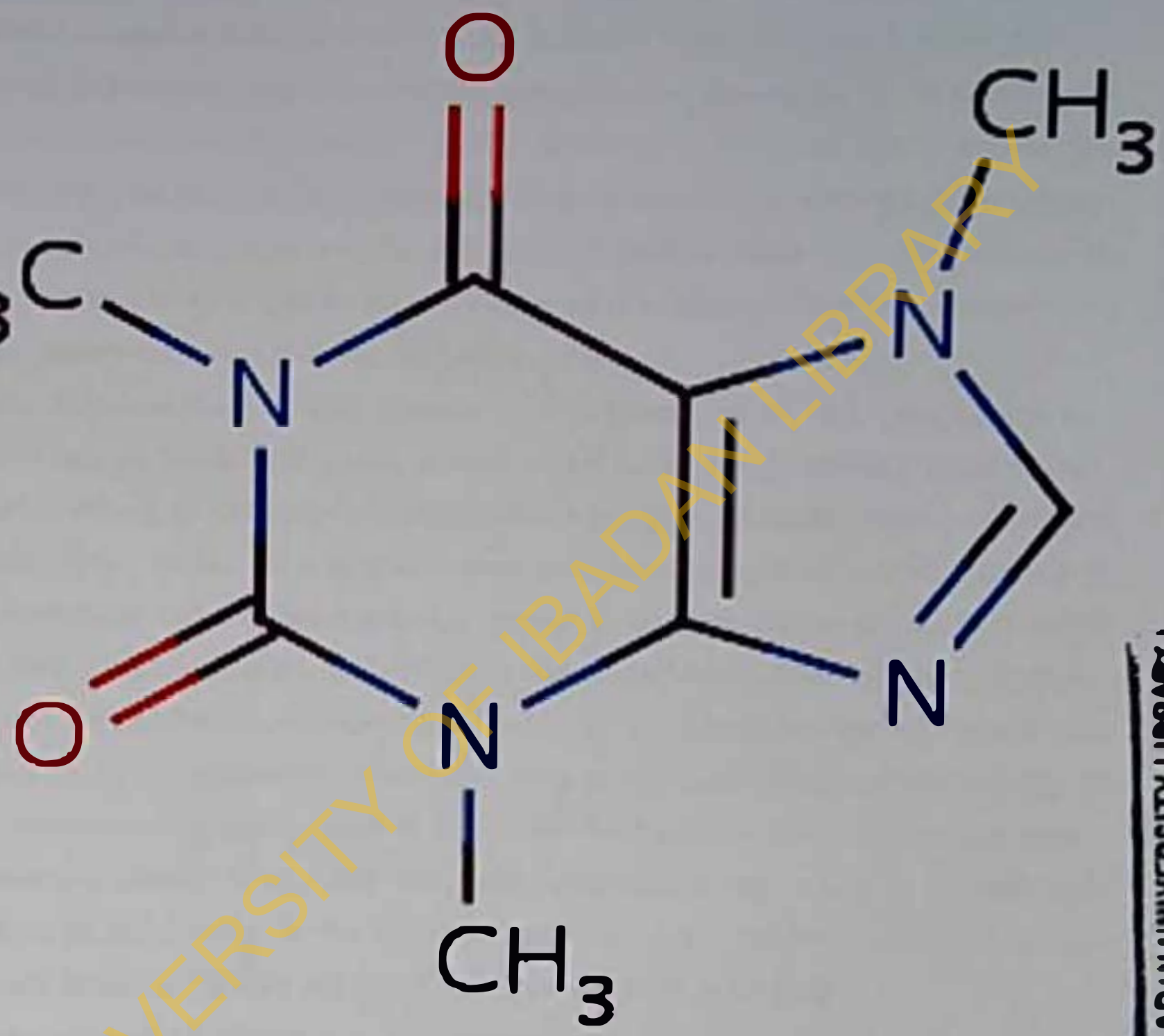


Figure 8 Structure of Caffeine (1,3,7-trimethylxanthine) modified from itech.Dickinson.edu The Role of Chemistry in History

2.3.2 Absorption and Distribution

Caffeine absorption from the gastrointestinal tract is rapid and reaches 99% within 45 minutes of ingestion (Blanchard and Sawers, 1983; Arnaud, 1993) in humans. Although it is almost completely absorbed in the pure form in animals (Arnaud, 1993) when taken as coffee the absorption is incomplete (Morgan *et. al.*, 1982). Caffeine is hydrophobic and is therefore able to pass through all biological membranes. Furthermore, there is no blood-brain barrier to caffeine in the adult or the fetal animal (Lachence *et. al.* 1983; Tanaka *et. al.*, 1984) and blood-to-plasma ratio is close to unity (McCall *et. al.* 1982), indicating limited plasma protein binding and free passage into blood cells. Similarly, there is no placental barrier to caffeine (Ikeda *et. al.* 1982; Kimmel *et. al.* 1984), thus high levels of caffeine have been observed in premature infants born to women who are heavy caffeine consumers (Khanna and Somain, 1984).

For caffeine doses lower than 10mg/kg, half life ranges from 0.7 to 1.2 hours in rats and mice, 3 to 5 hours in Monkeys (Bonati *et. al.*, 1984; 1985) and 2.5 to 4.5 hours in humans (Arnaud, 1987). While there is no difference in caffeine half-life in young and elderly humans (Blanchard and Sawers, 1983), the half-life is increased during the Neonatal period due to lower activities of some demethylation and acetylation pathways (Aranda *et. al.*, 1974; Carrier *et. al.*, 1988) and of cytochrome P-450 (Aranda *et. al.*, 1979). For these reasons, half-life of caffeine is about 100 hours in premature infants (Parsons and Neims, 1981) and 23-80 hours in the full-term newborn infants (Aranda *et. al.*, 1977; Le Guennec and Billon, 1987). As the pathways mature, the half life of caffeine decreases with postnatal age (Paire *et. al.*, 1988 and Pearlman *et. al.*, 1989). Other factors such as smoking (Murphy *et. al.*, 1988), use of oral contraceptives (Patwardhan *et. al.*, 1980) and pregnancy particularly in the last trimester (Aldridge *et. al.*, 1981; Brazier *et. al.*, 1983) reduce, increase and further increase the half life of caffeine respectively in the body.

2.3.3 Metabolism and Excretion

Studies indicate that caffeine is metabolized by the liver to form dimethyl- and monomethyl-xanthine, dimethyl and monomethyl uric acids, trimethyl- and dimethyl allantoin, and uracil derivatives (Arnaud, 1987; 1993). The dimethylation, C-8 oxidation and Uracil formation occur mostly in liver microsomes and the metabolic difference between rodents and humans is that, in the rat, 40% of the caffeine metabolites are trimethyl derivatives as compared with less than 6% in humans (Arnaud, 1985, 1993; Fredholm *et al.*, 1999). It was also shown that some metabolites of caffeine have marked pharmacological activity. Therefore, 1,3-

dimethylxanthine (theophylline) and 1,7-dimethylxanthine (paraxanthine) must be taken into account when considering the biological actions of caffeine-containing beverages (Fuhr *et al.*, 1996; Fredholm *et al.* 1999). In rodents, paraxanthine is the major metabolites in plasma (Miners and Birkett, 1996).

It has been observed that after long-term caffeine ingestion, the levels of theophylline in the brain of mice might be higher than those of caffeine during a substantial part of the day and usually higher than the levels of paraxanthine. It has been suggested that caffeine in the brain is metabolized partly via specific, local enzymatic pathways and that caffeine administration leads to high central nervous system (CNS) concentrations of Theophylline (Fredholm *et al.*,1999). It was also reported that dimethylation of caffeine to paraxanthine in rats appears to be predominantly catalysed by cytochrome P-450, whereas dimethylation to theophylline and theobromine may also take place via Flavin-containing monoxygenase (Chung and Cha, 1997). The local central nervous system concentrations of Theophylline or Paraxanthine have been observed to be higher after the ingestion of Caffeine (Benowitz *et. al.*, 1995). Fredholm *et. al.*, (1999) reported that Theophylline is three to five times more potent than Caffeine as an inhibitor of both Adenosine A1 and A2 receptors and Paraxanthine is also at least as potent as Caffeine (Fredholm *et. al.*, 1999; Benowitz *et. al.*, 1995). This then implies that the CNS effects of caffeine through adenosine receptor antagonism is dependent on its metabolism in the liver further implying that the observed differences in the effect of caffeine on various individuals may be due to differences in the ability of their livers to metabolize caffeine.

2.4 ACTIONS OF CAFFEINE ON DIFFERENT BODY SYSTEMS

2.4.1 Effects of Caffeine on the Central Nervous System

The effect of caffeine on cognitive and psychomotor function is the most extensively evaluated action largely because caffeine is consumed in many beverages and formulations for its psycho-stimulant effects. The documented effects of caffeine on the central nervous system showed a great deal of variation depending on dose of caffeine administered, the form (pure or in form of coffee or other beverages) in which it is administered and whether the experimental subjects were caffeine naive or habituated.

In single-dose experimental studies, doses as low as 1.8mg of caffeine had a significant effect compared to that of placebo (Mumford *et. al.*, 1994). Consumption of caffeine as low as ~0.2 mg/kg in the pure form improved mood and cognitive performance (Smit and Rogers, 2000), and

0.4-0.6 mg/kg as either pure caffeine or in coffee, tea, or energy drinks (EDs), increased alertness and improved mood and psychomotor performance (Quinlan *et. al.*, 2000; Smith, 2009). However, consumption of as little as 0.7-1.4 mg/kg caffeine was associated with increased anxiety, reduced psychomotor performance, and/or decreased hand steadiness in some subjects compared to placebo (Rogers *et. al.*, 2003). Adverse reactions such as anxiety (Smith *et. al.*, 2006) visual disturbances (Coren, 2002), increased hand tremor (Huma-yun *et. al.*, 1997), and impaired motor learning (Mednick *et al.*, 2008) were often reported to result from intakes of 2-3 mg/kg caffeine. However, many other studies did not display the adverse psychomotor effects from similar intakes (Smit and Rogers, 2000; Howard and Marczinski, 2010;). This wide variation in results has been attributed to polymorphisms in the genes coding for the adenosine and dopamine receptors, which mediate the effects of caffeine (Childs *et. al.*, 2008), as well as the CYP1A2 and NAT2 genes, which code for two enzymes important for caffeine metabolism.

Higher doses corresponding to ~4-7 mg/kg caused jitteriness, irritability, nausea, poor motor steadiness, palpitations, and panic attacks in individuals with psychological disorders, in addition to heightened anxiety (Rogers *et. al.*, 2008; Mastrakis *et. al.*, 2009;).

Frank life threatening toxicity have been observed with single day intakes of ~7-17 mg/kg caffeine as coffee, cola, or ED with effects including seizures, metabolic acidosis, tachycardia, and rhabdomyolysis (Trabulo *et. al.*, 2011; Monclmans *et. al.*, 2008). In an individual who ingested caffeine tablets ~143 mg/kg convulsions, cardiac arrest, and ultimately death resulted despite medical treatment (Shum *et. al.*, 1997), whereas another who ingested a similar amount had nausea, vomiting and cardiovascular collapse, but survived with medical treatment (Kapur and Smith, 2009). However, considerably lower doses ~2.4-14 mg/kg/day caffeine for one or more days, have been found to be toxic to subjects who had psychological disorders, their condition deteriorated after caffeine intake of with effects such as manic episodes, insomnia, aggression, delusions, paranoia, and seizures (Kaufman and Sachedo, 2003; Chelben *et al.*, 2008). Individuals who were not caffeine-habituated tended to have more adverse subjective effects than regular coffee consumers at given caffeine challenge dose. Objective measures (cognitive performance, BP) were similar in the two groups in some studies, but others found that habituated subjects performed better on cognitive assays, especially at the beginning of the test. Some investigators speculate that the beneficial effects of caffeine were merely a reversal of caffeine withdrawal effects such as headache, drowsiness, dysphoric mood, decreased alertness, fatigue, and flu-like

feeling (James, 1995; James and Gregg, 2004). This theory, however, was shown to be invalid, as caffeine induced similar, cognitive and psychomotor (and other) changes in habituated and non-habituated subjects (Smith *et. al.*, 2006).

The consumption of caffeine containing beverages in order to enhance alertness and ward off sleep has been justified by various studies which showed that caffeine treatment resulted in increased arousal, from intakes as low as ~0.6 mg/kg, with the response dose dependent up to ~8 mg/kg, for both habitual and non-habitual caffeine users (Rao *et. al.*, 2005; Seidl *et. al.*, 2000). Effect of caffeine intake on sleep occurred with doses ranging from ~1.4-17 mg/kg/day with effects ranging from delayed sleep onset, sleep disruption, shorter sleep duration, enhanced night time body temperature, melatonin suppression, and altered EEG activity being documented. Bonnet *et. al.* (2005) considered use of caffeine as a stimulant to be warranted, under medical supervision, when public health and safety personnel are responding to a disaster or when military personnel must engage in prolonged operations.

2.4.2 Effects of Caffeine on the Cardiovascular System

There is a dichotomous heart rate (HR) response to caffeine, with caffeine doses as low as 1 mg/kg in children and 1.4 mg/kg in adults causing slowing of the heart rate (Temple *et. al.*, 2010) while higher doses greater than 1.8 mg/kg caused increased HR. This response has been attributed to a vagally mediated bradycardia by baroreflex activation, and also a direct cardio-accelerator effect on the heart. Numerous studies show that there is tolerance to the cardiovascular effects of chronic caffeine consumption (Robertson *et. al.*, 1981) which have been attributed to upregulation of adenosine receptors (Biaggioni *et. al.*, 1991). This hypothesis seem to be supported by a study by Kennedy and Haskell, (2011)in which caffeine was found to decrease cerebral blood flow greatly in non-habitual consumers while having no significant effect on blood flow in habitual consumers. Their findings thus suggested that caffeine's effects are subject to tolerance.

A large number of epidemiologic studies have been done focusing on the relationship between dietary coffee and caffeine intake and cardiovascular function (Echeverri *et. al.*, 2010). Caffeine consumption increases blood pressure and contribute to cardiovascular mortality or morbidity (James, 2004) However, there are many inconsistencies in the results from the studies, contributing to the controversial problem of cardiovascular toxicity of caffeine (Mostofsky *et. al.*, 2014, Di Rocco *et. al.*, 2011). Case control studies have demonstrated a significant relationship between

coffee consumption and cardiovascular disease with an increased risk usually observed for an intake of five or more cups of coffee during the day (more than 5500 mg caffeine/day). Several studies demonstrated a direct effect of caffeine on the cardiovascular system, translating into an increase in the incidence of cardiac arrhythmias, increased heart rate, serum cholesterol and homocysteine, as well as triggered hypertension (Baylin *et al.*, 2006; Nawrot *et al.*, 2006). The effects of caffeine on blood pressure differ in habitual or nonhabitual caffeine drinkers, and this has been intensively investigated using different types of coffee (boiled, filtered, espresso, and decaffeinated). Caffeine intake was found to be positively associated with coronary artery disease (van Dam. 2008). Higher consumption of caffeine has also been associated with hypertension in women. (Bonita *et al.*, 2007). However, the effect on blood pressure is stronger among people who do not consume coffee on a regular basis and the pressor effect of caffeine was smaller if ingested through coffee (Nurminen *et al.*, 1999). Studies focusing on the consumption of decaffeinated coffee, tea, and non-paper-filtered coffee found no significant associations between coffee consumption and coronary heart disease (van Dam, 2008) In studies involving the consumption of decaffeinated and regular coffee intakes, the results were similar between individuals who were not usual coffee consumers and individuals that were decaffeinated-coffee consumers, thus, indicating that the blood pressure-rising effects are due to caffeine rather than other components of coffee (Bonita *et al.*, 2007). Caffeine is thought to exert this effect by adenosine receptor antagonism resulting in vasoconstriction and causing increased secretion of stress hormones in plasma, such as epinephrine, norepinephrine, and cortisol. The sympathetic nervous system is also activated by caffeine intake and can have an important role in the regulation of the cardiovascular system.

Caffeine overdose has been linked to coronary vasospasm, as well as to a variety of supraventricular and ventricular arrhythmias (Pelchovitz and Goldberger, 2011). Caffeine toxicity in cases of attempted suicide produces tachyarrhythmias, including supraventricular tachycardia, atrial fibrillation, ventricular tachycardia, and ventricular fibrillation. Risk of acute myocardial infarction in middle-aged men free from symptomatic coronary heart disease is increased by heavy consumption of caffeine-containing coffee (daily amounts exceeding 800 ml). Elevated concentrations of caffeine may have adverse local effects in the ischemic myocardium, by blocking adenosine receptors (Happonen *et al*, 2004)

2.4.3 Effects of Caffeine on the Reproductive system in Humans

Although conflicting evidence was found regarding the toxicity of caffeine to human gonads and reproduction, the weight of evidence indicates that there is a positive association with chronic caffeine intake. Epidemiological studies showed delayed conception or decreased fecundability in non-smoking men and women, and in smoking women whose only source of caffeine was coffee, with an intake of ≥300 mg/day (Jensen *et. al.*, 1998), in women with an intake of ≥500 mg/day (Bolumar *et. al.*, 1997), and in non-smoking women who consumed ≥301 mg/day. Florack *et. al.*, (1994) found that fecundability was decreased in women with an intake of 5 cups of tea/day (~4 mg/kg/day) and in men with an intake of >700 mg caffeine/day (10 mg/kg/day), but was increased in women with an intake of 400-700 mg caffeine per day. Curtis *et. al.* (1997) observed decreased fecundability among women who were coffee drinkers and among men who were heavy tea drinkers, but found no association with total caffeine intake. An intake of ≥ 1 cup coffee/day had no effect by itself, but enhanced the negative effect of alcohol on fecundability (Hakim *et. al.*, 1998).

Other caffeine associated adverse effects in women included an increased incidence of endometriosis with an intake of ≥300 mg/day (Berube *et. al.*, 1998), uterine leiomyomata in women <35 years old with an intake of ≥500 mg/day, decreased menses and cycle length with an intake of >300 mg/day (Fenster *et. al.*, 1999), increased premenstrual anxiety and mood changes with an intake of 241-320 mg/day (4-5 mg/kg/day) (Gold *et. al.*, 2007), and dystocia from a daily caffeine intake of 200-299 mg (3-5 mg/kg) (Kjaergaard *et. al.*, 2010). Several studies noted caffeine altered hormone levels in women including estradiol, progesterone, and/or sex hormone-binding globulins from an intake of 1-3 mg/kg/day (Kotsopoulos *et. al.*, 2009; Lucero *et. al.*, 2001; Fernini and Barrett-Connor, 1996).

Conversely, some studies found no relationship of caffeine intake with various reproductive parameters. Caffeine intake did not affect ovulatory disorder infertility (Chavarro *et. al.*, 2009). Kinney *et. al.* (2006; 2007) found no effect on age at menopause from an intake of >400 mg/day, and no effect on ovarian age during the reproductive years in women with an intake of ≥160 mg/day.

Several studies noted adverse reproductive/gonadal caffeine-related effects in males. An increase was seen in sperm aneuploidy for the X and Y chromosomes from an intake of ≥1 cup/day coffee (Robbins *et. al.*, 1997), sperm DNA double-strand breaks were increased with an intake of >308

mg/d caffeine (Schmid *et. al.*, 2007), and there was a weak association of "high" caffeine intake and sperm morphology alterations (Vine *et. al.*, 1997). Jensen *et. al.* (2000) observed that a high intake of cola (>14 bottles of 0.5-liter per week; ~1.4 mg/kg/day) and/or caffeine (>800 mg/day; 11 mg/kg/day) was associated with reduced sperm count. However, Kobeissi and Inhorn, (2007) concluded that caffeine intake was not an important risk factor for male infertility.

Ruder *et. al.*, (2009) concluded that exposures associated with oxidative stress and with evidence to influence the timing and maintenance of a viable pregnancy include alcohol, tobacco, and caffeine intake. Anderson *et. al.*, (2010) asserted that a person's time to pregnancy and their chance of having a healthy, live birth may be affected by factors including caffeine consumption. In a comprehensive review of caffeine effects, Nawrot *et. al.*, (2003) concluded that for healthy adults, caffeine intake up to 400 mg/d (6 mg/kg in a 65- kg person) is not associated with effects on male fertility, but recommended lower consumption for two 'at risk' subgroups: reproductive-aged women should consume ≤300 mg caffeine/day (4.6 mg/kg body weight for a 65-kg person) and children should consume ≤2.5 mg/kg body weight. Similarly, Sodeau *et. al.*, (2010) recommended that couples trying to conceive should limit their consumption to no more than 3 cups of coffee/day, despite the conflicting data of the effects of caffeine on reproduction in humans. Conversely, Peck *et. al.*, (2010) felt that the weight of evidence does not support a positive relationship between caffeine consumption and adverse reproductive or perinatal outcomes, due to the inability to rule out plausible alternative explanations for the observed associations, namely confounding by pregnancy symptoms and smoking, and by exposure measurement error.

2.4.4 Effects of Caffeine on glucose metabolism

The effect of caffeine on blood glucose remains a controversial topic of discussion amongst researchers with widely differing results observed on caffeine administration. Moreover, administration of caffeine in the pure form produces physiological effects which are at variance with those that occur on administration of substances such as coffee in which caffeine is a major constituent (Tunnicliffe and Shearer, 2008). The large numbers of studies investigating the effects of caffeine have used differing methodologies in human and animal subjects with differing routes and dosages of administration and duration of the experiments (Salahdeen and Alada 2009; Tofovic *et. al.*, 2001; Graham *et. al.*, 2001; Thong *et. al.*, 2002).

A large number of studies have reported that acute caffeine ingestion induces a decrease in insulin-mediated glucose uptake (Graham *et. al.*, 2001; Greer *et. al.*, 2001; Keijzers *et. al.*, 2002; Lee *et.*

al., 2005). It remains unknown whether this effect is mediated either entirely or in part by the small, albeit significant, increase in adrenaline concentration (0.6nM) following caffeine ingestion (Graham *et. al.*, 2001). Thong and Graham, (2002) showed that when caffeine was administered simultaneously with propranolol, the effect of caffeine was abolished suggesting that caffeine exerts its effect indirectly via adrenaline. A large body of evidence show that adrenaline, by activating the β-Adrenergic receptor, counteracts insulin stimulation of whole body glucose metabolism (Baron *et. al.*, 1987; Lankso *et. al.*,1992; Avogaro *et. al.*,1996; Asleson Jensen, 1998). Apart from this, the possibility also exists that caffeine may influence another prominent effect of Insulin, the inhibition of endogenous glucose production (EGP). Caffeine-induced increase in sympathetic activity may stimulate EGP (Clutter *et. al.*, 1980; Galster *et. al.*, 1981; Sacca *et. al.*, 1983). Thus, if the Caffeine exerts a general suppressive effect on insulin action (Battram *et. al.*, 2005), one would expect that EGP would be less inhibited by insulin i.e an increase in EGP would be observed. Caffeine has been shown to stimulate adrenaline release in vivo (Graham and Spriet, 1995; Greer *et. al.*, 1998; Thong, 2002) by increasing adrenal medullary secretion in response to direct stimulation (De Schaepdryver, *1959*) or indirectly by increasing central stimulation, causing increasing sympathetic outflow (Graham *et. al.*,2000).

Recently, Battram *et. al.*, (2007) examined the effects of caffeine and adrenaline (ADR) in concert to determine if their effects would be additive with respect to their impairment on whole body Insulin-mediated glucose disposal and therefore to elucidate the role of adrenaline in caffeine's actions. They found that the mechanisms by which CAF and ADR impair insulin actions while not being identical are likely to be closely related and share some common pathways. If they were acting via independent mechanisms a fully additive effect on whole body glucose disposal would have been observed. They have demonstrated that both caffeine and adrenaline independently elicit similar relative reductions in whole body glucose disposal in accordance with previous findings (Greer *et. al.*, 2001; Keijzers *et. al.*, 2002; Lee *et. al.*, 2005; Battram *et. al.*, 2005). While caffeine ingestion resulted in a plasma adrenaline concentration of 0.62nm, this concentration was 50% that achieved by adrenaline trial (1.2nm). Due to the fact that adrenaline impairs whole body glucose disposal in a dose-dependent manner (Deibert and Defronzo, 1980, Baron *et. al.*, 1987; Laurent *et. al.*, 1998), if adrenaline was solely responsible for caffeine's effect, one would expect a lesser response with CAF compared to ADR rather than a similar response. These findings suggest that the action of caffeine on insulin sensitivity are not solely governed by

indirect actions of adrenaline and that additional mechanisms are likely to be involved, a finding confirmed by earlier work of Battram *et. al.*, (2005).

Studies conducted on both rodents and humans have demonstrated an abolition of caffeine's effect on glucose metabolism when either the caffeine induced release of adrenaline is prevented (via adrenalectomy or due to tetraplegia) or the actions of adrenaline on Peripheral tissues is antagonized by adrenergic receptor antagonists (Strubelt, 1969; Sacco *et. al.*, 1975; Thong and Graham, 2002; Battram *et. al.*, 2007). This suggests that caffeine must elicit some adrenaline-independent effects on whole body glucose disposal.

With the present dose of caffeine, adenosine receptor antagonism is suggested to be the predominant mechanism by which caffeine elicits its effects (Fredholm, 1995). Caffeine, in addition to being an important constituent of the diet, is widely used either alone or in combination with other drugs as a therapeutic agent.

While several published studies have reported a hyperglycaemic effect of caffeine (Chenaskin & Ringsdorf, 1968; Wachman *et. al.*, 1970) and impairment of glucose tolerance in man, others have revealed either no difference between caffeine-treated subjects and controls or actual improvement in glucose tolerance (Feinberg *et. al.*, 1968; Daubresse *et. al.*, 1973). Epidemiologic studies have shown that coffee consumption can reduce the risk of type 2 diabetes (van Dam and Feskens, 2002; Tuomilehto *et. al.*, 2004). The results of most studies of the acute effects of caffeine ingestion on glucose metabolism and insulin sensitivity, as measured by using an oral-glucose-tolerance test or hyperinsulinaemic euglycemic or hypoglycemic clamp shortly after caffeine intake, are at odds with the epidemiologic study findings that long-term coffee consumption can increase insulin sensitivity and decrease diabetes risk. Lane *et al.*, (2004) cautioned that the consumption of caffeinated beverages by persons with diabetes could increase the risk of diabetes complications. This finding is consistent with findings in most studies in humans that glucose metabolism is impaired shortly after the ingestion of caffeine (Keijzers and Galan, 2002; Lane *et al.*, 2004, Pizziol *et. al.*, 1998), ground caffeinated coffee (Battram et. al., 2006), or instant caffeinated coffee (Johnston *et. al.*, 2003; Watchmann *et. al.*, 1970; Jankelson *et. al.*, 1967). A minority of these studies have found no impairment in glucose metabolism after ingestion of caffeine (Daubresse *et. al.*, 1973; Graham *et. al.*, 2000) or instant caffeinated coffee (Astrup *et. al.*, 1990; Kovacs *et. al.*, 2004).

Two human studies that distinguished between caffeinated and decaffeinated coffee suggest a possible resolution of the difference between caffeine's negative short-term effects on glucose metabolism and coffee's long-term ability to decrease diabetes risk. Naismith *et. al.* (1970) found that consumption of decaffeinated coffee for 14 days decreased blood glucose in healthy volunteers accustomed to consuming 560 mg caffeine/day. They used a crossover design without randomization, and the 20 volunteers served as their own controls. Bottram *et. al.* (2006), using a similardesign with randomization and 10 volunteers, found that the ingestion of caffeine increased plasma glucose and insulin more than did that of ground caffeinated coffee and also that the ingestion of ground caffeinated coffee increased plasma glucose more than did that of ground decaffeinated coffee, which actually decreased plasma glucose. Their findings suggest that there are non-caffeine compounds in coffee that counteract caffeine's acute impairment of glucose metabolism and hence contribute to the ability of long-term consumption of ground coffee to enhance glucose tolerance and insulin sensitivity. The findings of Bottram *et. al.*, (2006) also suggested that ground decaffeinated coffee has stronger potential than does ground caffeinated coffee to enhance insulin sensitivity and reduce diabetes risk over the long term. It is possible that tolerance develops to caffeine's impairment of glucose metabolism, although no evidence of this exists as yet in humans (Keijzers and Galan, 2002). In a randomized controlled trial, van Dam *et. al.*, (2004) found that such tolerance did not develop in a period of 4 weeks. Choi *et. al.*, (2002) found that rats fed caffeinated cola for 28 wk had insulin sensitivity significantly higher than that of controls, which suggested that such tolerance does develop in rats. Petrie *et al.*, (2004) and Robinson *et. al.*, (2004) found evidence that, when tolerance does develop in humans, it is reversed by ≤18 h of abstinence.

Evidences suggest that caffeine induces a decrease in insulin sensitivity primarily by diminishing the glucose uptake in skeletal muscle. Caffeine ingestion has been shown to increase glucose uptake in the liver in dogs (Pencek *et. al.*, 2004), and to increase it in dog skeletal muscle (Salahdeen and Alada, 2009) and decrease it in human skeletal muscle (Thong *et. al.*, 2002). Because caffeine is known to antagonize adenosine receptors, and adenosine is known to facilitate the action of insulin on the glucose uptake by adipocytes, it is possible that adenosine-receptor blockade is a key mechanism by which caffeine intake decreases insulin sensitivity (Wynne *et. al.*, 2004).

Two human studies that distinguished between caffeinated and decaffeinated coffee suggest a possible resolution of the difference between caffeine's negative short-term effects on glucose metabolism and coffee's long-term ability to decrease diabetes risk. Naismith *et. al* (1970) found that consumption of decaffeinated coffee for 14 days decreased blood glucose in healthy volunteers accustomed to consuming 560 mg caffeine/day. They used a crossover design without randomization, and the 20 volunteers served as their own controls. Battram *et. al* (2006), using a similardesign with randomization and 10 volunteers, found that the ingestion of caffeine increased plasma glucose and insulin more than did that of ground caffeinated coffee and also that the ingestion of ground caffeinated coffee increased plasma glucose more than did that of ground decaffeinated coffee, which actually decreased plasma glucose. Their findings suggest that there are non-caffeine compounds in coffee that counteract caffeine's acute impairment of glucose metabolism and hence contribute to the ability of long-term consumption of ground coffee to enhance glucose tolerance and insulin sensitivity. The findings of Battram *et. al.*, (2006) also suggested that ground decaffeinated coffee has stronger potential than does ground caffeinated coffee to enhance insulin sensitivity and reduce diabetes risk over the long term. It is possible that tolerance develops to caffeine's impairment of glucose metabolism, although no evidence of this exists as yet in humans (Keijzers and Galan, 2002). In a randomized controlled trial, van Dam *et. al.*, (2004) found that such tolerance did not develop in a period of 4 weeks. Choi *et. al.*, (2002) found that rats fed caffeinated coin for 28 wk had insulin sensitivity significantly higher than that of controls, which suggested that such tolerance does develop in rats. Petrie *et. al.*, (2004) and Robinson *et. al.*, (2004) found evidence that, when tolerance does develop in humans, it is reversed by ≤48 h of abstinence.

Evidences suggest that caffeine induces a decrease in insulin sensitivity primarily by diminishing the glucose uptake in skeletal muscle. Caffeine ingestion has been shown to increase glucose uptake in the liver in dogs (Pencek *et. al.*, 2004), and to increase it in dog skeletal muscle (Salahdeen and Alada, 2009) and decrease it in human skeletal muscle (Thong *et. al.*, 2002). Because caffeine is known to antagonize adenosine receptors, and adenosine is known to facilitate the action of insulin on the glucose uptake by adipocytes, it is possible that adenosine-receptor blockade is a key mechanism by which caffeine intake decreases insulin sensitivity (Wynne *et. al.*, 2004).

Evidences also suggest that caffeine acutely inhibits insulin sensitivity and glucose tolerance primarily by increasing epinephrine in humans. First, there are reports that caffeine increases circulating concentrations of epinephrine (Benowitz *et. al.*, 1995; Graham *et. al.*, 2000; Keijzers and Galan, 2002; Thong and Graham, 2002) and simultaneously decreases insulin sensitivity (Benowitz *et. al.*, 1995; Kijzers and Galan, 2002) in human subjects. Secondly, some studies in humans showed that adrenalin activates the β-adrenergic receptor and decreases insulin stimulation of whole-body glucose metabolism (Avogaro *et. al.*, 1996; Laaksо *et. al.*, 1992). Thirdly, reports exist that caffeine ingestion in humans with impaired epinephrine response has no effect on glucoseor insulin concentrations (van Soren *et. al.*, 1996). Fourth, Keijzers and De Galan, (2002) found that ingestion of caffeine by human subjects increased epinephrine and decreased insulin sensitivity, whereas ingestion of dipyridamole, an adenosine reuptake inhibitor, did not induce either of these changes. Fifth, Thong and Graham, (2002) found that insulin sensitivity in human subjects was reduced and plasma concentrations of insulin were increased when caffeine was administered alone but not when caffeine was administered together with propranolol, a nonselective β-adrenergic receptor blocker.

Vergauwen *et. al.*, (1997) found that adenosine receptor antagonism by caffeine sumulated rather than inhibited net glycogenolysis in a contracting isolated rat hindlimb perfusion. This study demonstrated that adenosine inhibits glycogenolysis in contracting oxidative muscle fibers and may be a potential modulator of carbohydrate metabolism. Furthermore, Raguso *et. al.*, (1996) used stable isotopes and indirect calorimetry to determine the effect of theophylline on substrate metabolism during 30 min of moderate submaximal exercise.

Three possible mechanisms through which the methylxanthines may exert their metabolic effects include increased intracellular Ca^{2+} release (Bianchi, 1961), inhibition of cAMP phosphodiesterase (Butcherand Sutherland, 1962), and antagonism of adenosine receptors (Sattin and Rall, 1970). It is now established that adenosine receptor antagonism is the most relevant mechanism in vivo (Fredholm, 1985) because pharmacological doses of methylxanthines (mM) rather than physiological doses (mM) are needed to elicit a Ca^{2+} or phosphodiesterase inhibition effect (Smellie *et. al.*, 1979). However recent findings tend to contradict these assertions, although a functional role for adenosine has been shown in isolated adipocytes (Joost *et al.*, 1986, Kurods *et al.*, 1987), cardiac muscle (Law *et. al.*, 1988)), and rodent skeletal muscle (Vergauwen *et. al.*, 1994, Han *et al.*, 1998), its role in insulin regulation of glucose transport in humans is unclear

since whole body insulin sensitivity (Keijzers *et. al., 2002*) and glucose uptake in the forearm (Natali *et. al., 1994*) were unaffected by infusion of dipyridamole (an adenosine reuptake inhibitor) and adenosine, respectively. Moreover, the presence in human skeletal muscle of the adenosine A_1 receptor, which is proposed to mediate adenosine interaction with insulin, is uncertain (Lynge *et. al., 2000*). Thong and Graham, (2002) therefore hypothesized that reduction in insulin action in vivo after caffeine ingestion was mediated by elevated epinephrine levels, and not by adenosine receptor antagonism. They administered caffeine in the presence and absence of a β-adrenergic receptor blocker, propranolol and observed that despite similar levels of epinephrine and caffeine, insulin and C-peptide concentrations were comparable to those observed in placebo in response to an oral glucose load, suggesting that the greater insulin response to caffeine was indeed secondary to elevated epinephrine levels. A large body of evidence indicates that elevated epinephrine levels acting via β-adrenergic receptors selectively induce whole body insulin resistance by transiently increasing hepatic glucose production and impairing glucose clearance by skeletal muscle (Deibert and DeFronzo, 1980; James *et. al.*, 1986; Baron *et. al.*, 1987; Lonkso *et. al.*, 1992; Avogaro *et. al.*, 1996). It has also been found that epinephrine enhances β-cell responsitivity to glucose during an intravenous glucose tolerance test (Avogaro *et. al.*, 1996). Thong and Graham, (2002) thus proposed that enhanced insulin response with caffeine ingestion was likely due to epinephrine's opposing actions on insulin-stimulated glucose clearance by peripheral tissues and, possibly, an increase in hepatic glucose output, in an effort to maintain glucose homeostasis. The data from the studies of Thong and Graham, (2002) did not support the notion that the insulin antagonistic effects of caffeine in vivo are mediated by adenosine receptor antagonism in skeletal muscle Instead, findings from the study and previous (Thong *et. al., 2002*) studies suggest that the negative effects associated with caffeine ingestion on insulin action are coupled to increased epinephrine production and its subsequent inhibition of insulin-mediated glucose uptake in skeletal muscle.

Studies on the effect of caffeine on glucose are many and varied. The following are just a few of such studies.

Greer *et. al.*, (2001) examined the effect of caffeine on whole-body glucose disposal in humans using a hyperinsulinemic-euglycemic clamp technique. The most significant finding was a 24% decrease in glucose uptake and a 35% decrease in carbohydrate storage after caffeine ingestion compared with placebo ingestion. Caffeine ingestion also resulted in higher plasma epinephrine levels than placebo ingestion ($P < 0.05$). The data thus supported their hypothesis that caffeine

ingestion would decrease glucose disposal and suggest that adenosine plays a role in regulating glucose disposal in resting humans

Graham *et. al.*, (2001) found that caffeine ingestion resulted in an increase ($P \leq 0.05$) in serum fatty acids, glycerol, and plasma epinephrine prior to the OGTT. During the OGTT, these parameters decreased to placebo levels. With caffeine the serum insulin and C- peptide concentrations were increased ($P \leq 0.001$) for the last 90 min of the OGTT and AUC were 60 and 37% greater ($P \leq 0.001$), respectively. However, the prolonged increase in insulin did not decrease blood glucose; in fact, the AUC for blood glucose was 24% greater ($P = 0.20$) in the caffeine group suggesting that caffeine ingestion may have resulted in insulin resistance.

During n 100-min euglycemic-hyperinsulinemic (100 microU/mL) clamp, whole-body glucose disposal was reduced ($P < 0.05$) in caffeine vs. placebo; the AUC over 100 min for insulin-stimulated glucose uptake in caffeine was reduced ~50% in rested and exer-cised muscle. Caffeine also reduced glycogen synthase activity before and during insulin infusion in both legs. Exercise increased insulin sensitivity of leg glucose uptake in both caffeine and placebo. Caffeine did not alter insulin sigoaliog in either leg. Plasma epinephrine and muscle cAMP coocentrations were increased with caffeine (Thong *et. al.*, 2002).

When overweight subjects were given caffeine a greater ($P \leq 0.05$) OGTT insulin response and a lower ($P < = 0.05$) insulin seasitivity mdex both before and after weight loss were observed. The proinsulin-insulin ratio indicated that neither weight loss nor caffeine affected the nature of the beta cell secretion of insulin. Thus, a nutrition and exercise intervention improved, whereas caffeine ingestion impaired, insulin-glucose homeostasis in obese men (Petrie *et. al.*, 2004).

Caffeine significantly increased scrum insulin, proinsulm, and C-peptide during the OGTT (75 g CHO) relative to placebo. Insulin AUC was 25% greater after caffeine than placebo even in the face of increased blood glucose in the caffeine trial. After caffeine ingestion, blood glucose remained elevated at 3 hr post-glucose load (8.9 ± 0.7 mmol/L) vs. baseline (6.7 ± 0.4 mmol/L). The insulin seasitivity index was lower (14%) aftercaffeine than after placebo ingestion (Robinson *et. al.*, 2004).

Caffeine increased glucose and insulin during the mixed-meal tolerance test (MMTT); no effect on the fasting levels of plasma, glucose, or insulin compared to placebo; the 2 hr AUC values

showed significant caffeine effects for both plasma glucose (P<0.04) and plasma insulin (P<0.01) responses to the MMTT. Lane *et al* 2004

Battram *et. al.* (2006) found that AUC for glucose and insulin were higher (P≤0.05) after ingestion of caffeine capsules than placebo or decaf during an oral glucose tolerance test (OGTT). A similar but less pronounced effect was seen after coffee intake. However, decaf coffee intake resulted in a 50% lower glucose response (P≤0.05) than placebo suggesting that the effects of pure caffeine and coffee was not identical thus providing explanation as to why acute pure caffeine ingestion impairs glucose tolerance while chronic coffee ingestion protects against type 2 diabetes.

Within the first hour of the glucose (75 g) test, glucose and insulin were higher for decaf than for placebo (P < 0.05); decaf yielded higher insulin than placebo and lower glucose and a higher insulin sensitivity index than caffeine (Greenberg *et. al.*, 2010).

2.4.5 Recent studies on effects of caffeine on glucose metabolism

Salahdeen and Alada, (2009) showed that caffeine increased glucose uptake in the canine hind limb at rest and during contraction and that the increased uptake was due to increased glucose extraction

Kolnes *et. Al.*, 2010 tested the hypothesis that caffeine and theophylline inhibit insulin-stimulated glucose uptake in skeletal muscles by incubating rat epitrochlearis muscles and soleus strips with insulin and different concentrations of caffeine and theophylline for measurement of glucose uptake, force development and PKB phosphorylation. They found that caffeine and theophylline completely blocked insulin-stimulated glucose uptake in both soleus and epitrochlearis muscles at 10 mM. Insulin-stimulated PKB Ser^{473} and Thr^{308} and GSK-3β Ser^{9} phosphorylation were also blocked by caffeine and theophylline Caffeine reduced and theophylline blocked insulin-stimulated glycogen synthase activation. Dantrolene (25 μM), a well-known inhibitor of Ca^{2+}-release, prevented caffeine-induced force development, but caffeine inhibited insulin-stimulated glucose uptake in the presence of dantrolene. Caffeine and theophylline reduced contraction-stimulated glucose uptake by about 50%, whereas contraction-stimulated glycogen breakdown was normal. They thus concluded that caffeine and theophylline block insulin-stimulated glucose uptake independently of Ca^{2+} release, and that the likely mechanism is via blockade of insulin-stimulated PI3-kinase/PKB activation. Caffeine and theophylline also reduced contraction-

stimulated glucose uptake, which occurs independently of PI3-kinase/PKB. They further hpothesised that caffeine and theophylline also inhibit glucose uptake in skeletal muscles via an additional and hitherto unknown molecule involved in GLUT4 translocation.

Egawa *et. al.*, (2011) investigated the effects of incubation with caffeine on insulin signaling in rat epitrochlearis muscle. Caffeine suppressed insulin-stimulated insulin receptor substrate (IRS)-1 Tyr612 phosphorylation in a dose- and time-depcadent manner. These responses were associated with inhibition of the insulin-stimulated phosphorylation of phosphatidylinositol 3-kinase (PI3K) Tyr458, Akt Ser473, and glycogen synthase kinase-3_ Ser9 and with inhibition of insulin-stimulated 3-*O*-methyl-D-glucose (3MG) transport but not with inhibition of the phosphorylation of insulin receptor-_ Tyrl 158/62/63. The results indicated that caffeine inhibits insulin signaling partly through the IKK/IRS-1 Ser307 pathway, via a Ca2_- and AMPK-independent mechanism in skeletal muscle serine phosphorylation; Akt; 5=-AMP-activated protein.

Sacramento *et. al.*, (2013) investigated whether the mechanism that contributes to the differences between acute and chronic caffeine effects on insulin sensitivity is mediated by altered insulin/AMPK signaling pathway in skeletal muscle. Experiments were performed in 3 months Wistar rats Glut-l expression decreased by 59.35% in high sucrose HSu animals, an effect not modified by chronic caffeine. In contrast, acute caffeine administration decreased significantly Glut4 expression at 0.5μM of caffeine ($p<0.01$). HSu and HF diets decreased significantly AMPKα1 expression by 70.45% and 33.93%. respectively. Chronic caffeine intake increased significantly AMPKα1 in HSu animals (42.54%) and in HF group AMPK α1 expression was restored to control levels. AMPK α1 activity decreased significantly in HF animals (44.74%), however chroniccaffeine intake did modify those values. In opposite, acute caffeine intake did not alter AMPK α1 expression. Insulin receptor decreased significantly in HF animals (59.43%) but chronic caffeine intake did not alter this value. Acute and chronic caffeine did not altered insulin receptor phosphor-Tyr1322 expression. In conclusion, the effect of chronic caffeine intake on insulin sensitivity is not mediated by altered insulin and/or AMPK signaling pathway. In cootrast, the effect of acute caffeine administration on insulin sensitivity seems to involve a decrease in Glut4 transporters. Sacramento *et. al.*, (2013) investigated the involvement ofadenosine receptors on insulin resistance induced by single-dose caffeine administration. They tested the effect of the administration of caffeine, and various odenosine receptor antagonists on whole-body insulin sensitivity. Acute caffeine decreased insulin sensitivity in a concentration dependcat manner an

effect that was mediated by A_1 and A_{2B} adenosine receptors. Additionally, acute caffeine administration significantly decreased Glut4, but not AMPK expression, in skeletal muscle. Based on their findings they suggested that insulin resistance induced by acute caffeine administration was mediated by A_1 and A_{2B} adenosine receptors.

Salahdeen *et al.*, (2014) evaluated the effect of chronic consumption of ethanol extract of kolanut on cardiovascular functions in rats treated over a period of six weeks. kolanut extract and caffeine reduced the contractile response to noradrenaline, and reduced the relaxation response to both acetylcholine and sodium nitroprusside. Since treatment with both kolanut extract and caffeine had similar characteristics between the two groups, they suggested that the action of kolanut extract was due to its caffeine content.

Rustenbeck *et. al.*, (2014) designed a controlled experimental study in order to confirm or refute the epidemiological evidence shows that chronic coffee consumption in humans is correlated with a lower incidence of type 2 diabetes mellitus. The animal model of type 2 diabetes used was high fat fed mice basically because of their short lifespan. Chronic coffee consumption retarded weight gain and improved glucose tolerance in a mouse model of type 2 diabetes and corresponding controls.

Coelho, (2014) in her PhD Thesis using prediabetic Wistar rats which were given to 35% of sucrose (high sucrose HSu) in drinking water over a period of 28 days, 0.5, 0.75 and 1g/l of caffeine given to control rats and in HSu model over a period of 12 weeks. Insulin sensitivity, basal glycemia, glucose tolerance, adipose tissue mass, Glut4 transporters and nitric oxide (NO) content in skeletal muscle and in the liver were determined. They found that caffeine restored insulin sensitivity and glucose tolerance in HSu rats. Caffeine (1g/l) restored Glut4 expression levels in skeletal muscle in HSu animals. Based on these results they suggested that caffeine could be used as a therapeutic tool for the treatment of prediabetes and prevention of T2DM.

Sacramento *et al.*, (2015) investigated the involvement of adenosine receptors and the mechanism behind their mediation of insulin resistance in skeletal muscle induced by single-dose caffeine administration. They tested the effect of the administration of caffeine, and various adenosine receptor antagonists on whole-body insulin sensitivity. Acute caffeine decreased insulin sensitivity in a concentration dependent manner an effect that was mediated by A_1 and

A_{2B} adenosine receptors. Additionally, acute caffeine administration significantly decreased Glut4, but not AMPK expression, in skeletal muscle. The results suggested that insulin resistance induced by acute caffeine administration was mediated by A_1 and A_{2B} adenosine receptors and that both Glut4 and NO may be downstream effectors involved in the insulin resistance induced by acute caffeine.

Kim *et al.* (2015) investigated the clinical changes induced by a high fat diet (HFD) and caffeine consumption in a rat model. The mean body weight of the HFD with caffeine (HFDC)-fed rat was decreased compared to that of the HFD-fed rat without caffeine. The levels of cholesterol, triglycerides (TGs), and free fatty acid, as well as the size of adipose tissue altered by HFD, were improved by caffeine consumpuon. caffeine might potenually inhibit HFD-induced obesity and we suggest possible biomarker candidates using MS-based metabolite profiling.

2. 5 Pharmacology of Kolanut

Cola Schott & Endl. (Sterculiaceae) is a genus of about 125 species of trees indigenous to the tropical rain-forest African region (Ratsch, 2005). Phylogenetically the genus was formerly classified in the family Malvaceae, subfamily Sterculiaiceae and was later transferred into the separate family Sterculiaceae. *Cola* is one of the largest in the family Sterculiaceae and is related to the South American genus *Theobroma*. It comprises of evergreen moderately sized trees often growing to a height of 20m with glossy ovoid leaves up to 30cm long. *Cola* species are found mostly in the relatively dry parts of the rain forest, although *Cola millenii* and *Cola gigantea* are widely distributed in wet and dry forest environments (Kuoame and Saconde, 2006; Olorode, 1984). Chevalier and Perrot, (1911) created the Subgenus *Eucola* containing five species of edible kolanuts – *Cola nitida* (important for trade), *Cola acuminata* (important for socio-cultural values), *Cola ballayi*, *Cola verticillata* and *Cola sphaerocarpa*. However, of these five, only Cola nitida and cola acuminata are known to be cultivated.

The mature fruit of *Cola* species is a nut known as kolanut (Duke, 2001). It has a bitter flavour and high caffeine content (Blades, 2000; Benjamin *et al.*, 1991). It is chewed in many West African cultures individually or in a group setting. It is often used ceremonially, presented to tribal chiefs or to guests. Chewing kolanut is thought to ease hunger pangs, and have sumulant and euphoriant

qualities effects similar to other xanthine containing herbs like cocoa and tea. However, the effects are distinctively different, producing a stronger state of euphoria and well being (Benjamin *et. al.*, 1991). They have stimulant effects on the central nervous system and heart and are widely consumed among Muslims as a substitute for alcoholic beverages which are prohibited. Kolanuts are used as a source of alkaloids in pharmaceutical preparations (Newall *et. al.*, 1996; Opeke, 1992).

The seed of kola comprises 13.5% water, 9.5% crude protein, 1.4% fat, 45% sugar and starch, 7.0% cellulose, 3.8% tannin and 3% ash. It is also rich in caffeine (2.8%) and theobromine (0.05%) (Purseglove, 1968). Chromatographic analysis of kola nuts have indicated the presence of phenolic constituents in quantities that are higher than those typical for many fruits. Whereas apples contain 0.1 – 2.0 g / 100 g fresh weight of polyphenolic compounds (van Buren, 1970), kola nuts contain an excess of 4.0 g/ 100 g fresh weight of polyphenolics. Many polyphenolic compounds are highly reactive with human body constituents and have an impact on metabolic processes. Nyamien *et. al.*, (2014) determined the polyphenol (flavonoids in particular) and caffeine content of various species of Cola nitida nuts from Côte d'Ivoire. Red Cola nitida (RCN); White Cola nitida (WCN); Purple Cola nitida (PCN) were extracted using different extraction solvents such as water, acidified water (0.01N citric acid), methanol, ethanol, acetone 60% and methanol/acetic acid 1% solution. Phytochemical screening showed that kola nuts contain antioxidants such as flavonoids, tannins and alkaloids in varying proportions according to the species. Spectrophotometric analysis revealed Phenolic contents are 26.76±0.54; 23.08±1.06 and 17.06±1.03 for WCN, RCN, and PCN respectively. Flavonoids, levels were 803.03±14.48, 697.13±12.76 and 647.76±21.16 for WCN, RCN and PCN respectively. Caffeine content determined by reverse-phase high performance liquid chromatography (HPLC) analysis indicated that concentrations are higher in RCN (10812.5±6.27 mg/kg FW). The findings suggest that kola seeds can be used as a possible source of antioxidant and caffeine for African populations and European industries.

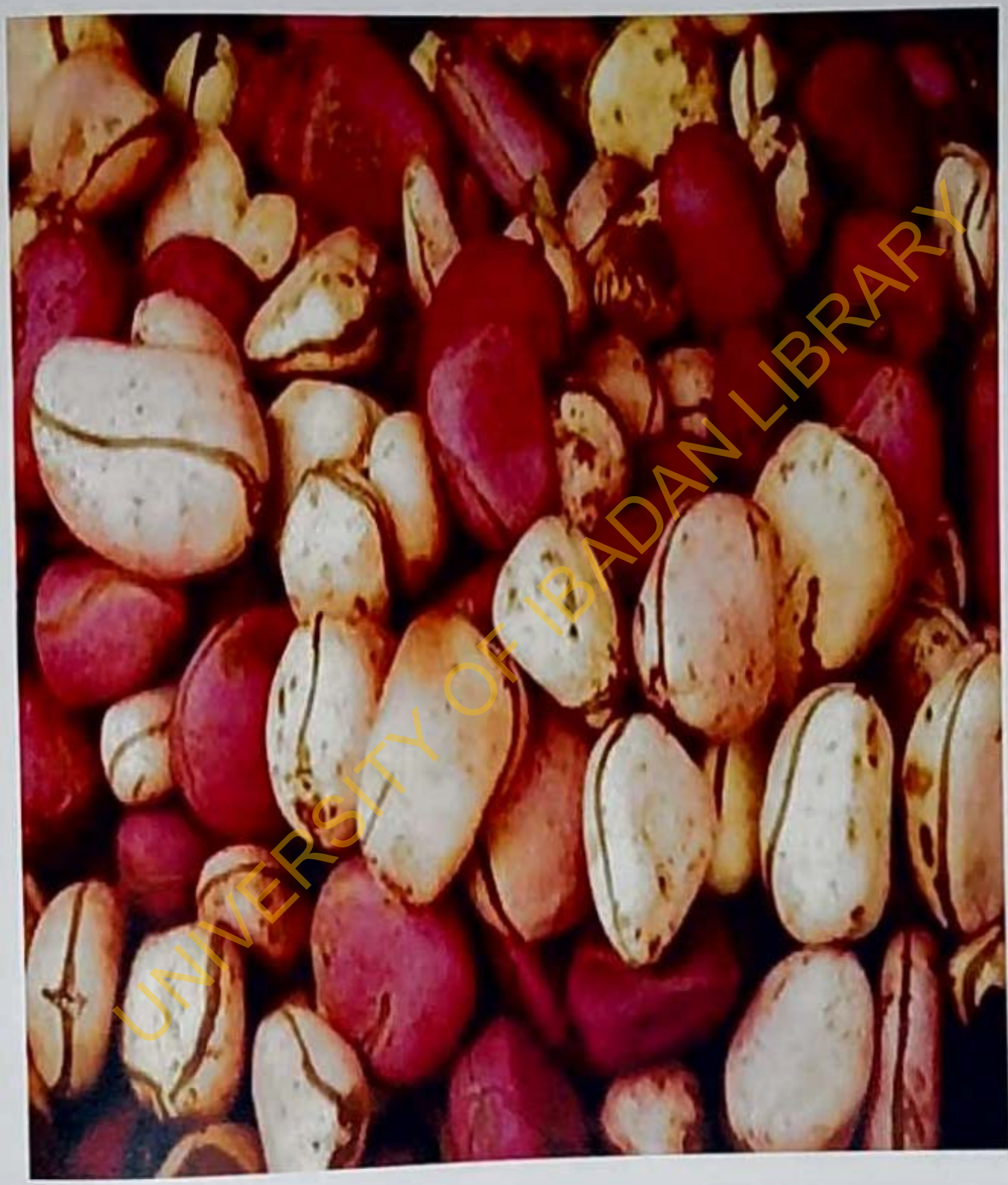


Figure 9 *Cola nitida* seeds (modified from alibaba.com)

Nyamieol *et. al.*, (2015) determined caffeine content from varieties of kola nuts (*Cola nitida*) using UV spectrophotometric method and also examined the effect of extraction solvent and solid-liquid ratio for the optimal caffeine extraction from kola nuts. Six solvents (Water, Ethanol 100%, Methanol 100%, methanol 50% in water, ethanol 50% in water and Water/Ethanol/Methanol (WEM) (2/1/1, v/v/v) and five solid/liquid (w/v) ratio (1/100, 3/100, 4/100, 5/100 and 6/100) were used to evaluate caffeine content and determine the effect of each solvent. Results showed that extraction solvent and solid/liquid ratio had significant effect on caffeine content with optimal extraction obtained with WEM solvent, solid/liquid of 3/100 and stirring at 150 trs/min applied for 20 hours. Caffeine content observed under the conditions was between 1.84% and 2.56%. The amount of caffeine extracted from kola nuts caffeine depended on the type of solvent and solid/liquid ratio used in the extraction. They recommended Kola nuts are a good source of caffeine particularly for beverage producers who are interested in other sources of caffeine other than the common Coffea canephora.

Odebunmi *et al* (2009) compared the proximate composition and the mineral content of kolmut (*Cola nitida*), bitter kola (*Garcinia cola*) and alligator pepper (*Aframomum melegueta*). The results show that *C. nitida* has the highest moisture, crude fat and crude fibre contents of 66.4, 5.71 and 7.13%, respecuvely. *A. melegueta* has non-detectable quantity of poussium and phosphorous but has the highest quantity of calcium (388 mg/Kg dry matter), magnesium (960 mg/Kg), iron (37.8 mg/Kg), zinc (32.93 mg/Kg) and manganese (68.53 mg/Kg). *C. nitida* had the highest content of potassium (3484.67 mg/Kg) and phosphorous (411.43 mg/Kg). *G. cola* also recorded the least content of all minemls except K and P which were absent in *A. melegueta*. Manganese was not detected in either of kola nut or bitter kola.

Muhammad and Fatima (2014) phytochemical analysis of red and white *Cola nitida* (kola nut) extracts were evaluated using qualitative and quantitative method. Thin layer chromatography (TLC) was used to evaluate the phenolic content. *Streptococcus anginosus* and *Pseudomonas aeruginosa*) obtained from stock cultures. Phytochemical screening revealed the presence of alkaloids, flavonoids, saponin, tannins, glycoside, steroids, volatile oil and balsams. Higher content of alkaloids, saponin and glycoside was obtained in the aqueous extract than the methanol for both red and white kola nut. The results of TLC analysis revealed the presence of quercetin and coumarin in red kola and kaemferol and coumarin in white kola respectively.

Analyses by Odebode, (1996) showed that the two major species (*acuminata and nitida*) differed markedly in the amount of total phenol and that differences also existed between different colour variants within the same species. The total phenol content was greater in *C. nitida* than *C. acuminata* . In *Cola nitida*, the quantity of total phenol in red nuts was up to three times that of white and pink nuts; but in *Cola acuminata* the difference was not significant. Investigations support the general view that *Cola nitida* is more astringent than *Cola acuminata*, because astringency is related to the phenolic content of fruits (Odebode, 1996). Atawodi *et. al.*, (1995) analysed both *C. acuminata* and *C. nitida* for their content of primary and secondary amines, and assessed for their relative methylating potential and found that seeds of both species contained high quantities of both primary and secondary amines. Methylating activity was significantly higher in kola nuts (170-490 µg/kg) than has ever been reported for a fresh plant product. The authors urge that the possible role of kola nut chewing in human cancer aetiology should be explored in countries where kola nuts are widely consumed as stimulants. Ibu *et. al.*, (1986) reported that both species of cola induced significant increases in gastric acid secretion They adviso sufferers from peptic ulcers to avoid eating kola nuts. Kola nuts are widely consumed in West Africa because they contain two alkaloids, caffeine and theobromine, which are powerful stimulants that counteract fatigue, suppress thirst and hunger, and are believed to enhance intellectual activity (Sundstrom. 1966; Nicholls, 1986).

Due to their unique bitter taste, kola nuts are effective for refreshing the mouth, and the twigs are used as "chewing sticks" to clean the teeth and gums (Lewis and Elvin-Lewis, 1985). Kola nuts are also used as a source of alkaloids in pharmaceutical preparations (Opeke, 1992). Large quantities of the nuts are exported to Europe and North America, where they are used chiefly for flavouring cola drinks such as Coca-Cola, which are refreshing or stimulating substitutes for tea or coffee (Irvine, 1956).

Socio-cultural values and uses

The Cola acuminata is more popular in the Igbo and Igedde tribes of eastern and middle regions of Nigeria, while the *cola nitida* is more common in the northern part of the country among the Hausa Fulani (Ibu *et. al.*, 1986). Chewing of kola nuts is a widespread habit in the Sub-Saharan countries of Africa, especially in northern Nigeria and Sudan. Kola chewing plays a similar social role to tea and coffee drinking or cigarette smoking in Western countries (Russell, 1955; Purseglove, 1968; Roscoparten, 1984). *C. acuminata* is widely used ceremonially and socially by

the people of West and Central Africa. At buth a kola tree may be planted for the new-born child. The child remains the lifelong owner of the tree. A kola tree is also often planted at the head of a grave as part of local death rites (Tindall, 1998). Russell, (1955) described cultural uses of kola in the Yorubaland of western Nigeria. He reported that the seed is normally kept in the house and an offering of kola forms part of the greeting to an honoured guest. The older the kola the more highly it is regarded, and white and pink nuts are kept for particularly favoured guests. The gift of kola and especially the splitting and sharing of kola nuts between two or more people signifies a special bond of friendship (Achebe 1958). Similarly, the sharing of kola nuts is a necessary prerequisite to business dealings that involve a strict etiquette in presenting, dividing, and eating of the fruits. Proposals of marriage may be made by a young man's presentation of kola nuts to the prospective bride's father and her acceptance or refusal may be conveyed by a reciprocal gift of nuts, with the meaning depending upon the quality and colour. Kola nuts presented by the bride's family signify fertility, productivity, prosperity, contentment and desire for the union (Sundstrom, 1966; Johnson and Johnson, 1976).

Kola figures prominently in religion and magic. It is used in divination and to learn the mind or intent of a god for healing the sick or against barrenness. It is often offered to Ifa along with goats and chicken in seeking the favour of the oracle. In some areas it is a component of an oath-taking process. In many societies the possession and use of kola nuts may be a symbol of wealth and prestige (Lovejoy, 1980).

Medicinal uses of kolanut

Traditionally, the leaves, twigs, flowers, fruits follicles, and the bark of both C. *nitida* and C. *acuminata* were used to prepare tonics as remedies for dysentery, coughs, diarrhoea, vomiting (Ayensu, 1978; Burkill, 1995) and chest complaints. The nuts have considerable potential for the development of new pharmaceuticals and foods (Fereday *et al.*, 1997). Extracts of *C. nitida* bark have been tested on various pathogenic bacteria (*Staphylococcus aureus*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, beta-haemolytic streptococci, *Escherichia coli* and *Neisseria gonorrhoeae)* (Ebana *et al.*, 1991), with the extracts showing inhibitory activity against all the organisms. Benie *et al.*, (1987) report that stem bark extracts of *C. nitida* inhibited the release of luteinizing hormone (LH) from rat pituitary cells and may therefore regulate gonadotropin release suggesting that it may be used as a natural fertility regulator.

Kolanuts are often used to treat whooping cough and asthma. The caffeine present acts as a bronchodilator, expanding the bronchial air passages (Jaycola, 2001; Kim, 2001). Kolanuts are also employed in the treatment of malaria and fever (Odugbemi, 2006). Experiments using animals indicate that kolanuts have analeptic and lipolytic properties and stimulate the secretion of gastric juices (Osim *et. al.*, 1991). Odugbemi, (2006) reported that the leaves of *Cola millenii* are used in the treatment of ringworm, scabies, gonorrhoea, dysentery and opthalmia.

Cola nitida has been used in folk medicine as an aphrodisiac, an appetite suppressant, to treat morning sickness, migraine headache, and indigestion (Esimone *et. al.*, 2007). It has also been applied directly to the skin to treat wounds and inflammation (Newall *et. al.*, 1996). The tree's bitter twig has been used as well, to clean the teeth and gums (Esimone *et. al.*, 2007). In Africa, duodenal and peptic ulcer is common among southern part of Africa, Burundi, Rwanda, and eastern Zaire, high land of Ethiopia, central Sudan and east Africa especially around Kilimanjaro Mountain. However, in Nigeria there is no record on the incidence of peptic ulcer, but seroprevalence of helicobacter pylori in patients with gastric and peptic ulcers was carried out in the western part of Nigeria. Of the 92% patients screened 41% represented with peptic ulcer disease. They are commonly used to counteract hunger and thirst, in some cases to control vomiting in pregnant women also as a principal stimulant to keep awake and withstand fatigue by students, drivers, and other menial workers (Chukwu *et. al.*, 2006). *Cola nitida* is not advised for individuals with stomach ulcers due both to its caffeine and its tannin content (Ibu *et. al.*, 1986, Newall *et. al.*, 1996).

2.5.1 Experimental studies on kolanut

Ajarem (1990) injected kolanut (*Cola nitida*) extract intraperitoneally (i.p) into mice and observed that while a medium dose of 5 mg/kg significantly increased the locomotor activity, a low dose (2.5 mg/kg) had no effect and a high dose (10 mg/kg) showed depressive effects on the locomotor activities. The results suggested that the Kolanut extract induced biphasic changes in the locomotor activity of mice depending on the dose and the treatment duration.

Osim *et. al.*, (1991) found that Kolanuts (*Cola Nitida* Alba) stimulated gastric acid secretion in cats to an extent that was greater than an equivalent amount of caffeine which had 42% of the potency of kola nuts in Inducing Acid Secretion. Furthermore, they found that atropine and cimetidine were effective in suppressing kolanut-Induced Acid Secretion. While this findings gave rise to the speculation that components in the kolanut other than caffeine may be the cause of the

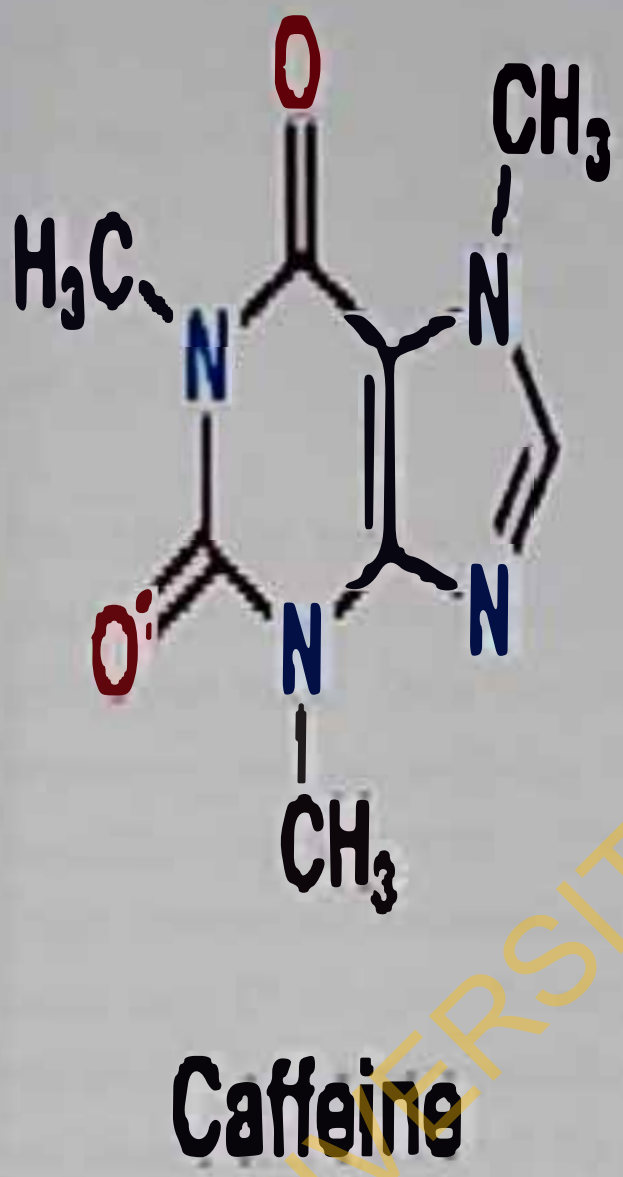
increased gastric acid secretion, they did not test the effect of decaffeinated kolanut on gastric acid secretion.

Oyedeji *et al.*, (2012) studied the effect of aqueous extract *Cola nitida* (Kola nut) on reproductive parameters of male albino rats. They found significant decrease in testosterone levels, sperm motility, sperm viability and sperm count relative to the control but there was no significant change in sperm morphology. The testicular histopathological study revealed that there was moderate to severe necrosis with the dissolution of numerous seminiferous tubules in testis.

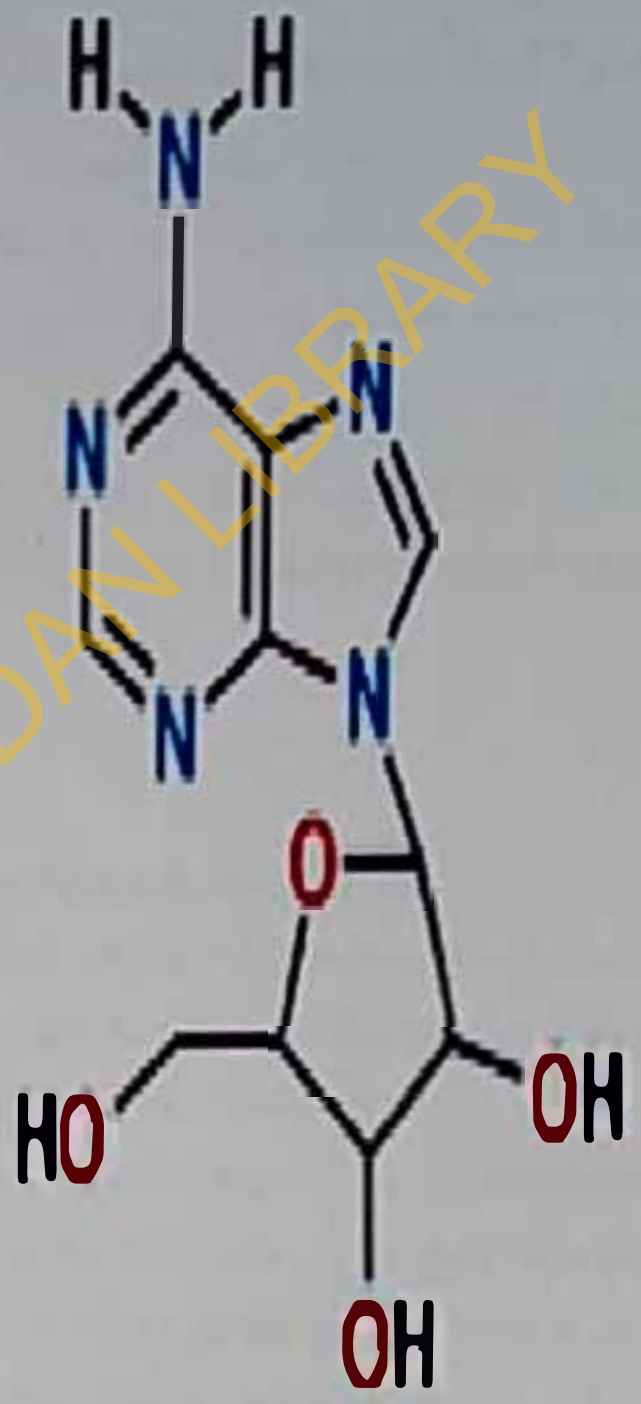
Salahdeen *et al.*, (2014) evaluated the effect of chronic consumption of ethanol extract of kolanut on cardiovascular functions in rats treated over a period of six weeks. Kolanut extract and caffeine reduced the contractile response to noradrenaline, and reduced the relaxation response to both acetylcholine and sodium nitroprusside. Since treatment with both kolanut extract and caffeine had similar characteristics between the two groups, they suggested that the action of kolanut extract was due to its caffeine content.

2.5 Adenosine and Adenosine Receptors

Adenosine is an endogenous nucleoside metabolite that is released from all tissues and cells including liver, pancreas, muscle and fat, particularly under stress, intense exercise, or during cell damage. It comprises a molecule of adenine attached by a glycosidic bound to a ribose sugar molecule. Being ubiquitous in nature, purines have evolved as important molecules for both intracellular and extracellular signaling, roles that are distinct from their activity related to energetic metabolism, as adenosine diphosphate (ADP) and adenosine triphosphate (ATP), and synthesis of nucleic acids (Khakh and Burnstock, 2009). Within the extracellular space, adenosine acts on four different G-protein coupled receptors that are classified as adenylyl cyclase inhibiting (A_1 and A_3) or adenylyl cyclase activating (A_{2A} and A_{2B}) (Tucker and Linden, 1993).

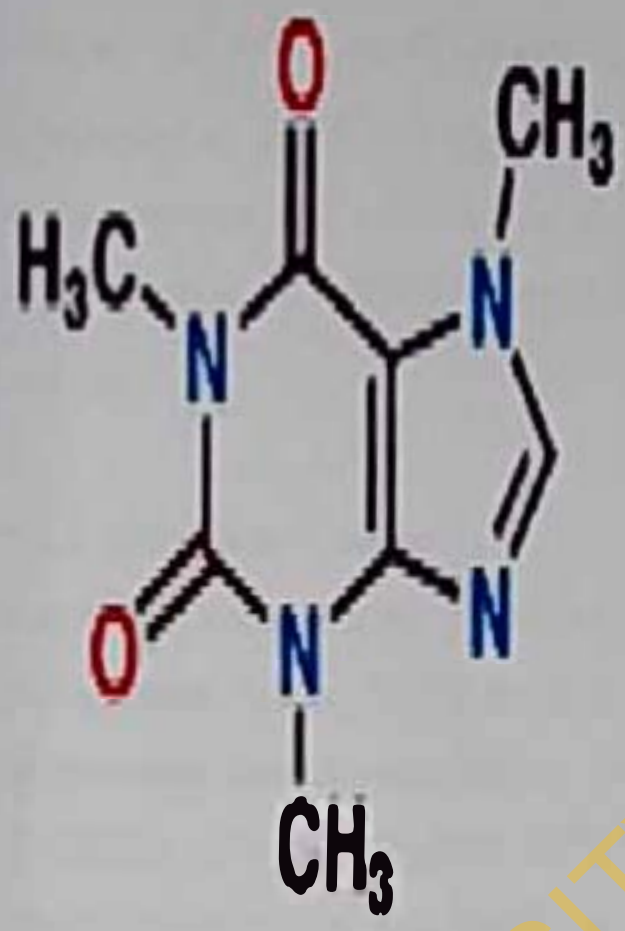


Caffeine

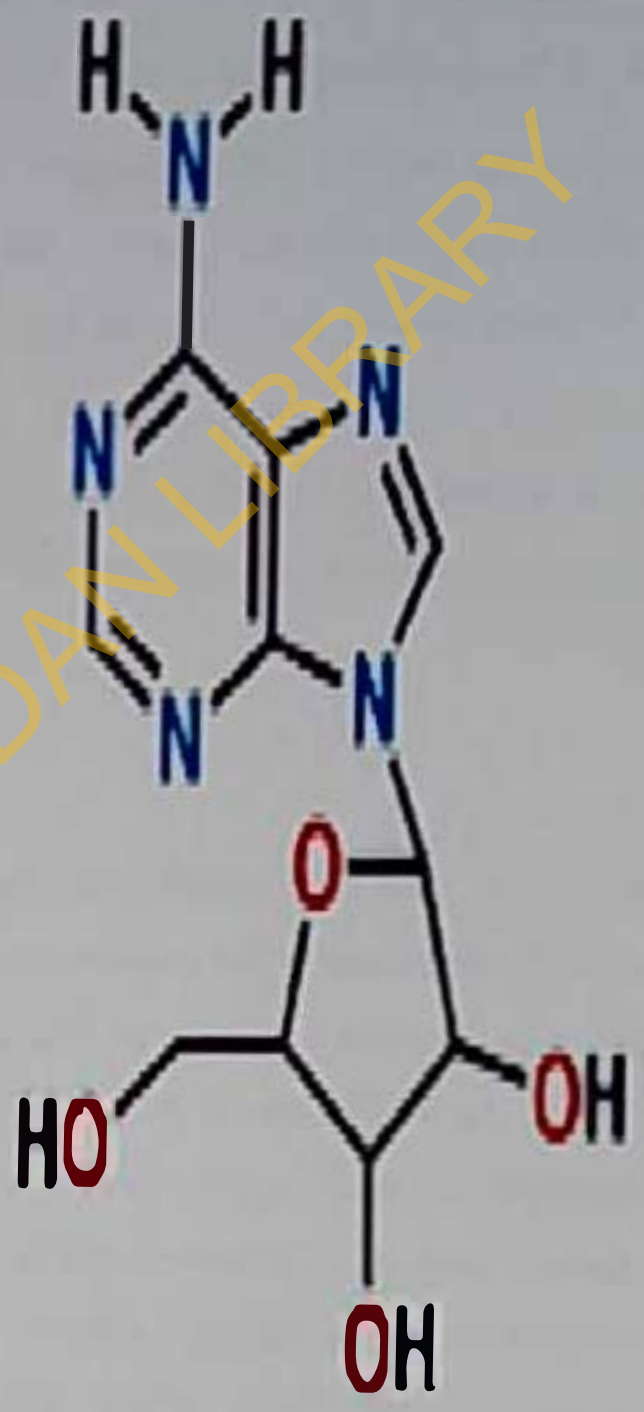


Adenosine

Figure 10. Similarity of Caffeine and Adenosine (Source Caffeine and adenosine, e.g Wikipedia commons)



Caffeine



Adenosine

Figure 10 Similarity of Caffeine and Adenosine (Source Caffeine and adenosine by Wikipedia commons)

In general, adenosine has been found to act in concert with other hormones or neurotransmitters in either an inhibitory or a stimulatory way. The myriad modulatory actions of adenosine suggest that: 1) adenosine may simultaneously produce multiple effects within the same cell; and 2) activation of A1 receptors may lead to either a decrease or an increase in the coupling of other receptors to their G proteins (Linden, 1991). Signaling through the A_1AR is also central to glucose tolerance and insulin clearance. Faulhaber-Walter *et al.*, (2011) showed that elimination of A_1AR from mice at young age (8 weeks) on standard diet leads to delayed plasma glucose and insulin clearance. The effect was sustained at older ages (20-29 weeks) on both regular and high fat diet. The overall effect of adenosine signaling through A_1AR results in improved insulin sensitivity (Vannucci *et. al.*, 1989; Vannucci *et. al.*, 1992; Green *et. al.*, 1997; Dong *et. al.*, 2001; Schoelch *et. al.*, 2004;), and pronounced reductions of plasma free fatty acids, glycerol, and triglycerides (Johansson *et. al.*, 2007). Overall, A_1AR is important for insulin sensitivity, glucose homeostasis and lipolysis.

2.6.1 Adenosine receptors

Adenosine receptors are a class of specific purinergic receptors with adenosine as the endogenous ligand. There are four adenosine receptor subtypes among vertebrates, which have been cloned and characterized to date: adenosine A1, A_{2A}, A_{2B} and A3 receptors that belong to the G-protein coupled receptors (GPCRs) family (Fredholm *et. al.*, 1994, Fredholm *et. al.*, 2001). These receptors are also known as P1 receptors (adenosine selective) distinct from P2 receptors which are ATP selective (Burnstock, 1978). Neuromodulation by adenosine is exerted through activation of high-affinity adenosine receptors (A1 and A2A) which are probably of physiological importance, and of low-affinity adenosine receptors (A2B), which might be relevant in pathological conditions. The A3R is a high-affinity receptor in humans, but it has a low density in most tissues (Ribeiro & Sebastião, 2010).

Adenosine Receptors in Organs and Tissues Involved in Glucose Metabolism

2.6.2 Adenosine receptors in the pancreas

Stimulation of the A1 receptor on the β-cell inhibits insulin secretion (Bertrand *et al.*, 1989). Using two stable P2receptor agonists, α,β-methylene ATP and ADPβS, which are more specific for the P2X and the P2Y receptor agonists, respectively, Petit and colleagues have shown that both of these receptors exist on the β-cell (Petit *et al.*, 1998). Their action is to potentiate glucose-stimulated insulin secretion. Of the purinergic receptors only the A1 has been shown to be important in pharmacological action in the β-cell, as it is antagonized by the group of compounds known as the methylxanthines. Neuromodulation by adenosine is exerted through activation of high-affinity adeoosine receptors (A1 and A2A) which are probably of physiological importance, and of low-affinity adenosine receptors (A2B), which might be relevant in pathological conditions. Campbell and Taylor, (1982) showed that adenosine and its modified analogues, 2-deoxyadenosine and N^6-phenylisopropyladenosine, strongly inhibited insulin release from rat islets, probably because of their ability to inhibit the accumulation of cyclic AMP. They proposed that the βcells, like many other tissues, may possess two different sites at which adenosine nucleosides interact to produce their biological effects. Bacher, (1982) investigated the effect of adenosine-5'-N-ethylcarboxamide, (NECA), a long-lasting adenosine derivative with pronounced vasoactivity on glucagon and insulin release from the in situ isolated blood perfused pancreas in the anesthetized dog. The found insulin release was inhibited by NECA at low concentrations, but significantly increased at higher concentrations of the adenosine analogue. Similar effects were observed with infusion of adenosine at 10^{-7} and 10^{-6} mol/l. Aminophylline (10^{-4} mol/l) produced a 10-fold attenuation of the actions of NECA. They therefore proposed that glucagon release at low concentrations of NECA and adenosine in contrast to that of insulin release at high concentrations may represent a local pancreatic regulatory mechanism of adenosine in glucose homeostasis.

Similarly, Bertrand *et al.*, (1989), used mouse islets to study the effects of adenosine and its stable analogue L-N6-phenylisopropyladenosine (L-PIA) on pancreatic beta-cell function. They found that a high concentration (500 μM), adenosine augmented glucose-induced electrical activity in beta-cells and potentiated insulin release. These effects were prevented by the inhibitor of nucleoside transport nitrobenzylthioguanosine. At a lower concentration (50 μM), adenosine caused a small and transient inhibition of glucose-induced electrical activity and insulin release. L-PIA (10 μM) slightly and transiently inhibited insulin release, 45Ca efflux and 86Rb efflux from

islet cells, and decreased electrical activity in beta-cells. When adenylate cyclase was stimulated by forskolin in the presence of 15 mM glucose, insulin release was strongly augmented. Under these conditions, L-PIA and adenosine (with nitrobenzylthioguanosine) caused a sustained inhibition. No such inhibition was observed when insulin release was potentiated by dibutyryl adenosine 3',5'-cyclic monophosphate (cAMP). These data are consistent with the existence of A1 purinergic receptors on mouse beta-cells. They could mainly serve to attenuate the amplification of insulin release brought about by agents acting via cAMP. Chapal et al studied the effects of analogue of adenosine, 5'-N ethyl carbox untido adenosine (NECA) on glucagon secretion in vitro, using the isolated pancreas of the rat perfused in the presence of glucose (2.8 mM). NECA provoked a peak of glucagon secretion, comparable to those previously obtained with adenosine. The effect was concentration-dependent and appeared at nanomolar concentrations. The EC50 was approximately 4 x 10-8 M. Theophylline (50 pM) considerably decreased the peak of glucagon secretion induced by 1.65 gM NECA and totally suppressed the effect of 16.5 nM NECA indicating the involvement of an adenosine receptor and provide evidence for an adenosine receptor of the A2-subtype being involved in glucagon secretion

2.6.3 Adenosine receptors in the skeletal muscles

All four adenosine receptors are known to be expressed in muscles, with the expression of A1AR being the highest (LaNoue and Martin, 1994; Johansson *et al.*, 2007). Since adenosine can activate A1AR with EC50 values in the range between 10 nM to 1 μM (Fredholm *et al.*, 2001; Hasko *et al.*, 2008), adenosine signaling in skeletal muscles is predominantly attributed to the A1AR. In muscle tissue activation of adenosine signaling leads to reduction of insulin sensitivity, measured by the ability of insulin to inhibit glycolysis. Insulin activation in muscle tissue is not only associated with inhibition of glycolysis but with transport of glucose and activation of glycogen synthesis. Reports show that adenosine had an effect on only glycolysis (Espinal *et al.*, 1983; Budohoski *et al.*, 1984; Challis *et al.*, 1984) and glucose transport by stimulation of the insulin sensitive glucose transporters GLUT4 (Vanoucci *et al.*, 1992); glycogen synthesis was not affected by adenosine (Challis *et al.*, 1984). In isolated soleus muscles from rats, it was found that depletion of adenosine (by adenosine deaminase) in the surrounding media improved insulin sensitivity. The observed effect on insulin sensitivity was due to decrease in the concentration of

insulin necessary to activate glycolysis (Espinal *et. al.*, 1983). Using the same model and adenosine analogs, reduction in insulin sensitivity was observed when insulin levels were reduced to half of the maximum dose required to stimulate glycolysis in the muscle (Budohoski *et. al.*, 1984). Non-specific adenosine receptor antagonists (methyl xanthines) reversed the inhibitory effect of adenosine on insulin signaling (Budohoski *et. al.*, 1984). Interestingly, A_1AR selective agonist (ARA) in the gastrocnemius muscle resulted in the amelioration of insulin sensitivity measured by improvement of glucose infusion rate and reduction of FFA levels in obese rats (Schoelch *et. al.*, 2004). The pattern observed does not contradict the earlier mentioned observations (Espinal *et al.*, 1983), as here the authors used an A_1AR specific agonist. More recent studies, using euglycemic hyperinsulinemic clamp and A_1AR selective antagonist (BWA1433, selective for A_1 at low doses) reported that adenosine signaling through A_1AR improves overall body glucose clearance in obese rats (Crist *et. al.*, 1998). In addition, with the help of radioactively labeled glucose, this group determined that there is a tissue specificity of glucose clearance under hyperinsulinemic conditions. In the gastrocnemius (fast and slow twitching fibers) and the soleus muscles (slow twitching) of rats there was an improvement of glucose uptake in obese animals after one week of treatment with A_1AR antagonist. In lean animals, however, glucose uptake under one week of treatment was slightly but significantly lowered (Crist *et. al.*, 1998). On the other hand, inhibition of A_1AR by selective antagonist has also been reported to improve overall body glucose tolerance (Xu *et. al.*, 1998). This observation contradicts the studies that have shown an improved insulin sensitivity and overall glucose clearance as a result of activation of A_1AR by specific agonists (Vannucci *et. al.*, 1989; Vannucci *et al.*, 1992; Green *et al.*, 1997; Crist *et. al.*, 1998; Schoelch *et al.*, 2004). It is possible then, that in muscle, these agonists activate the A_2 adenosine receptors, an effect that can oppose the A_1AR signaling. Overall, signaling by adenosine using pharmacological reagents improves glucoseclearance (Crist *et. al.*, 1998; Xu *et. al.*, 1998), but may result in a tissue specific insulin resistance (Crist *et. al.*, 1998).

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2.5.4 Adenosine receptors in the adipose tissue

Joost and Steinfelder, (1983) investigated the effects of theophylline on insulin receptors and insulin action in isolated rat adipocytes and found that theophylline reduced insulin binding by decreasing receptor affinity. Basal as well as insulin-stimulated glucose transport were inhibited by much smaller theophylline concentrations (0.15-0.6 mM) than those necessary to reduce insulin binding and to lower ATP levels (1-4.8 mM), or to stimulate lipolysis (0.3-2.4 mM). Insulin fully antagonized the effect of theophylline on lipolysis but failed to reverse the inhibition of glucose transport completely. Their results suggest that (a) theophylline impairs insulin action at a post-receptor level and, at higher concentrations, by a decrease of receptor binding, (b) the reduction of insulin receptor affinity probably reflects ATP depletion of the adipocyte, and (c) the xanthine inhibits glucose transport independently from its effects on lipolysis.

2.6.5 Adenosine receptors in the Liver

González-Benitez *et. al.*, (2002) identified the adenosine receptor subtypes involved in the regulation of hepatic glycogen metabolism in isolated hepatocytes by challenging them with adenosine A_1, A_{2A}, and A_3receptor-selective agonists. They found that in isolated rat hepatocytes activation of the adenosine A_1 receptor triggered Ca^{2+}-mediated glycogenolysis, activation of the adenosine A_{2A} receptor stimulated cAMP-mediated gluconeogenesis, and activation of the adenosine A_3 receptor increased cytosolic [Ca^{2+}], and decreased cAMP with minor changes in glycogen metabolism.

2.7 GLUCOSE TOLERANCE TEST

The glucose tolerance test (GTT) is a measure of the body's ability to clear a standardized glucose load from the blood. The assessment of glucose tolerance is an important part of the investigation of a variety of metabolic and endocrine disorders. The glucose tolerance test is used in clinical practice and research to identify individuals with normal or impaired glucose tolerance and patients with type 2 diabetes. It is the only means of identifying impaired glucose tolerance. There are three principal means by which the glucose load is introduced, the oral, intravenous and intra-peritoneal routes. While the first two are used in both humans and animals, the intra-peritoneal route is used exclusively in animals.

2.7.1 The Oral Glucose Tolerance Test (OGTT)

The oral glucose tolerance test (OGTT), the most commonly used method for evaluating whole body glucose tolerance, is a method in which the glucose load is introduced into the body through the mouth and therefore has to be absorbed from the intestinal tract i.e. this test also takes into account intestinal aspects of glucose absorption. Animals are made to undergo fasting for 14 to 16 hrs, a glucose solution is administered by oral gavage (Hedrich, 2004), and blood glucose along with plasma or serum insulin concentration is measured at different time points in a period of 2 to 3 hrs. Plasma glucose responses during the OGTT are the results of peripheral glucose utilization and hepatic glucose production (Mitrakou *et. al.*, 1990). Plasma glucose and insulin responses during the test are indicators of the ability of pancreatic cells to secrete insulin and the sensitivity of tissues to insulin (Reaven *et. al.*, 1993), the OGTT has also been often used to evaluate β-cell function and insulin resistance (Giorda and Appendino, 1993; Matsui *et. al.*, 1996; Huang *et. al.*, 1998;). Some epidemiological studies (Haffner *et. al.*, 1996; Haffner *et. al.*, 1997) have used fasting plasma insulin concentrations as an index of insulin resistance and the 30min ratio of changes in plasma insulin and glucose have been used as an index of β-cell function. Stumvoll *et. al.*, (2000) demonstrated that an individual's insulin sensitivity and β-cell function could be determined from BMI and values for plasma glucose and insulin obtained during an OGTT. While the Ins30/Gluc30 ratio, which is widely used as an index of β-cell function did not correlate well with actually measured cell function (Mitrakou *et. al.*, 1992; Haffner *et. al.*, 1995; Henriksen *et. al.*, 1997), fasting and 120-min plasma insulin concentrations, which are commonly used as indicators of insulin resistance, were found to correlate well with β-cell function.

In clinical practice it is usual to give the loading dose of glucose by mouth, and a precise evaluation of glucose tolerance from the blood sugar values then obtained is difficult, as absorption of glucose from the gut is not completed for a variable time. The rising and falling limbs of the curve do not, therefore, reflect the true rates of either absorption or disposal, since the two processes occur simultaneously over a period of time.

Procedures of glucose tolerance testing

In order to ensure uniformity and reproducibility of the procedure, the following guidelines must be adhered to.

(i) The subjects should fast for at least eight hours and no more than sixteen hours before commencement of testing.

(ii) Time of testing

The tests should be conducted between 7:00 am and 10:00 am to avoid circadian variation in glucose tolerance.

(iii) Size of glucose load

A standardized loading dose of glucose (1.75g/Kg) which takes into account the body weight should be given particularly in children or when performing the procedure in animals.

The area under the curve (AUC) is proportional to the total amount of drug absorbed by the body or the total amount of drug that reaches the blood circulation. The AUC during the OGTT represents glucose that comes from hepatic glucose production and used glucose.

A number of formulas for insulin sensitivity index (ISI) obtained from OGTT (ISI_{OGTT}) have been developed to assess insulin sensitivity. The product of the glucose area under the plasma glucose curve and insulin area under the plasma insulin curve has been used as an index of insulin resistance (Levine and Haft, 1970; Myllynen *et. al.*, 1987). Berson and Yalow (1959, 1960), were the first to suggest the use of the product of the area under the curves (AUCs) for glucose and insulin as an index of whole-body insulin sensitivity. More recently, Belfiore *et. al.*, (1998) proposed a hyperbolic function conversion of the product of the glucose and insulin (AUC) to derive an index of insulin sensitivity. However, the correlation between ISI_{OGTT} and those obtained from glucose clamp (ISI_{clamp}) in previous studies may not correlate well (Matsuda and DeFronzo, 1999; Stumvoll *et. al.*, 2000; Katz *et. al.*, 2000). This is because the glucose clamp study is designed for measuring peripheral glucose utilization (Bergman *et. al.*, 1985), whereas plasma glucose responses during the OGTT are the results of peripheral glucose utilization and hepatic glucose production (Mitrakou *et. al.*, 1990). The area under the glucose curve during the OGTT represents glucose that comes from hepatic glucose production and unused glucose.

In order to correct for the abnormalities, Soonthornpun *et. al.* (2003) developed a new equation. ISI_{OGTT}, [1.9/6 x body weight (kg) x fasting plasma glucose (mmol/liter) + 520 - 1.9/18 x body weight x $AUC_{glucose}$ (mmol/h.liter) – urinary glucose (mmol)/1.8] + [$AUC_{insulin}$ (pmol/h.liter) x body weight], which would represent peripheral glucose utilization only. They tested the equation with ISI_{clamp} and found that Pearson's correlation coefficient between ISI_{OGTT} and ISI_{clamp} was

0.869 ($P < 0.0001$) and concluded that ISI_{OGTT} derived from their equation was more suitable than others for assessing insulin sensitivity in subjects with normal glucose tolerance.

2.7.2 The Intravenous Glucose Tolerance Test (IVGTT)

The test consists of injecting I.V. a bolus of glucose and frequently sampling the glucose and insulin plasma concentrations afterwards, for a period of about three hours. Glucose solution (0.33 g Glucose/kg body weight) is rapidly injected (over a period less than 3 minutes) through one arm line. Blood samples are taken for analysis at intervals through the contra-lateral arm vein. Following the intravenous administration of glucose, the maximal hyperglycemia is immediate and the subsequent fall in blood glucose is not influenced by simultaneous absorption as occurs in the oral glucose tolemnce test (Duncan, 1955).

2.7.3 The Intra-peritoneal Glucose Tolerance Test (IPGTT)

The intraperitoneal glucose tolerance test (IPGTT) measures the clearance of an intraperitonally injected glucose load into peripheral tissues. The administration of glucose via an intraperitoneal injection means that there is no incretin response that is known to significantly potentiate the glucose-mediated insulin response. Compared to the oral glucose tolerance test the IPGTT does not address mechanisms participating in glucose absorption from the intestine and hence can be considered a more "artificial" test than the OGTT. This procedure is performed exactly as the OGTT, except that the 20% aqueous glucose solution is administered by i.p. injection using a 25-G × 5/8-in. needle (e.g., Terumo) and the syringes, containing the calculated volume of glucose, should be prepared for each animal with the needles already attached and voided of dead volume. Similarly to oral administration, a 2 g glucose/kg dose is used, with an i.p. injection volume of 10 µl/g body weight.

In a typical intraperitoneal glucose tolerance test, initial peak glucose values should be at least two-fold higher than the baseline value, but can be ten-fold higher or more, greatly depending on the particulars of the experimentalset-up, including the genetic background of the mice. Increased AUC in an IPGTT indicates whole-body IR, in which skeletal muscle plays a major role. A significant increase in peak glucose (715-730) may be indicative of β-cell dysfunction; simultaneous insulin measurement is useful to support this.

Homeostatic Model Assessment (HOMA)

The homeostatic model assessment (HOMA) is a mathematical model that takes into account several parameters known to effect glucose homeostasis. It relies on experimental human data, and allows for values of insulin sensitivity and β-cell function to be obtained when simultaneous fasting plasma glucose and fasting insulin concentrations are known (Manhews *et. al.*, 1985; Levy *et. al.*, 1998; Wallace *et. al.*, 2004). The solutions for the full model have highly significant correlation to euglycemic clamp results in humans (Wallace *et. al.*, 2004). Due to their nonlinear nature, they are best calculated using a computerized program available at *http://www.dtu.ox.ac.uk/*. Two simple formulas can be used to approximate the HOMA indices, one for insulin resistance (HOMA-IR) and one for β-cell function (HOMA-%B). This protocol describes these two simple formulas. It should be noted that the validation of HOMA on mice is lacking, so these indices should be used with caution until such validation becomes available.

Calculate the HOMA-IR and HOMA-%B results according to the following two equations: where FPI is the fasting plasma insulin concentration (μU/ml) and FPG is the fasting plasma glucose concentration (mg/dl).

2.8 Type 2 Diabetes Mellitus

Type 2 diabetes mellitus (T2DM) can be said to a disease in which there is a relative decrease in insulin secretion in contradistinction to type I diabetes mellitus (T1DM) in which there is an absolute decrease in insulin secretion. Whereas type 2 diabetes mellitus used to be known as adult onset diabetes and type 1 diabetes as juvenile diabetes, recent trends have given the lie to this nomenclature. T2DM now affects children and has become a major pediatric problem arising from the increasingly sedentary lifestyle of children particularly in the developed world. T2DM is the most common form of diabetes, accounting for 90% - 95% of all diagnosed cases in adults. The incidence of T2DM has reached epidemic proportions worldwide (Zimmet *et. al.*, 2001). T2DM results from the interaction between a genetic predisposition and behavioral and environmental risk factors. It is currently thought that T2DM develops only in individuals who have a genetic predisposition for defects in the pancreatic beta cells that secrete insulin (Ha[illegible], 1992). The natural course of the disease begins with normal glucose homeostasis in individuals that have the genetic predisposition, who develop insulin resistance (a state of reduced responsiveness to normal

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circulating levels of insulin), a so consequence of life style factors such as diet, obesity, or physical inactivity. In order to overcome the insulin resistance there is increased secretion of insulin by the pancreas to maintain glucose transport into cells and normal glucose levels in blood. The hyperinsulinemia causes further loss of insulin receptor sensitivity thereby worsening the insulin resistance. As a result, insulin can no longer inhibit lipolysis in the adipose tissue and levels of free fatty acids and glycerol rise in the plasma. The increase of plasma FFA results in their elevated uptake by the liver which leads to their oxidation and consequent accumulation of acetyl coenzyme A (Acetyl CoA). The elevated levels of Acetyl CoA in the liver stimulate the rate limiting enzymes for gluconeogenesis (pyruvate carboxylase and phosphoenolpyruvate carboxykinase) and glycogenolysis (glucose-6-phosphatase) leading to production of more glucose, which in turn results in the production of more insulin by the pancreas (Kovacs and Stumvoll, 2005). On the other hand, accumulation of FFA in the liver gives rise to non-alcoholic fatty liver disease. In that sense, improper glucose homeostasis can cause tissue damage and whole body deterioration (McGarry, 2002). Over time, the underlying beta-cell dysfunction appears, and insulin secretion diminishes to levels that can no longer maintain normal regulation of blood glucose in blood and tissue. Abnormally high glucose values usually appear first after meals, when carbohydrate intake challenges the glucose regulatory system. The sequence of development of these abnormalities, the causes of failure of the pancreatic beta-cell and the nature of the signals from the insulin resistant tissues that fail to induce an appropriate beta-cell response remain the subject of ongoing debate and research in both the adult and the pediatric literature. However, there is a general consensus that IR with compensatory hyperinsulinemia is the earliest abnormality with the subsequent step being impairment in insulin secretion, resulting in hyperglycemia and overt clinical diabetes (Gungor *et. al*., 2005). Although the genetic basis of type 2 diabetes has yet to be identified, there is strong evidence that such modifiable risk factors as obesity and physical inactivity are the main non genetic determinants of the disease (Manson *et al*., 1991; Stern, 1991).

Insulin controls glucose homeostasis through three coordinated mechanisms

1. suppression of hepatic glucose production (HGP),
2. stimulation of glucose uptake by the splanchnic (hepatic plus gastrointestinal) tissues
3. stimulation of glucose uptake by peripheral tissues.

Glucose uptake, in turn, depends on two major metabolic pathways: glucose oxidation and glucose storage (or nonoxidative glucose disposal). Each of these processes may be a cause of the insulin resistance in NIDDM.

HGP

In the immediate post prandial period, insulin is released into the portal vein and binds to specific receptors on the hepatocyte and suppress HGP. If this does not occur, there will be two inputs of glucose into the body (the liver and the ingested glucose from the gastrointestinal tract), and marked hyperglycemia will develop (DeFronzo *et. al.*, 2005).

Splanchnic (hepatic) glucose uptake

A second potential mechanism that might account for the impairment in insulin action during the insulin clamp is a decrease in glucose uptake by the liver

Peripheral (muscle) glucose uptake,

As a result of glucose oxidation (DeFronzo *et. al.*, 1985) and glycogen formation (Bogardus, 1984) are impaired in T2DM subjects, an excessive amount of glucose is converted to lacate (DeFronzo *et. al.*, 1985), which is subsequently released and can serve as a substrate to drive glucoocogenesis by the liver (Consoli *et. al.*, 1987). This accelerated Cori cycle activity provides an important mechanism that sustains the accelerated rate of HGP in diabetic individuals with well-established fasting hyperglycemia. The increased glucose uptake by peripheral tissues is due to the mass action effect of hyperglycemia, which passively drives glucose into cells (DeFronzo and Ferrannini, 1987; Cherrington *et. al.*, 1987).

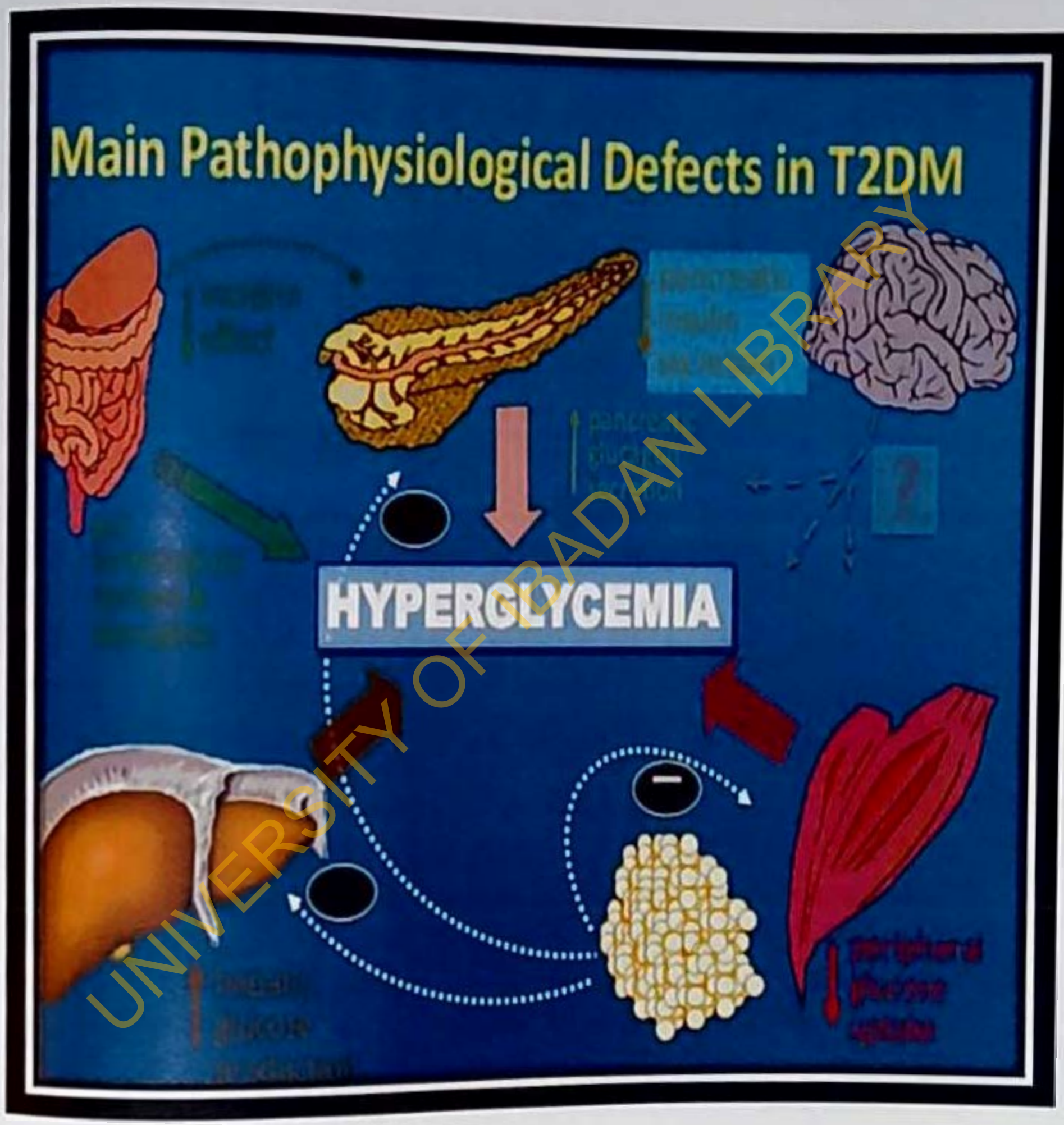

Figure 11 Pathophysiology of type 2 diabetes mellitus (Adapted from Inzucchi SF, Sherwin RS: in Cell Medicine 2011

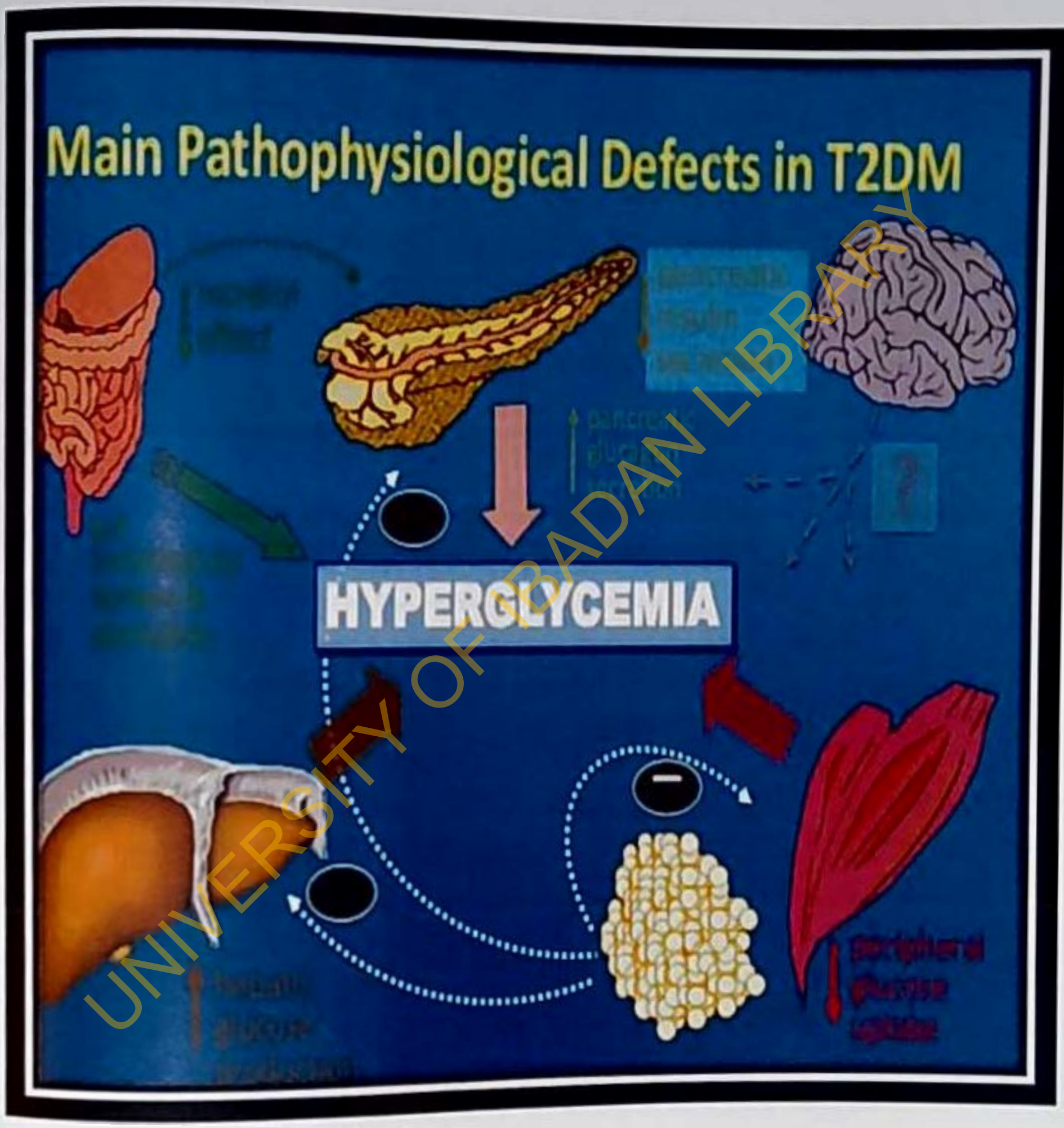

Figure 11 Pathophysiology of type 2 diabetes mellitus (Adapted from Inzucchi SF, Sherwin RS: in Cell Medicine 2011

Glucose clearance from the circulation depends on insulin and insulin receptor signaling in adipose and muscle tissue. Under certain physiological conditions such as obesity and/or type II diabetes, insulin is unable to effectively clear blood glucose. This inability of insulin to clear glucose from the circulation constitutes insulin resistance (McGarry, 2002). As a result, insulin can no longer inhibit lipolysis in the adipose tissue and levels of free fatty acids and glycerol rise in the plasma. The increase of plasma FFA results in their elevated uptake by the liver which leads to their oxidation and consequent accumulation of acetyl coenzyme A (Acetyl CoA). The elevated levels of Acetyl CoA in the liver stimulate the rate limiting enzymes for gluconeogenesis (pyruvate carboxylase and phosphoenolpyruvate carboxykinase) and glycogenolysis (glucose 6 phosphatase) leading to production of more glucose, which in turn results in the production of more insulin by the pancreas (Kovacs and Stumvoll, 2005). On the other hand, accumulation of FFA in the liver gives rise to non-alcoholic fatty liver disease. In that sense, improper glucose homeostasis can cause tissue damage and whole body deterioration (McGarry, 2002).

Tuomilehto *et. al.*, (2004) demonstrated that type 2 diabetes mellitus can be prevented by changes in the lifestyles of high-risk subjects. They found that middle-aged, overweight subjects with impaired glucose tolerance who received individualized counseling aimed at reducing weight, total intake of fat, and intake of saturated fat and increasing intake of fiber and physical activity had reduced risk of diabetes by 58 percent ($P<0.001$). The reduction in the incidence of diabetes was directly associated with the changes in lifestyle There is no doubt that the diet is an aspect of lifestyle that affects the development of type 2 diabetes mellitus. Caffeine is one of the most widely consumed components of diet which has been extensively studied. However, opinion remain divided on whether caffeine is harmful or beneficial to persons suffering from T2DM While some investigators reported that ingestion of caffeine by lean, obese, or type 2 diabetic individuals, 1-h prior to an oral glucose tolerance test (OGTT) resulted in transient glucose intolerance mainly by reduced whole body glucose predominantly at the level of the skeletal muscle (Thong *et. al.*, 2002), epidemiological studies demonstrate that chronic coffee ingestion decreases the risk of developing type 2 diabetes (Yamaji *et. al.*, 2004). Salazar-Martinez *et. al.*, (2002) reported that the consumption of approximately 6 cups of coffee per day decreased the relative risk of developing type 2 diabetes in both men and women by 54 and 29%, respectively.

CHAPTER THREE

3.0 MATERIALS AND METHODS

The following materials were used for the study

(a) Animals

(i) Male Wistar rats weighing between 235- 350g.

(ii) Male Swiss mice for the toxicity study.

(b) Drugs/kits

(i) Caffeine, 99% (Alfa Aesar USA)

(ii) 50% Dextrose solution (Dana pharmaceuticals Nigeria)

(iii) Rat insulin (ELISA) kit (Crystal Chem USA)

(iv) Glycogen Standard

(v) 30% KOH

(vi) 88% Formic acid

(vii) Cone HCl

(viii) Anthione reagent

(ix) 95% Ethanol

(x) Glycogen Synthase (ELISA) kit (Cusabio China)

(xi) Glycogen Phosphorylase (ELISA) kit (Cusabio China)

3.1 PLANT MATERIAL AND EXTRACTION.

Kolanut (Cola nitida) was obtained from Ilobu market in Osun State, Nigeria. The seeds were authenticated at the Department of Biology, Ladoke Akintola University of Technology (LAUTECH) Ogbomoso by Dr. Ogunkunle and a sample number LHO 442 was deposited in the LAUTECH herbarium. The kolanut seeds were cut into thin pieces and air dried away from direct sunlight. The dried seeds were then ground into powder and 500 gram of the powder dissolved in 2 litres of 70% ethanol for 72hrs (Murray and Hansen, 1995). The solution was filtered with 125 mm filter paper and the resulting filtrate concentrated with rotary evaporator.

Extraction Yield Fraction (E.Y.F) was calculated.

E.Y.F= weight of extract yield/ initial weight of sample $\times 100 = 58.5/500 \times 100 = 11.7\%$

3.2 PREPARATION OF DECAFFEINATED EXTRACT.

Indirect method of decaffeination was used. Briefly, 500g of the Cola nitida powder was dissolved in 2500 ml of distilled water for 72 hrs. The solution was sieved and the residue left to dry while 70% dichloromethane (DCM) was added to the solution and left for 24 hours in order to remove the caffeine from the solution. The resulting mixture was separated using a separation flask on a retort stand nod the residue in the distilled water was remixed with the initial residue and dissolved in 70% Ethanol, for 72 hrs and filtered (Murray and Hansen, 1995). The filtrate was then concentrated with the rotary evaporator as above.

3.3 TOXICITY STUDIES

Acute toxicity of the kolanut extract was evaluated using tweny-five male Swiss mice (weight range between 25 and 35 grams). Following a 24 hour fast, the mice were divided into five groups of 11 mice per group. The mice in the groups 1,2,3,4, and 5 were given 1mg/Kg, 100mg/Kg, 1000mg/Kg, 2000mg/Kg and 3000mg/Kg of the extract respectively. Observation for 72hrs after administration of the extract showed no mortality in any of the five groups.

3.4 GAS CHROMATOGRAPHY- MASS SPECTROSCOPY (GC-MS) ANALYSIS OF KOLANUT EXTRACTS

The GC-MS analysis of the crude and decaffeinated kolanut extracts was carried out using a Hewlett Packard Gas chromatograph (Model 6890 series) equipped with a flame ionisation detector and Hewlett Packard 7683 series injectors. MS transfer line temperature of 250°C. The GC was equipped with a fused silica capillary column-HP-5MS (30x0.25 mm), film thickness 1.0µm. The oven temperature was held at 50°C for 5 minutes holding times raised from 50 to 250°C at a rate of 2°C/min, employing helium gas (99.999%) as a carrier gas at a constant flow rate of 22 cm/s. 1.0mg/ml of extract (1mg dissolved in 1ml absolute alcohol) at a split ratio of 1:30 was injected. MS analysis was carried out on Agilent Technology Network Mass Spectrometer (Model 5973 series) coupled to Hewlett Packard gas chromatograph (Model 6890 series) equipped with NIST08 library software database. Mass spectra were taken at 70eV/200°C; a scanning rate of 1 scan/s. Identification of compounds was conducted using the database of NIST08 library. The mass spectra of the unknown compounds were compared to those of the known compounds stored in the software.

3.5 PREPARATION OF STOCK SOLUTION

Stock solutions of Caffeine (CAF), Kolanut (EEK) and Decaffeinated Kolanut (DEK) were prepared by dissolving 0.45g each of the CAF powder, EEK and the DEK in 100ml of distilled water. The above concentration was chosen so that no rat would have to be given more than 0.5ml of the dissolved abstract daily in order to receive 6mg/kg of the drug of extract. The dose of 6mg/kg caffeine and extracts administered corresponds to the amount of caffeine ingested in 3-4 cups of coffee which has been found to have significant effects in prevention of type 2 diabetes (Salazar-Martinez *et. al.*, 2004).

3.6 Body Weight

The rats were weighed weekly for eight weeks using an electronic digital balance. Weight gain was accessed for each rat by subtracting the initial weight from the final weight after eight weeks of drug and extract administration.

3.7 Food Intake/consumption.

All the animals in the four groups were fed ad libitum with rat pellets obtained from Ladokun feeds Nigeria limited Ibadan. Average daily food intake of the rats was determined by assessment of the weight of food consumed during a week of ad libitum feeding. The maximum average daily food intake was then increased by 50% and presented to the animals daily. Daily food intake was determined by subtracting the weight of food remaining from the weight of the food initially presented. The food intake was assessed daily for a period of six weeks commencing two weeks after commencement of the treatment.

3.8 Water Consumption/Intake

All the animals in the four groups were given water ad libitum. Average daily water intake of the rats was determined by assessment of the volume of water consumed during a week of ad libitum water consumption. The average maximum daily water consumption was then increased by 50% and presented to the animals daily. Daily water intake was determined by subtracting the volume of water remaining from the volume of water initially presented. The water intake was assessed daily for a period of six weeks commencing two weeks after commencement of the treatment.

Table 1: Experimental design of the study

Acute

Group	No of rats	Treatment
(I)Control	12	Six rats were each given 0.3 mL of distilled water by oral gavage and blood collected for oral glucose tolemnce test and determination of insulin. Another set of six rats were each given 0.3 mL of distilled water by oral gavage, sacrificed and used for liver glycogen, glycogen synthase and glycogen phosphorylase determination.
(II)Caffeine	12	Six rats were each given CAF 6 mg/kg by oral gavage and blood collected for oral glucose tolerance test and determination of insulin. Another set of six rats were each given CAF 6 mg/kg by oral gavage sacrificed and used for liver glycogen, glycogen synthase and glycogen phosphorylase determination.
(III)EEK	12	Six rats were each given EEK 6mg/kg by oral gavage and blood collected for oral glucose tolerance test and determination of insulin. Another set of six rats were each given EEK 6 mg/kg by oral gavage, sacrificed and used for liver glycogen, glycogen synthase and glycogen phosphorylase determination.
(IV)DEK	12	Six rats were each given DEK 6 mg/kg by oral gavage and blood collected for oral glucose tolerance test and determination of insulin. Another set of six rats were each given DEK 6 mg/kg by oral gavage sacrificed and used for liver glycogen, glycogen synthase and glycogen phosphorylase determination.

Table 2 Experimental design (Chronic)

Group	No of rats	Treatment
(I)Control	12	Six rats were each given 0.3 mL of distilled water by oral gavage daily for eight weeks. A day after completion of treatment, blood collected for oral glucose tolerance test and determination of insulin. Another set of six rats which had also undergone treatment with 0.3 mL distilled water for eight weeks, were used for liver glycogen, glycogen synthase and glycogen phosphorylase determination.
(II)Caffeine	12	Six rats were each given CAF 6 mg/kg by oral gavage daily for eight weeks. A day after completion of treatment, blood collected for oral glucose tolerance test and determination of insulin. Another set of six rats which had also undergone the above treatment with CAF for eight weeks, were used for liver glycogen, glycogen synthase and glycogen phosphorylase determination.
(III)EEK	12	Six rats were each given EEK 6 mg/kg by oral gavagedaily for eight weeks. A day after completion of treatment, blood collected for oral glucose tolerance test and determination of insulin. Another six rats which had also undergone the above treatment with EEK for eight weeks, were used for liver glycogen, glycogen synthase and glycogen phosphorylase determination.
(IV)DEK	12	Six rats were each given DEK 6 mg/kg by oral gavage daily for eight weeks. A day after completion of treatment, blood collected for oral glucose tolerance test and determination of insulin. Another six rats which had also undergone the above treatment with DEK for eight weeks, were used for liver glycogen, glycogen synthase and glycogen Phosphorylase determination.

3.9 EXPERIMENTAL DESIGN

Male Wistar rats weighing 235-350 g were used for the study. The study was carried out under acute and chronic conditions viz: tests carried out immediately after administration of the extracts and tests carried out after 8 weeks of administration of extracts

3.10 Experimental Procedure for the acute studies

3.10.1 Group I Control

After an overnight fast, each of the six rats was given 0.3 mL of distilled water by oral gavage. Thirty minutes after the distilled water was given, a blood sample was taken corresponding to 0minute. Thereafter, 50% glucose solution 1.75 g/kg (Perfumi *et al.*, 1991) was given to each rat and blood samples were taken at 30, 60, 90 and 120 minutes after the glucose load for glucose tolerance test and plasma insulin determination.

Another subgroup of six rats were each given 0.3 mL of distilled water by oral gavage. Thirty minutes after the distilled water was given 50% glucose solution 1.75 g/kg was given to each rat. One hour after glucose loading, the rats were sacrificed and their livers harvested for glycogen content, glycogen synthase and glycogen phosphorylase determination.

3.10.2 Group II Caffeine treated group

After an overnight fast, each of the six rats was given CAF 6 mg/kg by oral gavage. Thirty minutes after the CAF was given, a blood sample was taken corresponding to 0minute Thereafter, 50% glucose solution 1.75 g/kg (Perfumi *et al.*, 1991) was given to each rat and blood samples were taken at 30, 60, 90 and 120 minutes after the glucose load for glucose tolerance test and plasma insulin determination.

Another subgroup of six rats were each given caffeine 6 mg/kg by oral gavage. Thirty minutes after the caffeine was given 50% glucose solution 1.75 g/kg was given to each rat. One hour after glucose loading, the rats were sacrificed and their livers harvested for glycogen content, glycogen synthase and glycogen phosphorylase determination.

3.10.3 Group III Ethanol extract of kolanut (EEK) treated rats

After an overnight fast, each of the six rats was given EEK 6mg/kg by oral gavage. Thirty minutes after the EEK was given, a blood sample was taken corresponding to 0 minute. Thereafter, 50% glucose solution 1.75 g/kg (Perfumi *et al.*, 1991) was given to each rat and blood samples were

taken at 30, 60, 90 and 120minutes after the glucose load for glucose tolerance test and plasma insulin determination.

Another subgroup of six rats were each given EEK 6mg/kg by oral gavage. Thirty minutes after the EEK was given 50% glucose solution 1.75 g/kg was given to each rat. One hour after glucose loading, the rats were sacrificed and their livers harvested for glycogen content, glycogen synthase and glycogen phosphorylase determination.

3.10.4 Group IV Decaffeinated ethanol extract of kolanut (DEK) treated rats

After an overnight fast, each of the six rats was given DEK 6 mg/kg by oral gavage. Thirty minutes after the DEK was given, a blood sample was taken corresponding to 0minute. Thereafter, 50% glucose solution 1.75 g/kg (Perfumi *et al*., 1991) was given to each rat and blood samples were taken at 30, 60, 90 and 120minutes after the glucose load for glucose tolerance test and plasma insulin.

Another six rats were each given DEK 6 mg/kg by oral gavage. Thirty minutes after the DEK was given 50% glucose solution 1.75 g/kg was given to each rat. One hour after glucose loading, the rats were sacrificed and their livers harvested for glycogen content, glycogen synthase and glycogen phosphorylase determination.

3.11 Experimental Procedure for chronic studies

3.11.1 Group I Control

Six rats were each given 0.3 mL of distilled water by oral gavage daily for eight weeks. At the end of the treatment period, after an overnight fast, following light anaesthesia with thiopentone sodium 40mg/ml, a blood sample corresponding to 0 minute was taken by retro-orbital puncture from each of the six rats. Thereafter, 50% glucose solution 1.75 g/kg was given to each rat by oral gavage and blood samples were taken by retro-orbital puncture at 30, 60, 90 and 120 minutes after the glucose load for glucose tolerance test and plasma insulin determination.

Another set of six rats which had also undergone treatment with 0.3 mL of distilled water for eight weeks, were subjected to an overnight fast. On the morning of the procedure, each rat was given 50% glucose solution 1.75 g/kg by oral gavage. One hour after glucose loading, the rats were sacrificed by cervical dislocation and their livers harvested for glycogen content, glycogen synthase and glycogen phosphorylase determination.

3.11.2 Group II Caffeine treated

Six rats were each given caffeine 6 mg/kg by oral gavage daily for eight weeks. At the end of the treatment period, after an overnight fast, following light anaesthesia with thiopentone sodium 40 mg/Kg, a blood sample corresponding to 0 minute was taken by retro-orbital puncture from each of the six rats. Thereafter, 50% glucose solution 1.75g/kg was given to each rat and blood samples were taken for glucose tolerance test and plasma insulin determination at 30, 60, 90 and 120 minutes after the glucose load.

Another set of six rats which had also undergone treatment with caffeine for eight weeks, were subjected to an overnight fast. On the morning of the procedure, each rat was given 50% glucose solution 1.75 g/kg. One hour after glucose loading, the rats were sacrificed and their livers harvested for glycogen content, glycogen synthase and glycogen phosphorylase determination.

3.11.3 Group III EEK treated

Six rats were each given EEK 6 mg/kg by oral gavage daily for eight weeks. At the end of the treatment period, after an overnight fast, following light anaesthesia with thiopentone sodium 40mg/ml a blood sample corresponding to 0 minute was taken by retro-orbital puncture from each of the six rats. Thereafter, 50% glucose solution 1.75 g/kg was given to each rat and blood samples were taken at 30, 60, 90 and 120 minutes after the glucose load for glucose tolerance test and plasma insulin determination.

Another set of six rats which had also undergone treatment with EEK for eight weeks, were subjected to an overnight fast. On the morning of the procedure, each rat was given 50% glucose solution 1.75 g/kg. One hour after glucose loading, the rats were sacrificed and their livers harvested for glycogen content, glycogen synthase and glycogen phosphorylase determination.

3.11.4 Group IV DEK treated

Six rats were each given DEK 6 mg/kg by oral gavage daily for eight weeks. At the end of the treatment period, after an overnight fast, following light anaesthesia with thiopentone sodium 40mg/ml a blood sample corresponding to 0 minute was taken by retro-orbital puncture from each of the six rats. Thereafter, 50% glucose solution 1.75g/kg was given to each rat and blood samples were taken at 30, 60, 90 and 120minutes after the glucose load or glucose tolerance test and plasma insulin determination.

Another set of six rats which had also undergone treatment with DEK for eight weeks, were subjected to an overnight fast. On the morning of the procedure, each rat was given 50% glucose solution 1.75 g/kg. One hour after glucose loading, the rats were sacrificed and their livers harvested for glycogen content, glycogen synthase and glycogen phosphorylase determination.

3.12 Oral Glucose Tolerance Test (OGTT)

The procedure was carried out between the hours of 7:00 am and 10:00 am in all groups (Jarret *et al.*, 1972). Each rat was first anaesthetized with 40mg/kg thiopentone sodium intraperitoneally. Then by retro-orbital sinus puncture, a blood sample approximately 0.5ml was taken into EDTA bottles and kept in ice packs. A loading dose of 1.75g/Kg 50% glucose (Dana pharmaceuticals, Lagos) was given to each animal by oral gavage. Thereafter, blood samples were taken at 30, 60, 90 and 120 minutes after the glucose loading. Plasma was separated and stored at -20 ^{0}C after spinning the blood samples at 3000 g for 15 minutes. Plasma glucose and plasma insulin were determined for each sample. The mean values for each time point were used to plot a graph of plasma glucose concentration against time to obtain the glucose tolerance curve.

3.13 Determination of plasma glucose

After collection of the blood samples in EDTA Eppendorf bottles, the plasma was extracted after centrifuging the samples at 3000 g for 15 minutes. The plasma glucose was determined by the glucose oxidase method (Trinder, 1969).

Principles of the blood glucose determination by the glucose oxidase method.

In the presence of glucose oxidase, glucose is oxidized to gluconic acid and hydrogen peroxide. The hydrogen peroxide in the presence of peroxidase enzyme oxidizes a suitable oxygen acceptor to give chromogenic oxidation, the intensity of which is proportional to the amount of glucose initially present. The intensity of the colour developed is measured using a spectrophotometer.

1- Glucose + O_2 + Glucose Oxidase $\Longrightarrow$ Gluconic acid + H_2O_2

2- H_2O_2 + peroxidase $\Longrightarrow$ $H_2O + O_2$

3- O_2 + oxygen acceptor $\Longrightarrow$ chromogen

3.14 Determination of Plasma Insulin

The blood samples obtained during the oral glucose tolerance test were centrifuged at 3.000 revs/minute for 15 minutes after which the plasma was extracted with Pasteur pipettes into Eppendorf bottles and stored at -20°C for assay at a later date. Plasma insulin coocentrations were determined with ELISA kits specific for rodent insulin (Crystal Chem Inc USA).

Principles of the Assay

1. The insulin in the plasma sample and the prepared standards bind to the guinea pig anti-insulin antibody coated on the microplate well. The microplate was then incubated for 2 hours at 4°C Subsequent washing removes any unbound material.
2. Horse radish peroxidase-conjugated anti-insulin antibody is then bound to the guinea pig anti insulin antibody/sample insulin complex immobilized to the microplate well. A second washing is done to remove excess POD conjugate.
3. 3,3',5, 5' tetramethylbenzidine substrate solution (TMB) was added to the bound peroxidase conjugate to cause a reaction .
4. The absorbance is measured with aid of the ELISA plate reader at A_{450}-A_{630}
5. Insulin concentration is determined by reading off a curve plotted from the standard values (Figure 11).

3.15 Determination of Liver Glycogen

Liver glycogen determination was done by the Anthrone method (Jermyn, 1975). Briefly, one gram (1g) of each of the liver were carefully weighed and kept in glass flasks. To this, 10ml of 30% Potassium Hydroxide (KOH) was added in an Erlenmeyer flask and heated for 20 minutes until the tissue dissolved. After allowing the solution to cool, 4ml of it was measured into a test tube and 5ml of 95% ethanol added to it. The resulting solution was centrifuged at 3000 revolutions for 15 minutes and the supernatant discarded leaving a white precipitate. The glycogen precipitate was then dissolved in 2ml of distilled water. From the glycogen solution, 0.5ml aliquots were measured into clean sample bottles and 0.5ml of concentrated HCl, 0.5ml of formic acid (88%) and 4ml anthrone reagent were added to it in a stepwise manner. 0.5ml of distilled water treated in a similar manner as above formed the blank. A standard curve was drawn by preparing several dilutions of the glycogen standard (mg/ml) which were similarly treated as above. All the tubes were then heated in a boiling water bath for 10 minutes and then allowed to cool. Aliquots of the

samples were then poured into cuvettes after allowing bubbles to disperse and absorbance read at 630nm on a spectrophotometer against blank. The corresponding absorbance is read off standard curve constructed from values for the glycogen standard (Figure 12).

Glycogen content was calculated using the formula below

mg Glycogen/100g Fresh liver = mg glycogen/ml x 10/4 x 2/0.5 x 100/liver wet weight

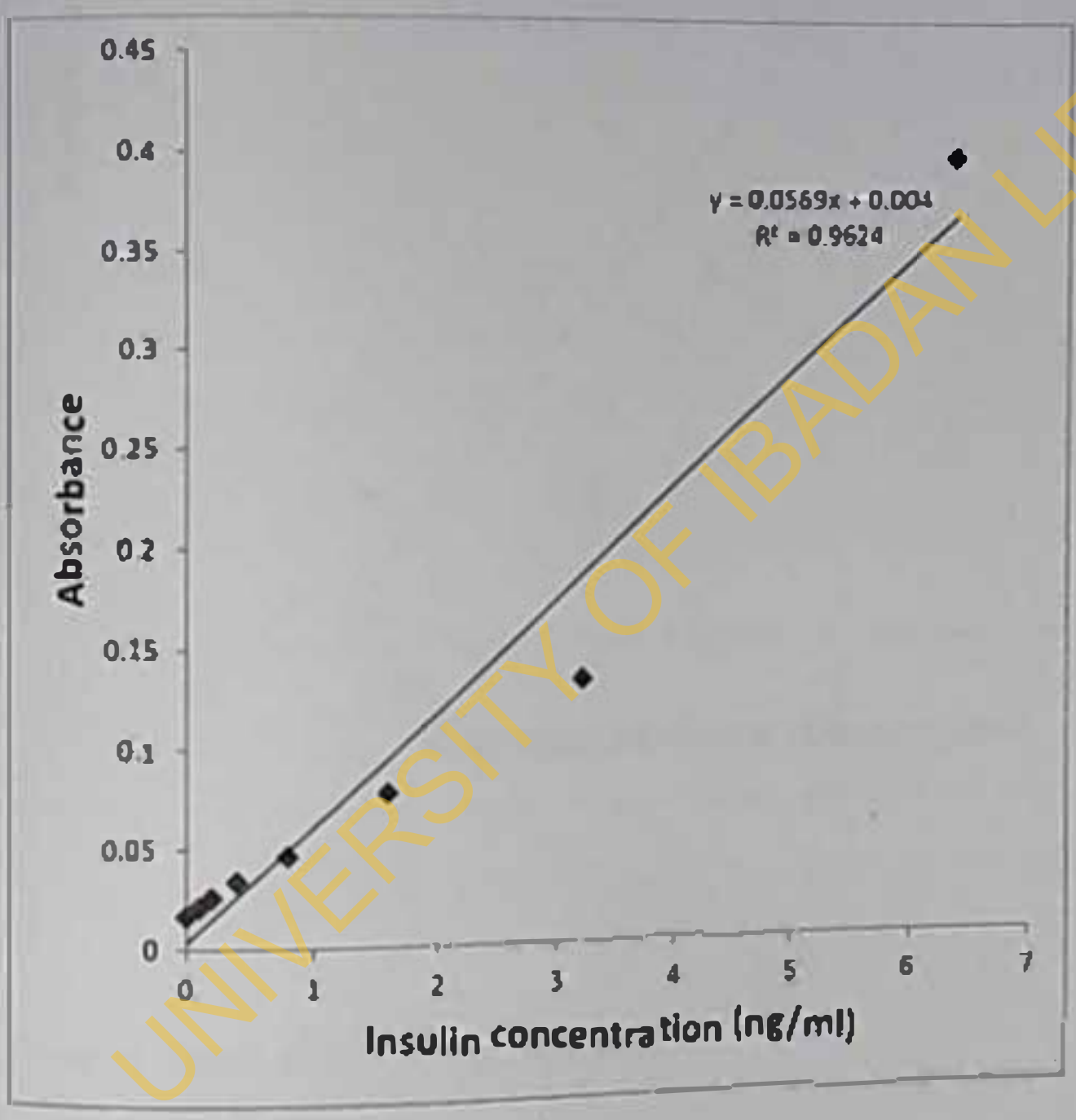

Figure 12: Standard curve for Insulin

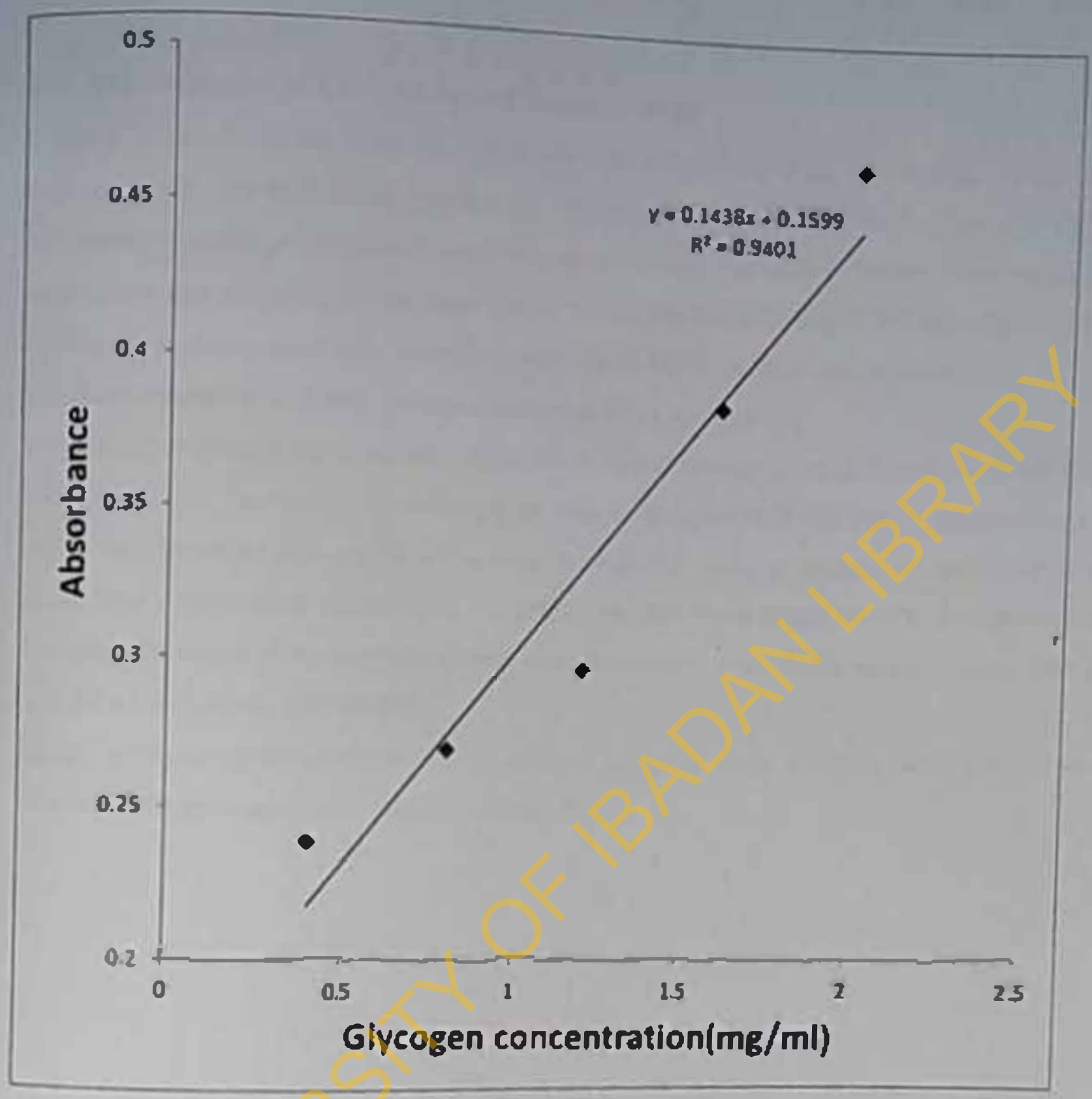

Figure 13: Standard curve for glycogen content

3.16 Determination of Liver glycogen synthase activity

0.1gram of liver tissue was weighed with digital scale and homogenised in 1ml of phosphate buffer solution on ice. Thereafter, the homogenate was centrifuged at 3000g for 15 minutes at 4°C in a refrigerated centrifuge. The supernatant was decanted into Eppendorf bottles in ice packs. The supernatant was subjected to two freeze thaw cycles and re-centrifuged. The glycogen synthase activity in the supernatant was determined with aid of ELISA kits (Cusabio China).

3.17 Determination of Liver glycogen phosphorylase activity

0.1gram of liver tissue was weighed with digital scale and homogenised in 1ml of phosphate buffer solution on ice. Thereafter, the homogenate was centrifuged at 3000g for 15 minutes and the supernatant decanted into eppendorf bottles in ice packs. The supernatant was subjected to two freeze thaw cycles and re-centrifuged. The final supernatant was stored at -20°C for the assay at a later date. Glycogen phosphorylase activity was determined with ELISA kits (Cusabio China)

3.18 STATISTICAL ANALYSIS.

Results were expressed as mean± SEM, and were analyzed using ANOVA and Student's t-test. The level of significance was accepted at $P<0.05$.

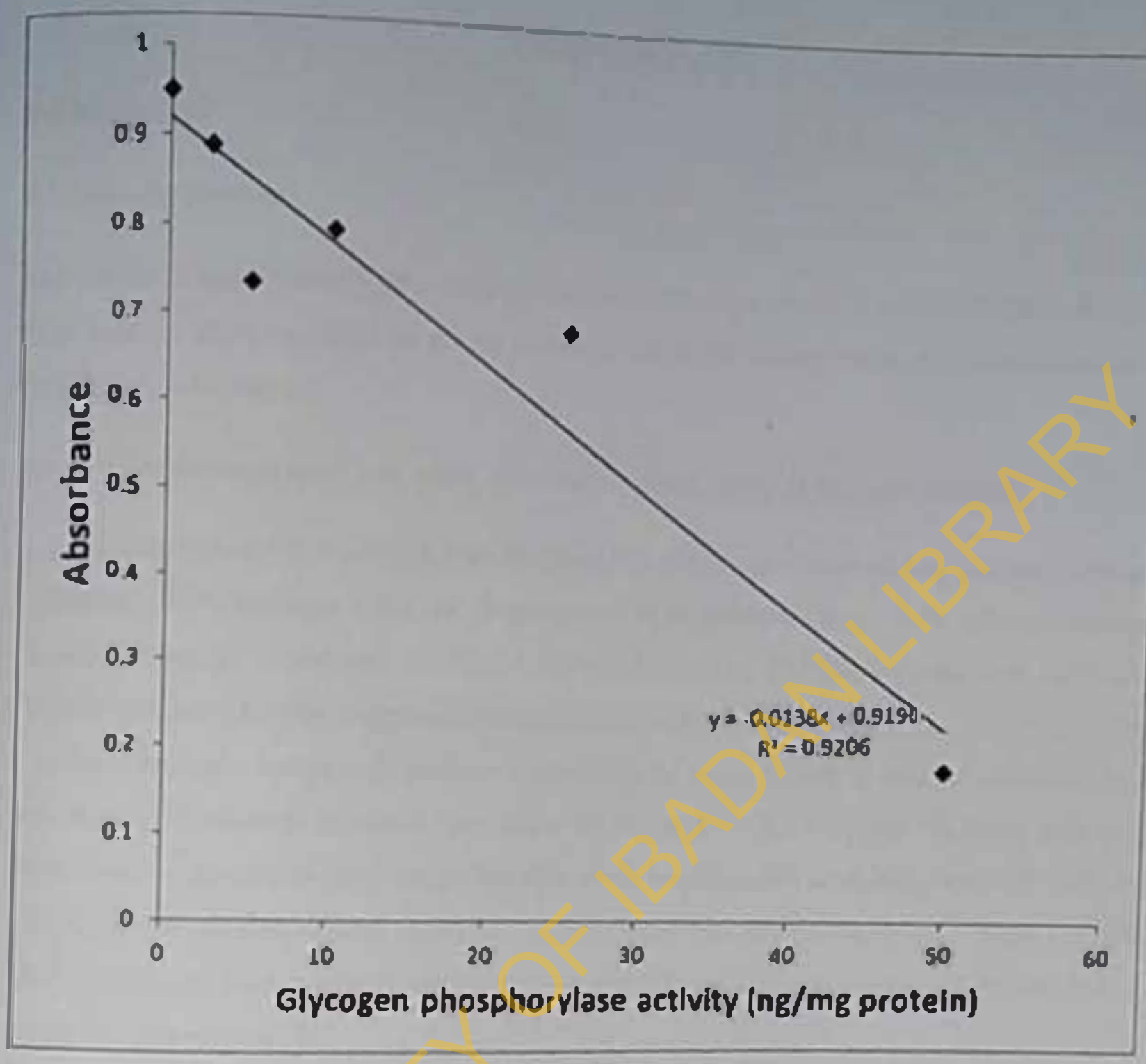

Figure 14: Standard curve for glycogen phosphorylase activity

CHAPTER FOUR

4.0 RESULTS

4.1 Toxicity studies

The results of acute toxicity after administration of ethanol extracts of kolanut (EEK) showed that there was no death recorded on giving graded doses of the extract ranging in concentration from 1mg/Kg to 3000 mg/Kg.

4.2 Gas chromatography and mass spectroscopy (GC-MS) of kolanut extracts

GC-MS analysis of the crude and decaffeinated kolaout extracts showed that the extract prepared contained 17.5% caffeine while the decaffeinated kolanut extract had 3.31% caffeine content. In the ethanol extract of kolanut, 39 compounds represented by 39 distinct peaks were identified by GC-MS (Figure 15). The compounds identified through the NIST08 database are listed in Tables 3 and 4. The major compounds present in the ethanolic crude extract of kolanut identified by GC-MS were caffeine with retention time (RT): 19.601 and 19.761 of Total: 50.569% and quality: 96%. Other components identified in the EEK were hexadecanoic acid, ethyl ester (RT: 20.43), 9, 12 Octadecadienoic acid, ethyl ester (RT: 22.353), 9-Octadecadienoic acid, ethyl ester, ethyl oleate (RT:22.422), cyclohexanone, 2-methyl-5-(1-methylethenyl) Octadee9-enoic acid decanoic acid, 10-(2-hexylcyclopropyl).

Similarly, analysis of the decaffeinated extract of kolanut (DEK) (Figure 16) showed caffeine concentration of 3.31% and quality: 96 with 34 distinct peaks representing 32 compounds. The major compound identified in the DEK was methyl 9, 10, methyllene-hexadecanoat with RT: 20.59, Total: 29.736% and qunlity 89

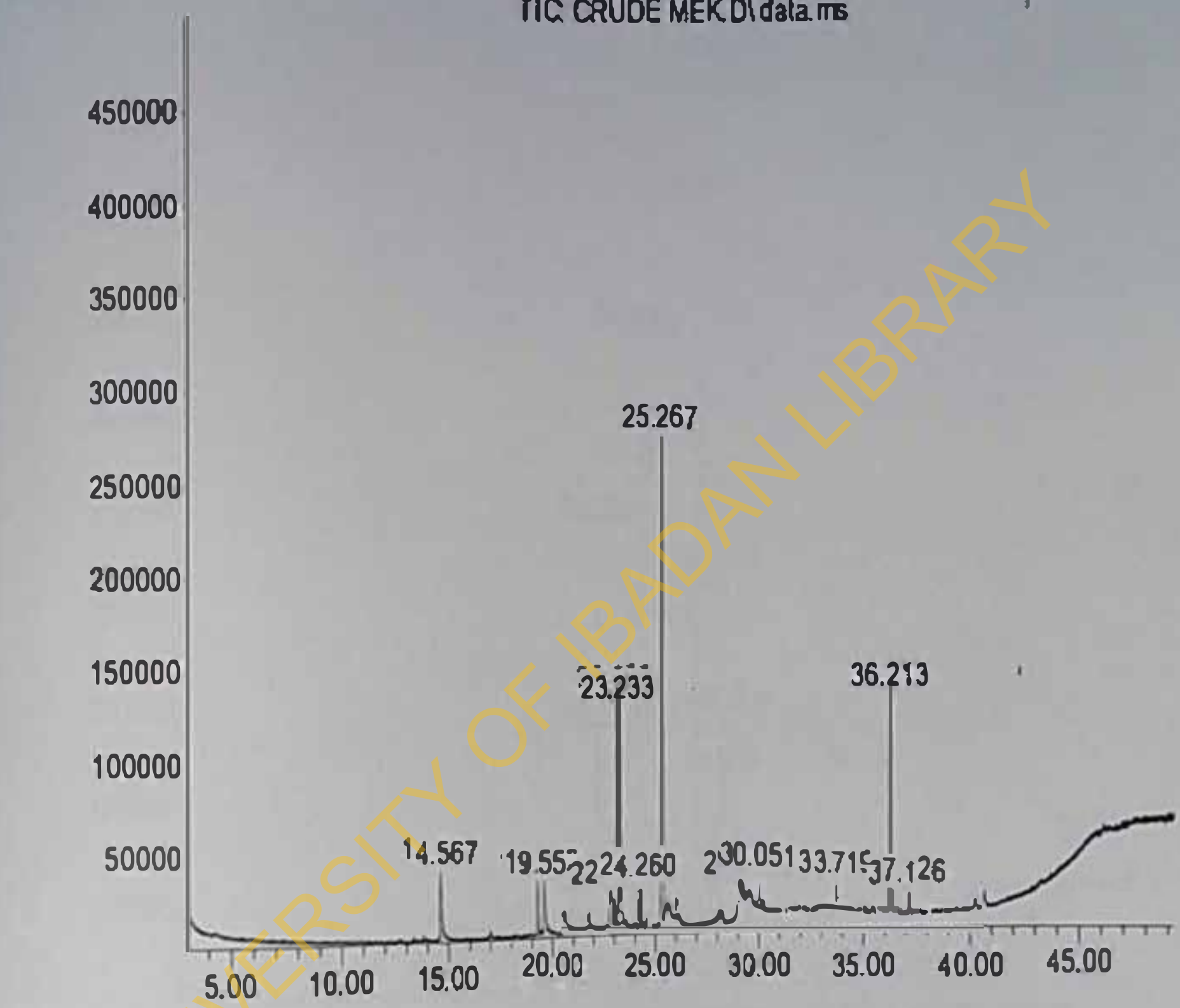

Figure 15: GC-MS chromatogram of ethanol extract of kolanut (EEK)

X axis shows retention time for each compound

Peaks represent each compound detected

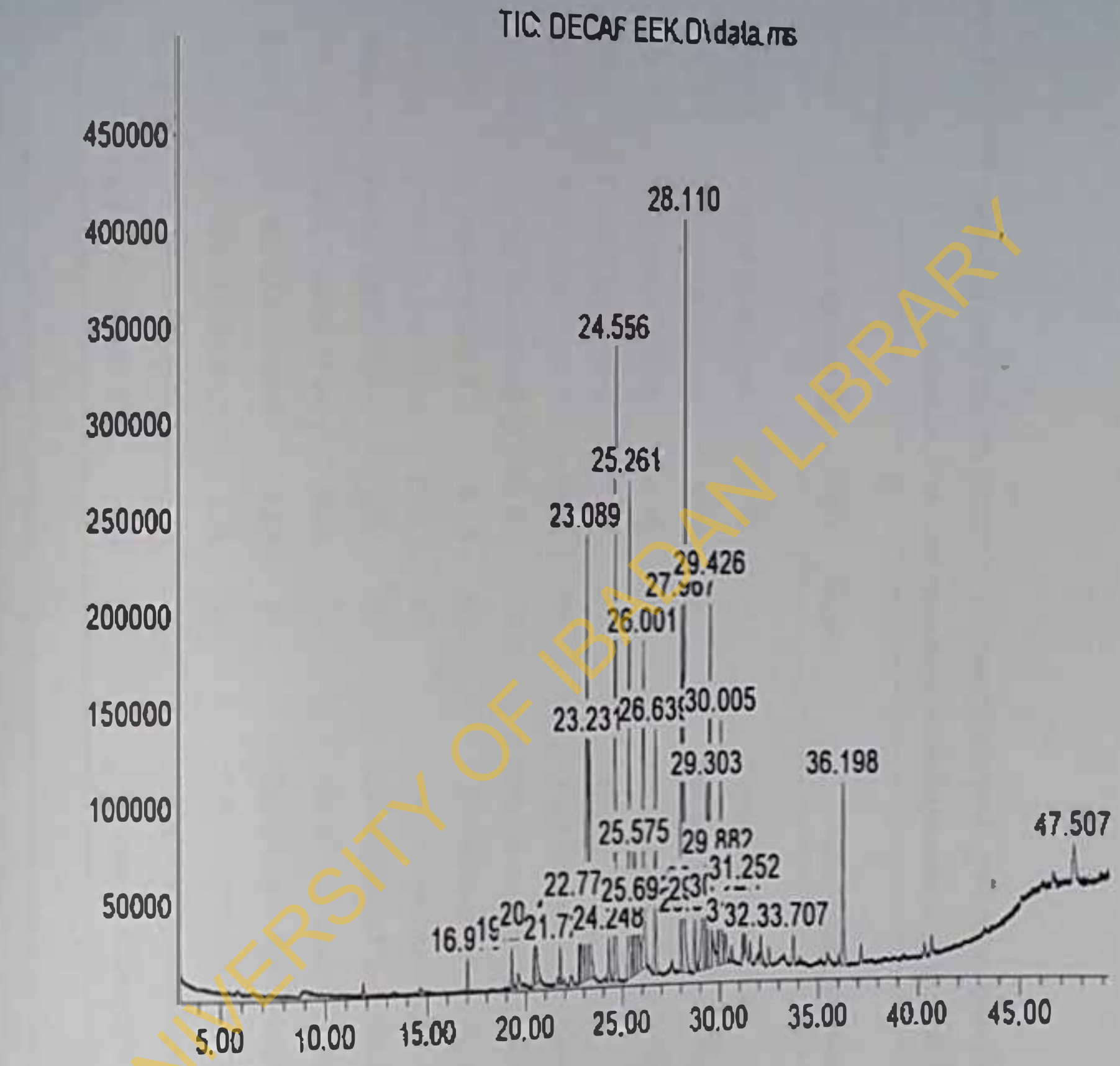

Figure 16: GC-MS chromatogram of decaffeinated ethanol extract of kolanut (DEEK)

Table 3: GC-MS analysis of crude Ethanol Extract of Kolanut (EEK) showing the compounds identified by mass spectra database, retention time, total percentage and relative quality of compounds

PK no	Retention time	Mass spectra data	% total	Qual	Identified compound
1.	14.565	178990 000057-09-0	11.90	43	Cetrimonium Bromide
2.	19.240	141185 1000282-82-8	9.00	53	Thiophene-2-acetic acid, dodec-9-y-nyl ester
3.	19.555	112764 000112-69-6	11.50	43	1-Hexadecanamine, N,N-dimethyl
4.	22.793	55116 000058-08-2	17.47	93	Caffeine
5.	23.091	87666 006740-88-1	55.71	91	Cyclohexanone, 2-(2-chlorophenyl)- 2-(methylamino)-, (.+/-.)-
6.	23.234	150263 1000309-086	46.93	78	Phthalic acid, cyclohexylmethyl butyl ester
7.	24.258	161704 000084-78-6	8.13	83	1,2-Benzenedicarboxylic acid, butyl octyl ester
8.	25.265	119605 017851-53-5	29.8	83	1,2-Benzenedicarboxylic acid, buty 83 12-methylpropyl ester
9.	29.185	199698 002122-26-1	2.8	53	Chloromethyl 7-chlorododecanoate
10.	29.225	122847 1000130-84-8	0.61	58	(S)(+)-Z-13-Methyl-11-pentadecen-1 -ol acetate
11.	30.049	217985 055162-62-3	1.89	91	Tetracontane, 3,5,24-trimethyl-
12.	33.717	217985 055162-61-3	1.75	91	Tetracontane, 3,5,24-trimethyl-
13.	36.211	178619 1000308-98-5	13.55	86	Phthalic acid, 2-ethylhexyl isohex yl ester
14.	37.127	47644 017301-29-0	1.42	53	Undecane, 3,7-dimethyl-
Total					

Table 4: GC-MS analysis of Decaffeinated Ethanol Extract of Kolanut (DEEK) showing the compounds identified by mass spectra, database, retention time, total percentage and relative quality of compounds

PK no	Retention time	Mass spectra data	% total	Qual	Identified compound
1.	16.974	58109000629-59-4	0.43	78	Tetradecane
2.	19.234	118091 1000293-64-1	0.46	53	2-Thiopheneacetic acid, 2-isopropo xyphenyl ester
3.	20.407	19134 005625-46-7	0.72	53	3,6-Dimethylpiperazine-2,5-dione
4.	20.487	25224 038487-86-4	1.12	52	Benzonitrile, 2-amino-4-chloro-
5.	21.729	58109 000629-59-4	0.51	80	Tetradecane
6.	22.725	55118 000058-08-2	0.86	93	Caffeine
7.	22.753	55116 000058-08-2	0.44	95	Caffeine
8.	22.770	55119 00005808-2	1.99	94	Caffeine
9.	23.091	87665 006740-88-1	8.38	91	Cyclohexanone, 2-(2-chlorophenyl)- 2-(methylamino)-, (+/-)-
10.	23.228	161711 000085-69-8	4.33	78	2-Benzenedicarboxylic acid, buty l 2-ethylhexyl ester
11.	24.247	150277 1000308-94-3	0.65	78	Phthalic acid, isobutyl cyclohexyl methyl ester
12.	24.556	113705 005129-60-2	9.3	98	Pentadecanoic acid, 14-methyl-, methyl ester
13.	25.259	119605 017851-53-5	8.4	83	1,2-Benzenedicarboxylic acid, buty l 2-methylpropyl ester
14.	25.551	102726 000057-10-3	1.77	99	n-Hexadecanoic acid Tridecanoic acid
15.	25.574	102726 000057-10-3	2.3	99	n-Hexadecanoic acid Tridecanoic acid
16.	25.694	102726 000057-10-3	2.3	99	n-Hexadecanoic acid Pentadecanoic acid
17.	26.003	70179 000627-90-7	5.9	72	Tridecanoic acid, ethyl ester Ethyl tridecanoate

18.	26.638	135402	1000336-62-4	3.6	58	i-Propyl 14-methyl-pentadecanoate n-Hexadecanoic acid
19.	27.966	132282	002566-97-4	6.1	99	9,12-Octadecadienoic acid, methyl ester, (E,E)-
20.	28.109	133702	013481-95-3	12.6	99	10-Octadecenoic acid, methyl ester
21.	28.664	135381	000112-61-8	0.8	95	Octadecanoic acid, methyl ester
22.	29.065	108922	056554-35-9	2.7	83	9,17-Octadecadienal, (Z)- Z-7-Tetradecenoic acid
23.	29.190	121324	1000130-90-4	0.4	72	12-Methyl-E,E-2,13-octadecadien-1- ol
24.	29.305	142890	007619-08-1	3.4	95	9,12-Octadecadienoic acid, ethyl ester 2-Chloroethyl linoleate
25.	29.425	122814	1000245-71-9	6.8	49	E-11-Hexadecenoic acid, ethyl ester
26.	29.883	5953	002808-75-5	1.7	62	1-Methyl-2-methylenecyclohexane
27.	30.003	11317	004925-71-7	4.5	93	9-Oxabicyclo[6.1.0]nonane, cis- Oleic Acid
28.	30.243	119881	014811-95-1	0.9	78	1,19-Eicosadiene 8-Hexadecenal, 14-methyl-, (Z)-
29.	31.250	10583	000766-53-0	0.3	76	Bicyclo[2.2.2]octane, 2-methyl-
30.	31.250	101000	1000159-93-4	0.9	52	Pentanoic acid, 10-undecenyl ester
31.	32.040	110397	002423-10-1	0.5	87	9-Octadecenal, (Z)-
32.	33.705	37511	062183-55-5	0.6	53	Octane, 3-ethyl-2,7-dimethyl-
33.	36.200	178619	1000308-98-5	3.3	86	Phthalic acid, 2-ethylhexyl isohexyl ester
34.	47.506	121324	1000130-90-4	1.05	58	12-Methyl-E,E-2,13-octadecadien-1- ol

Total

4.3 The effects of Caffeine, Kolanut and Decaffeinated Kolanut extracts on body weight in male Wistar rats.

Figure 17 shows the average weight gain in the four groups after eight weeks of treatment with the various extracts. All the treated groups showed less average weight gain compared to the control with the caffeine treated group showing the least weight gain but there was no significant reduction in weight gain compared to the control group.

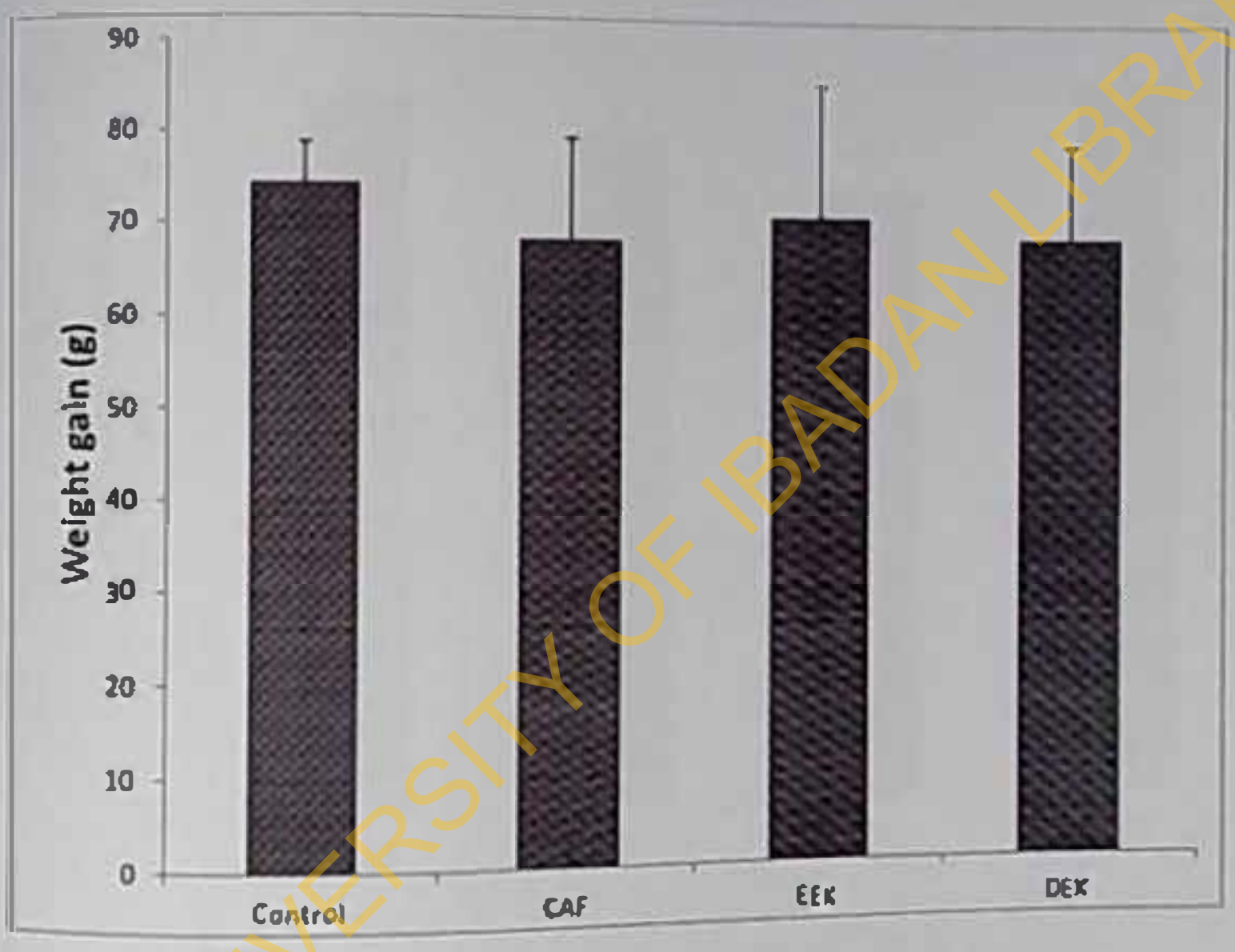

Figure 17: Average weight gain in caffeine, EEK, and DEEK treated rats (n=6)

4.4 The effects of Caffeine, EEK and DEK on food consumption in rats.

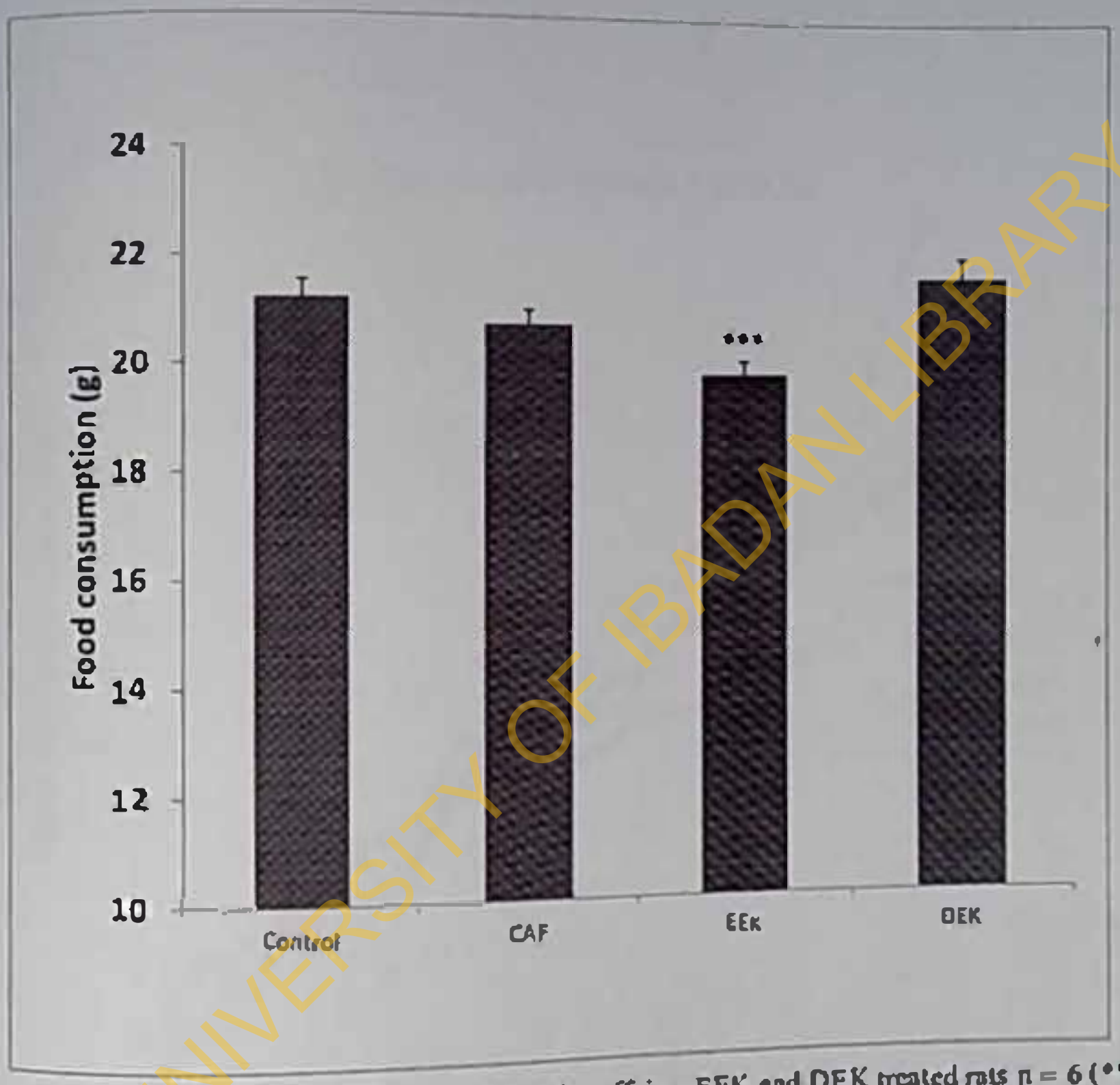

Figure 18: Average daily food consumption in caffeine, EEK and DEK treated rats n = 6 (*** = p< 0.001)

4.5 The effects of Caffeine, EEK and DEK on water intake in rats.

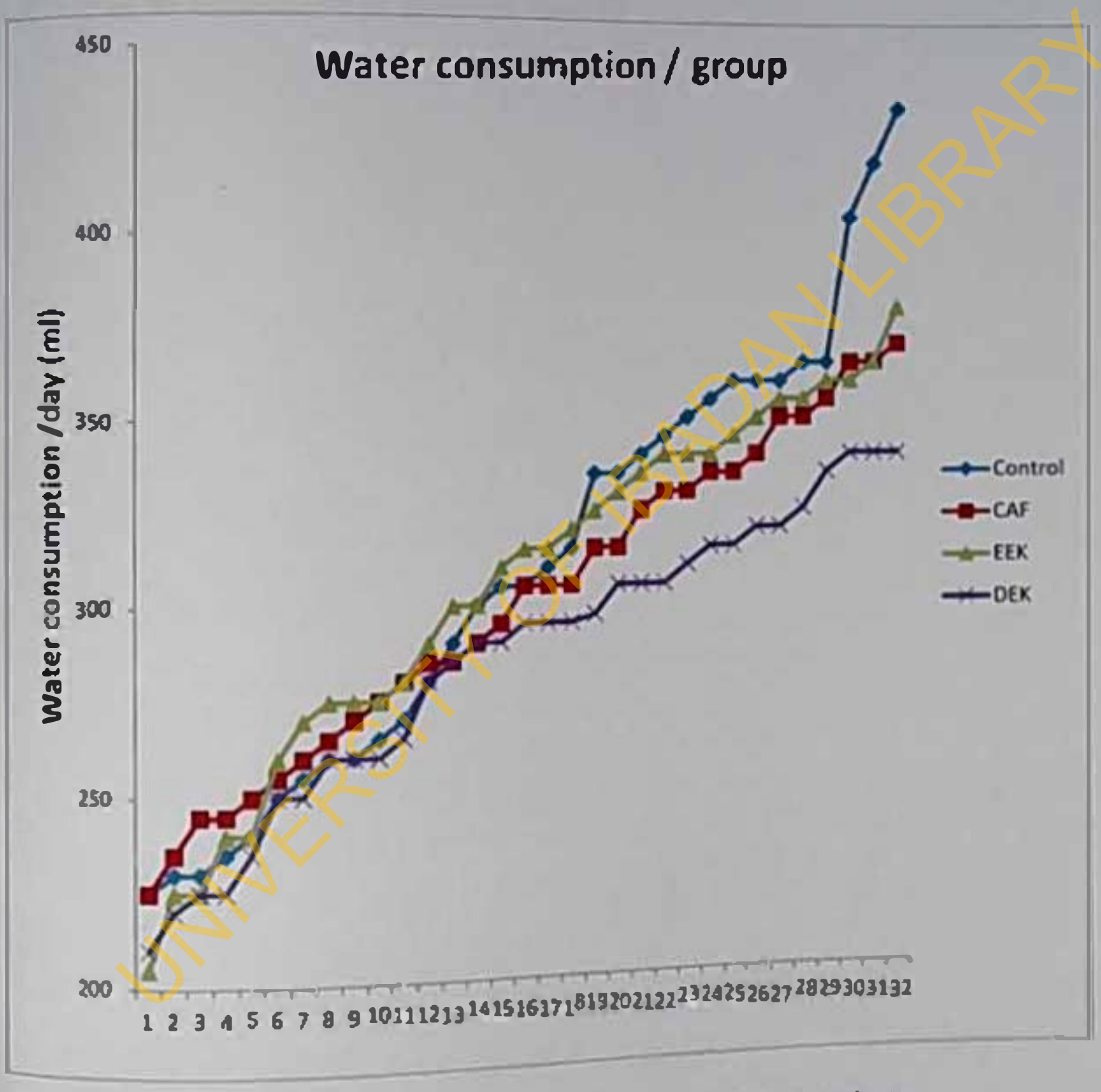

Figure 19: Daily water consumption in Control, CAF, EEK and DEK treated rats

CAF

4.6 Effects of Caffeine, EEK and DEK on Oral Glucose Tolerance (Acute).

Figures 20 and 21 show the effect of acute administration caffeine, EEK and DEEK on glucose tolerance test. Glucose disposal of the oral glucose load was significantly reduced (as evidenced by persistently raised blood glucose levels) in the caffeine and kolanut treated animals compared to the control. There was no significant difference in glucose load disposal in the decaffeinated treated group of animals. The persistence of glucose in circulation is more clearly shown in the area under the curve (AUC) for glucose in figure 21

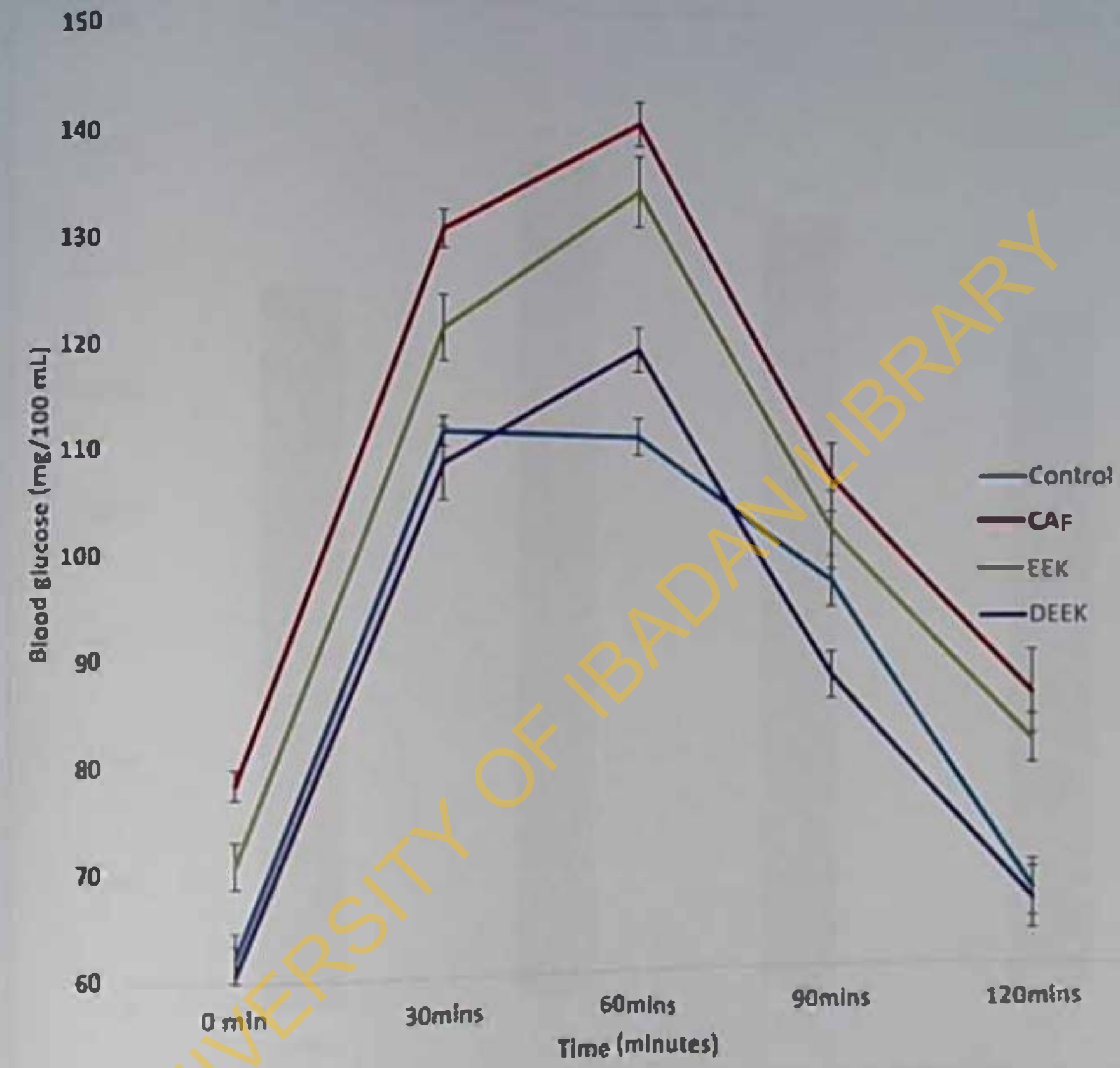

Figure 20: OGTT in CAF, EEK and DEEK treated rat (Acute)

Figure 21 AUC during OGTT in rats after acute treatment n = 6 (••• p < 0.001, ••• p < 0.0001)

4.7 Effects of Caffeine, EEK and DEK on Oral Glucose Tolerance (Chronic).

Figures 22 and 23 show the effect of chronic administration of caffeine, EEK and DEEK on glucose tolerance test. Glucose disposal remained markedly reduced in the caffeine treated animals but was increased in kolanut extract treated rats compared to the control.

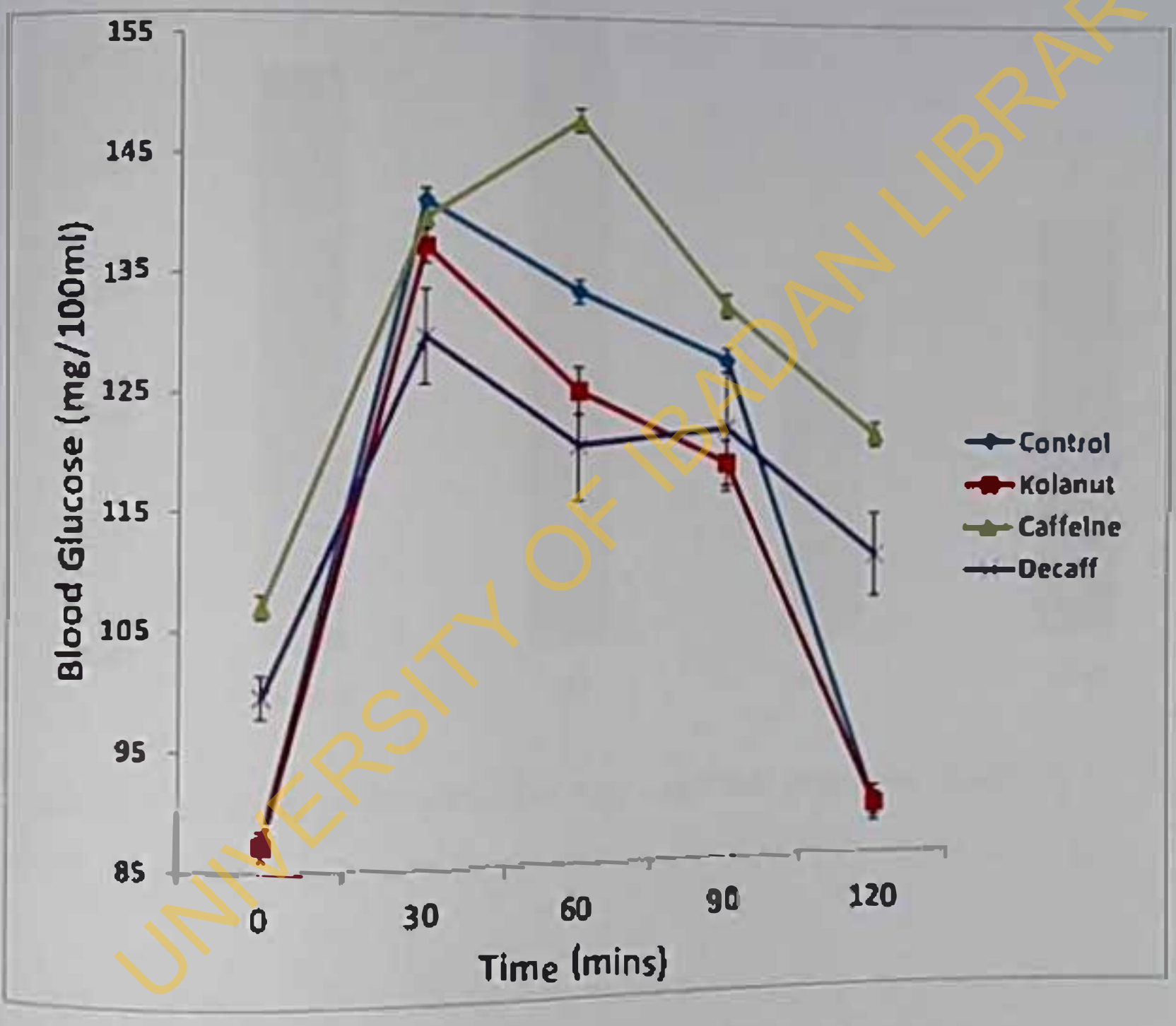

Figure 22 OGTT in caffeine, EEK and DEK treated rat (Chronic) n = 6.

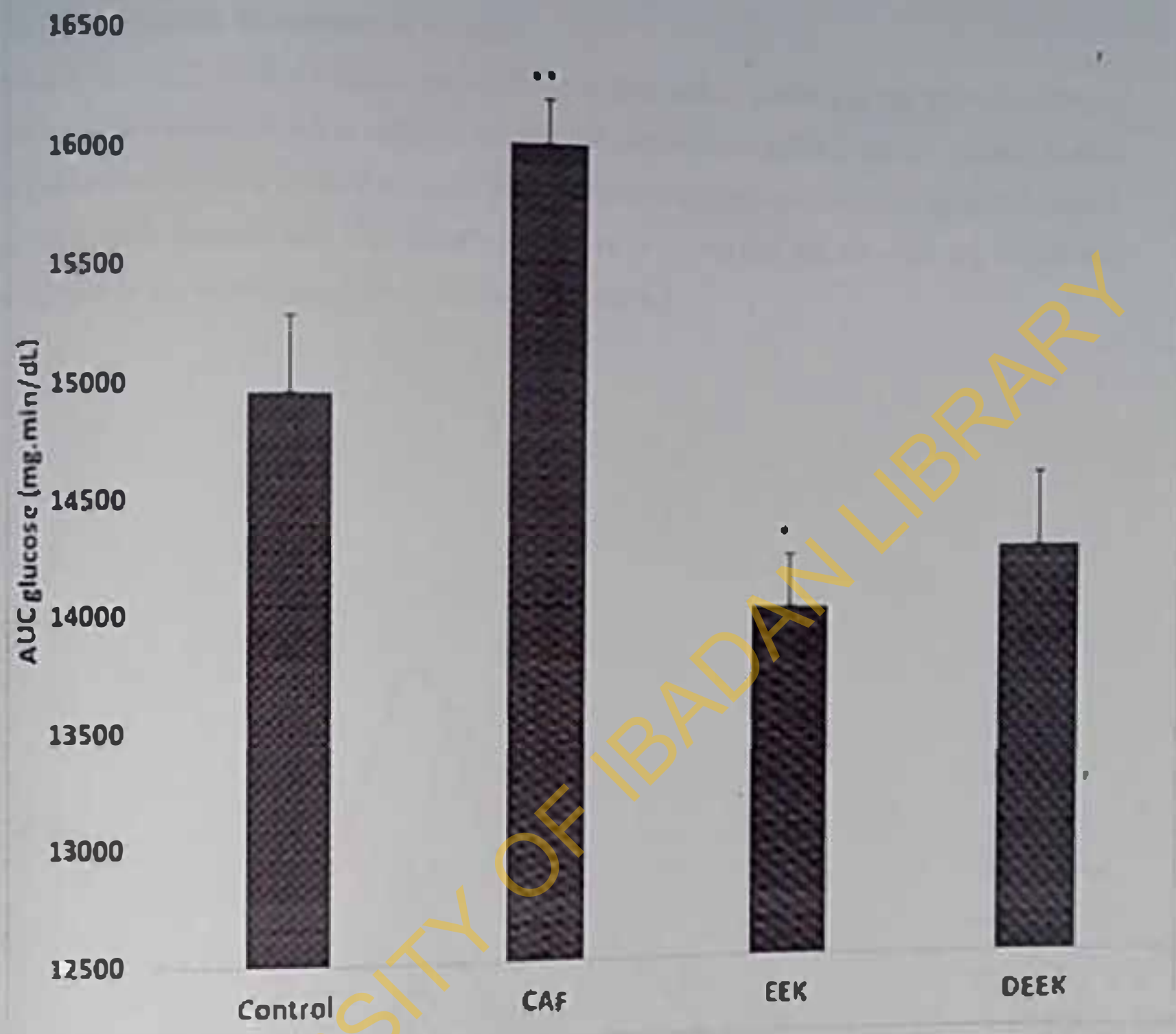

Figure 22 AUC during OGTT in Caffeine, EEK and DEEK treated rats (chronic) n = 6 (** = p<0.01, * = p< 0.05)

4.8 Plasma insulin concentration (acute)

Figures 24 and 25 show the insulin response to oral glucose load during the oral glucose tolerance test on acute administration of caffeine, kolanut and decaffeinated kolanut extract. Administration of caffeine and kolanut resulted in significantly raised levels of plasma insulin compared to control in response to glucose load. The decaffeinated kolanut treated rats did not show any significant difference in the insulin response compared to the control.

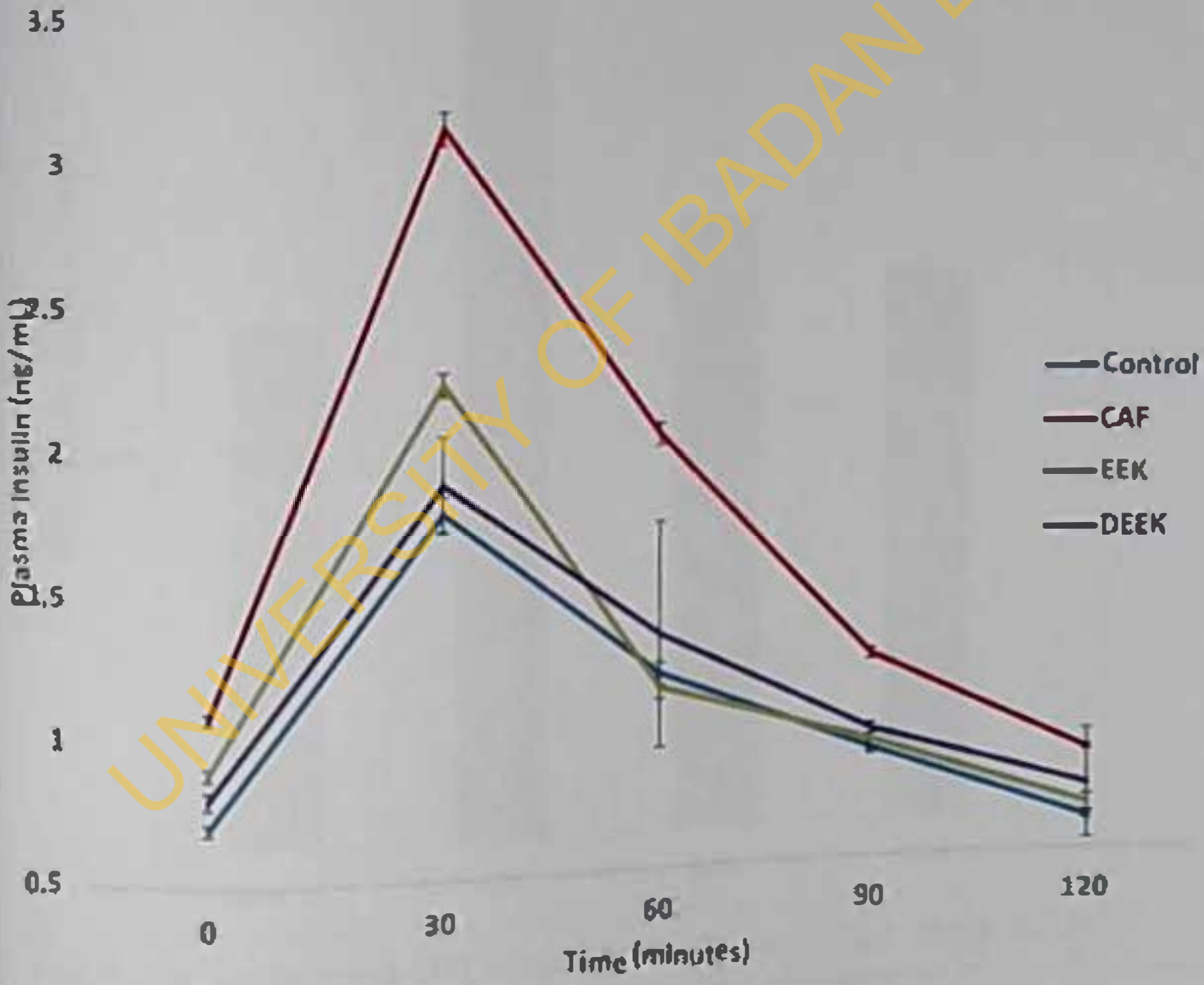

Figure 24 Plasma insulin levels during OGTT in rats (acute) n = 6

4.9 Plasma insulin concentration (chronic)

Figures 26 and 27 show the insulin response to oral glucose load during the oral glucose tolerance test on chronic administration of caffeine, kolanut and decaffeinated kolanut extract. While administration of caffeine over a period of eight weeks resulted in significantly ($P< 0.001$) raised levels of plasma insulin compared to control in response to glucose load, the chronic administration of EEK significantly ($P<0.05$) lowered the insulin level. The decaffeinated kolanut treated rats did not show any significant difference in the insulin response compared to the control.

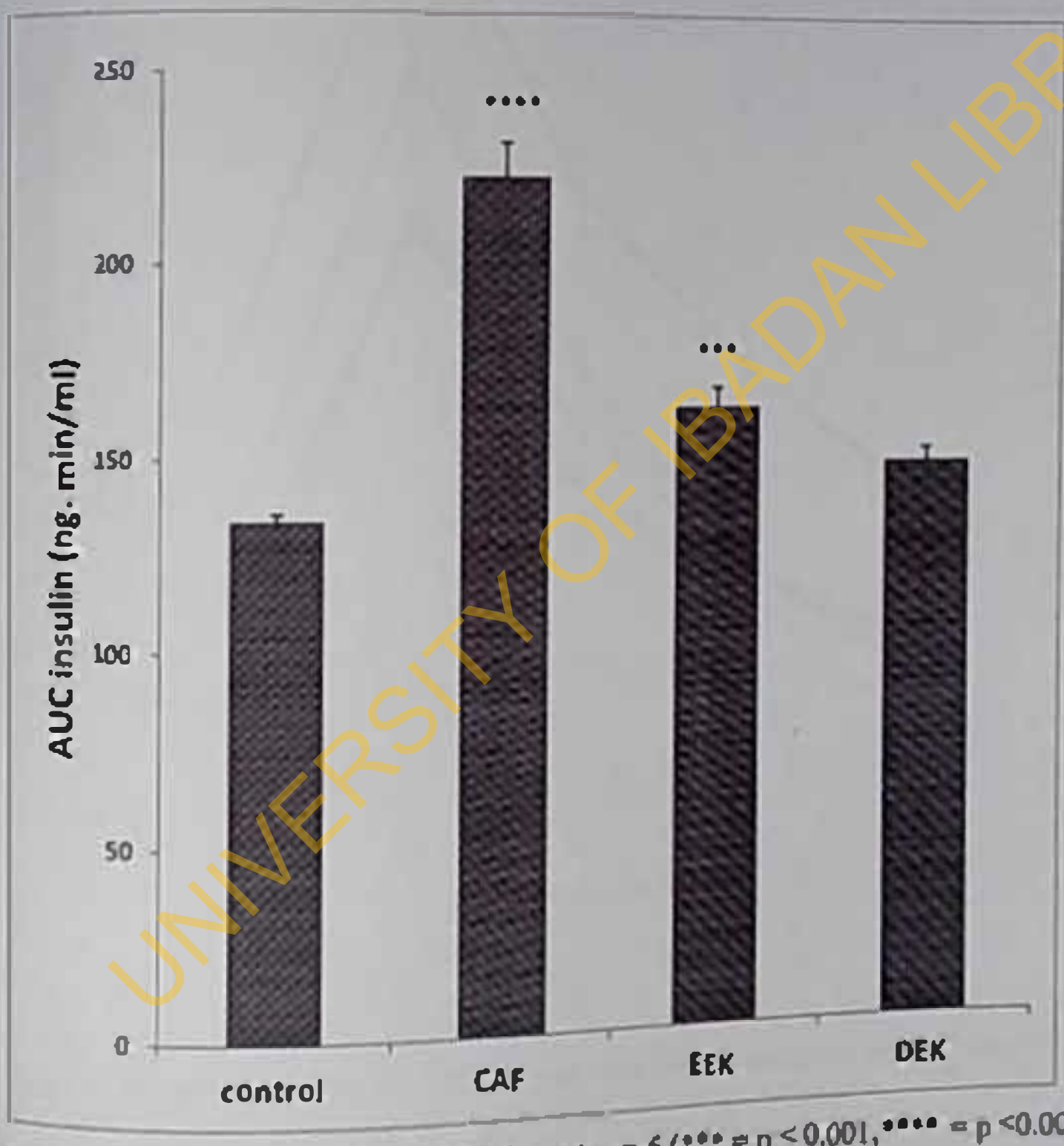

Figure 25 AUC insulin during OGTT (acute) n = 6 (*** = p < 0.001, **** = p <0.0001)

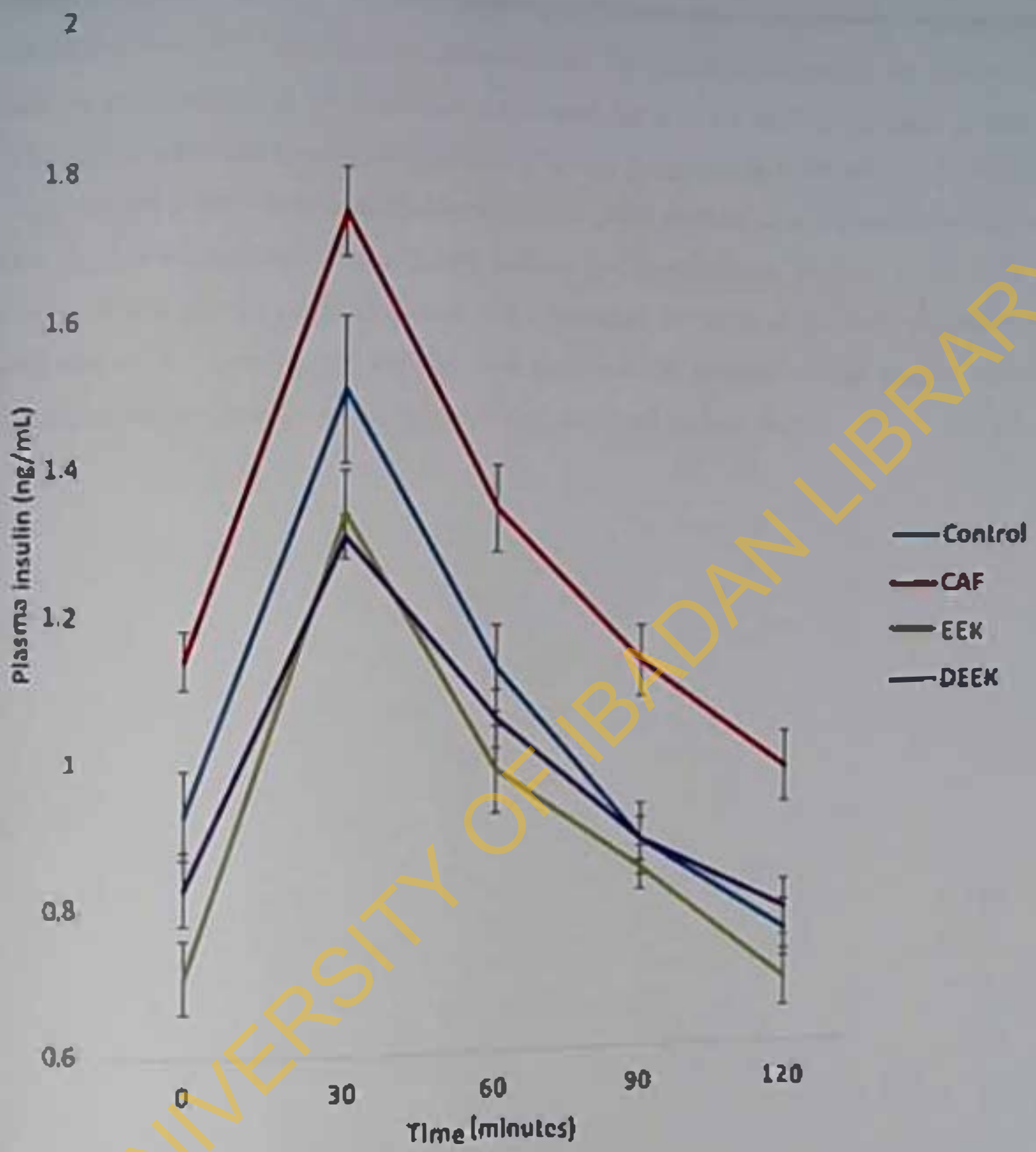

Figure 26 Plasma insulin during OGTT in rats after chronic treatment with extracts n = 6.

4.10 Liver Glycogen Content.

Figures 28 and 29 show the liver glycogen content after acute and chronic administration of caffeine, kolanut and decaffeinated kolanut extract. The glycogen content of rats following acute treatment with caffeine and with kolanut were significantly lower than the glycogen content of the control group while there was no significant difference in the glycogen content of the decaffeinated kolanut treated group when compared to the control group upon acute administration of the extract. After chronic administration of caffeine, kolanut and decaffeinated kolanut, it was only in the kolanut extract treated group that there was significant elevation of the liver glycogen content compared to the control, the caffeine and decaffeinated kolanut treated groups showed no significant difference in liver glycogen con tent compared to the control.

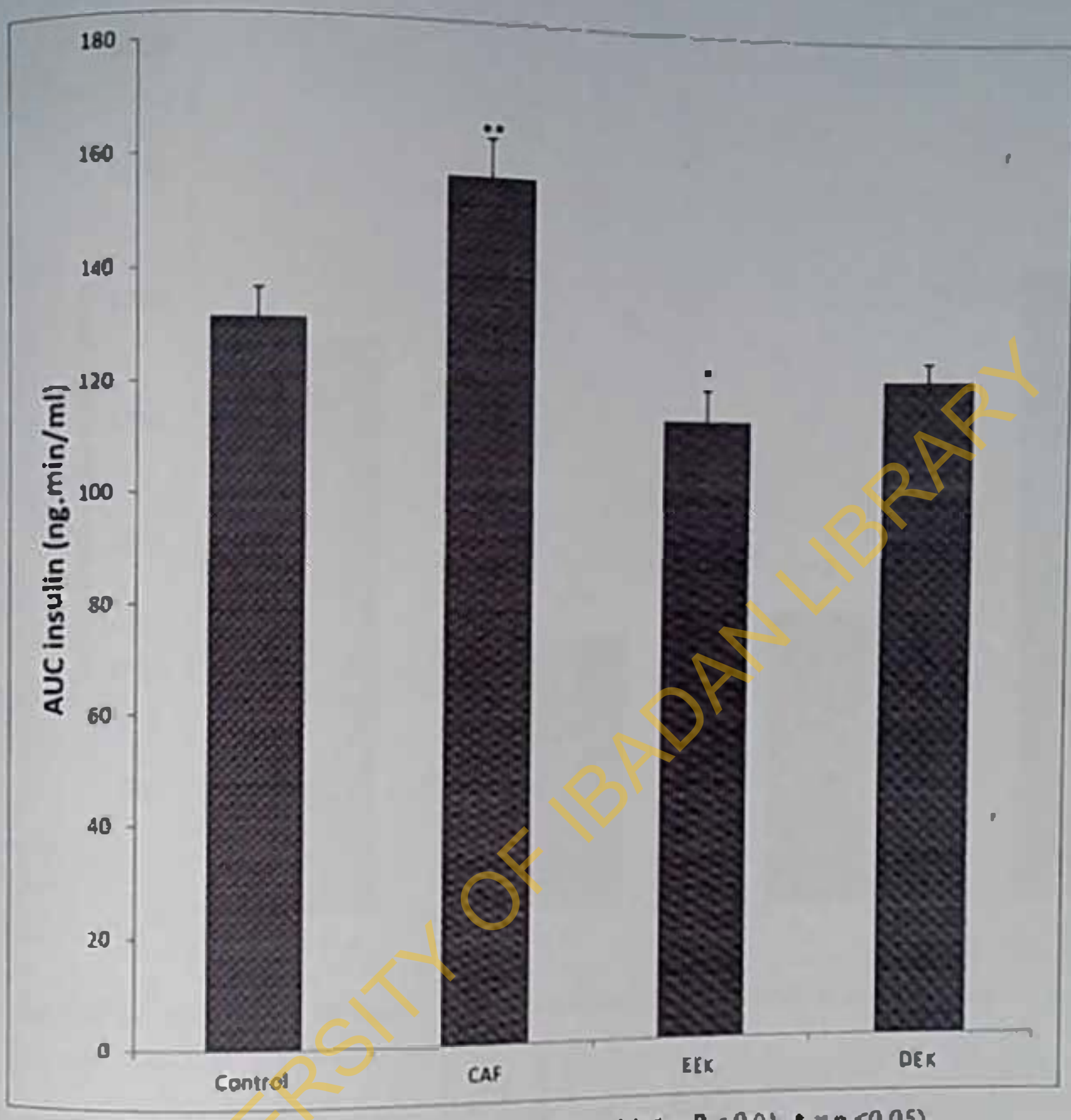

Figure 27 AUC insulin during OGTT (Chronic) n = 6 (** = P < 0.01, * = p <0.05)

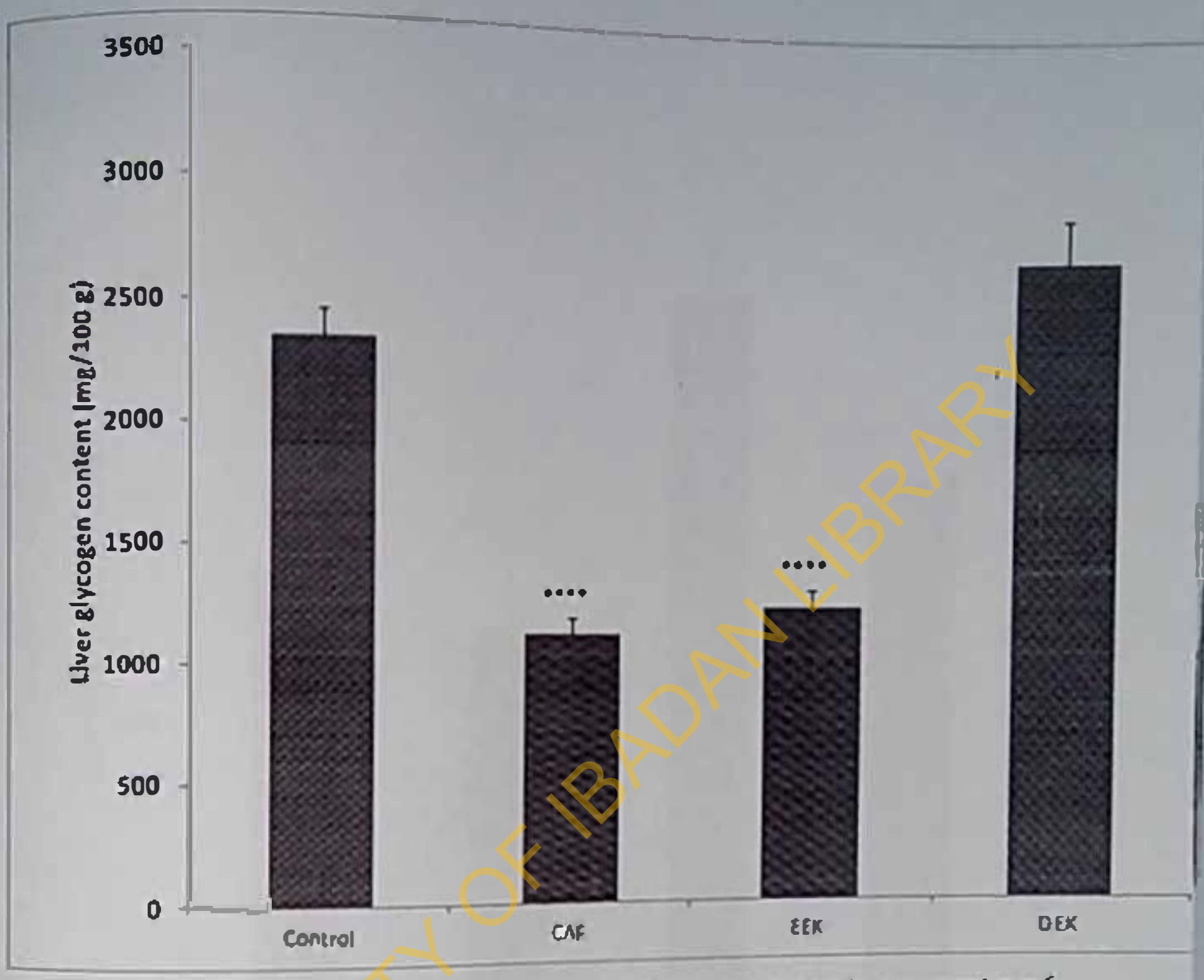

Figure 28 Liver glycogen content in Caffein, EEK and DEK rats after acute treatment n = 6 (*** = p < 0.001, **** = p < 0.0001)

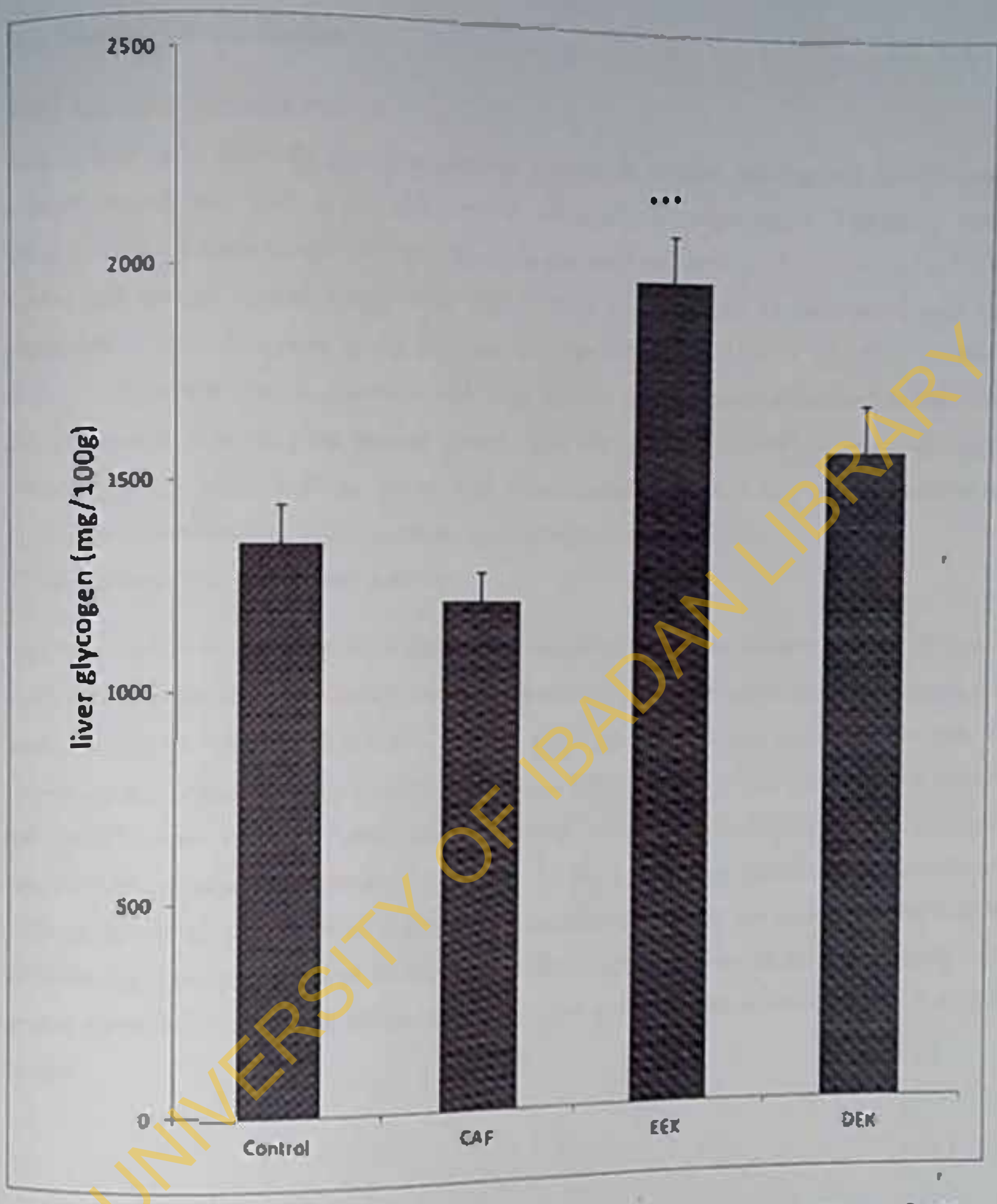

Figure 29: Liver glycogen content in rats after Chronic treatment n = 6, (*** = p < 0.001)

4.11 Enzyme Activity Studies.

4.11.1 Glycogen synthase activity

Figures 30 and 31 show the glycogen synthase activity in caffeine, kolanut and decaffeinated kolanut treated rats after acute and chronic administration respectively. Following acute administration of extracts and caffeine, the glycogen synthase activity in the liver of caffeine treated and kolanut treated groups were significantly less than that of the control with the decaffeinated kolanut treated group showing no significant difference in glycogen synthase activity compared to control. However, following chronic administration of caffeine kolanut and decaffeinated kolanut, only the kolanut treated group showed a significantly increased glycogen synthase activity while caffeine treated and decaffeinated kolanut treated groups showed no significant difference in glycogen synthase activity compared to the control.

4.11.2 Glycogen Phosphorylase Activity

Figures 32 and 33 show the glycogen phosphorylase activity in caffeine, kolanut and decaffeinated kolanut treated rats after acute and chronic administration of extracts respectively. Following acute administration of extracts and caffeine, only the glycogen phosphorylase activity in the liver of kolanut treated groups was significantly greater than that of the control with the caffeine treated and decaffeinated kolanut treated group showing no significant difference in glycogen phosphorylase concentration compared to control. However, following chronic administration of caffeine kolnnut and decaffeinatedkolanut, only the kolanut treated group showed a significantly decreased glycogen phosphorylase activity while caffeine treated and decaffeinated kolanut treated groups showed no significant difference in glycogen phosphorylase activity compared to the control.

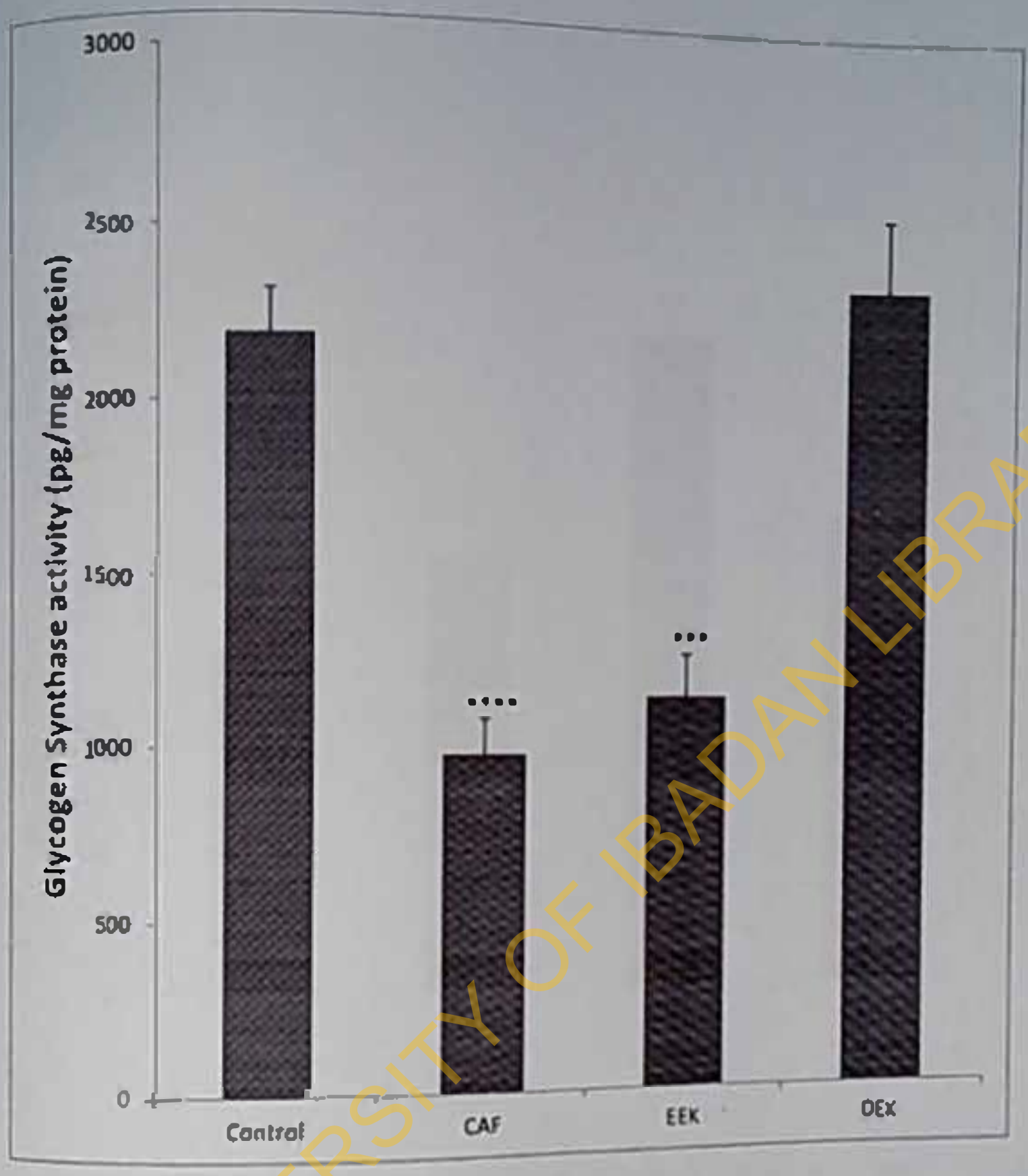

Figure 30: Glycogen synthase concentration in glucose challenged rats after acute treatment n = 6, (**** = p < 0.0001, *** = p < 0.001)

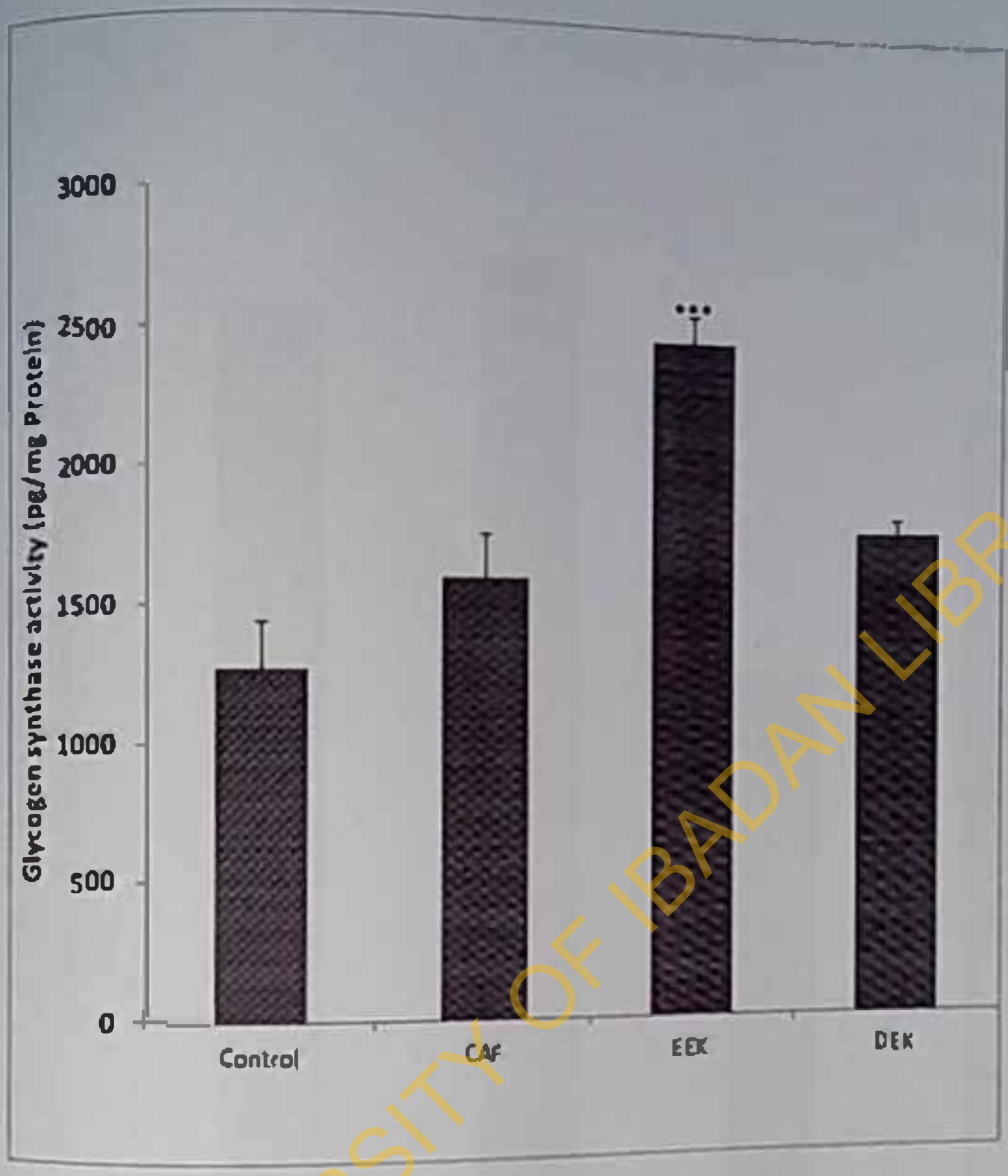

Figure 31: Glycogen synthase concentration in glucose challenged rats Chronic treatment n = 6 (*** = p < 0.001)

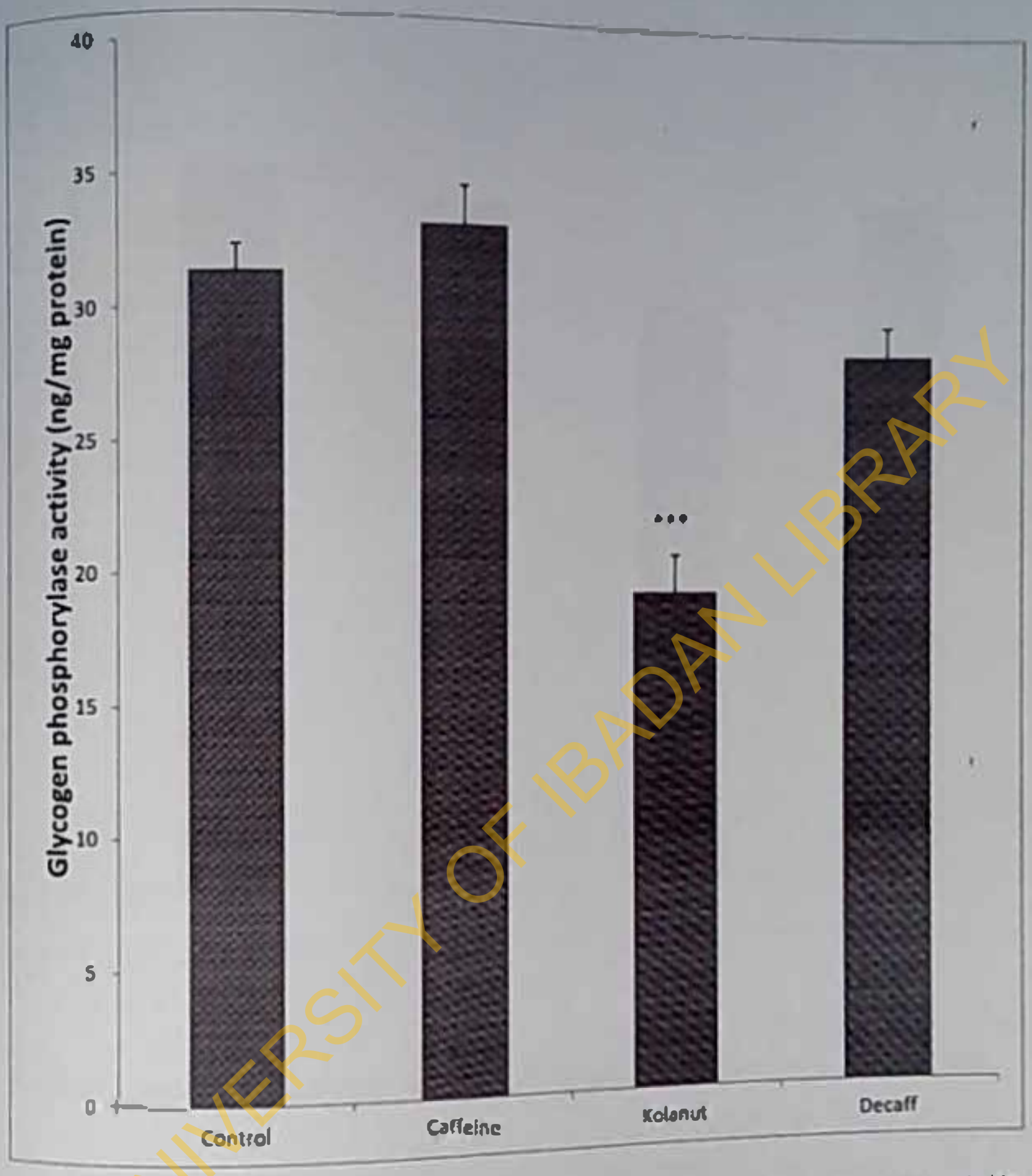

Figure 32: Glycogen phosphorylase concentration in glucose challenged rats (Acute) n = 6, (*** = P < 0001)

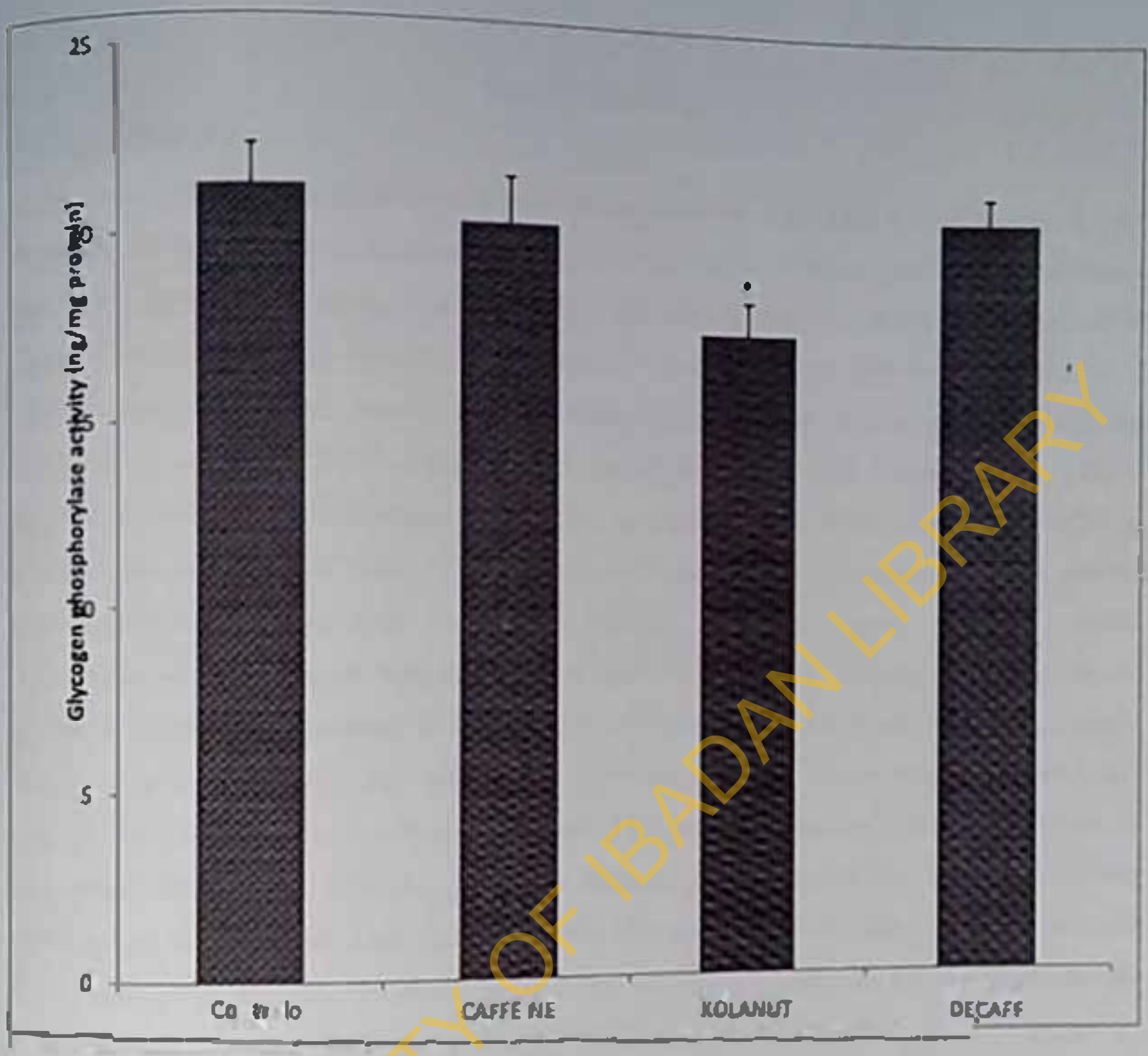

Figure 33: Glycogen phosphorylose in glucose challenged rats (Chronic) n= 6, (* = p < 0.05)

CHAPTER FIVE

5.0 DISCUSSION

In this study, gas chromatography-mass spectrometry analysis of the EEK and DEK prepared for this study showed that the EEK contained 17.5% caffeine while the decaffeinated kolanut extract had 3.29% caffeine with about 97% quality. The caffeine concentration in the kolanut extract is higher than that previously reported by Oguluga. (1975) and Somonn. (1973) who reported 0. 5 and 7% respectively. It is however much lower than the concentration of 51% reported recently by Salahdeen *et. al.*, (2014, 2015). Adeyeye *et. al.*, (2007) reported 0.6-3%, Nyamien *et. al.*, (2015) reported 1.84-2.56% concentration of caffeine in kolanut. This wide variation in caffeine concentration in extracts of *Cola nitida* could have arisen from the methods of analysis and the state of the kolanut extract used. For instance, Oguluga. (1975) used spectrophotomeuy, while Nyamien *et. al.*, (2014) used high performance lipid chromatography (HPLC) to determine the caffeine concentration of kolanut. Other factors such as time and period of collection, geographical origin, climatic conditions and methods of preparation of the extract could influence the concentration of the active constituents particularly alkaloids and phenolic compounds present in the kolanut (Hicks *et. al.*, 1996; Arogba, 1999). Interestingly, the caffeineconcentration in tea and coffee ranges between 1-4% and 1-2% respectively (Kaplan *et. al.*, 1974). It must however be noted that kolanut is not made up of caffeine alone. Othersignificant biologically active compounds are present in kolanut. Apart from the caffeine. GC-MS analysis of the extracts used in this study showed that there are 38 other compounds in the EEK while there are 32 other compounds in the DEK following decaffeination (Salahdeen *et. al.*, 2015). Some of these compounds are the polyphenols which are Chlorogenic acid, Quinic acid, Tannic acid, Catechin, Epicatechin, Gentisic acid and Rubutin (Odebode, 1996), all various forms of flavonoids which are known to have significant biological activity (Matsumoto *et. al.*, 2014). Interestingly, in this study, even the decaffeinated kolanut still had as much as 3.29% caffeine. In other words, a decaffeinated kolanut may still be able to exhibit some actions of caffeine especially when administered for a protracted period.

The absence of mortality following the acute toxicity study of our extracts is consistent with previous reports by Salahdeen and Alada, (2009) who also reported no mortality at 3000mg/Kg.

The dosage of 6mg/Kg used in this study, represents the amount of caffeine estimated to be contained in 3 cups of coffee being consumed by a 70Kg man (Donovan and De Vane, 2001).

Effects of caffeine, EEK and DEK on body weight, food consumption and water intake.

In the present study there was no difference in the weight gained after eight weeks of treatment with caffeine, EEK and DEK. Other investigators have however found that there was a reduction in the rate of weight gain in caffeine treated animals (Zheng *et. al.*, 2004) and humans (Conway *et. al.*, 2003 Greenberg *et. al.*, 2005 Lopez-Garcia *et. al.*, 2006). The weight loss was attributed to increased thermogenesis, lipolysis and fat oxidation induced by caffeine. Salahdeen *et. al.*, 2015 also observed an increase in body water loss through excessive urination in caffeine treated rats and suggested the reduced weight gain could have been a result of the fluid lost from the body since it is well established that at least 60% of total body weight is water. Caffeine is a well known mild diuretic (Wemple *et. al.*, 1997; Zhang *et. al.*, 2014).

In spite of the lack of observable reduction in weight gain in all treatment groups in this study, there was decrease in food consumption by the EEK treated rats. Other investigators have reported that chronic consumption of kolanut significantly decreased body weight and food intake (Ikegwonu *et. al.*, 1981; Umoren *et. al.*, 2009). The present experiments lasted eight weeks whereas those of Ikegwonu, (1981) lasted 18 weeks and that of Umoren, (2009) was just over a period of four weeks. Moreover, significantly higher doses of the kolanut extract were used by both investigators. It is quite conceivable that extending the treatment to twelve or even sixteen weeks may lead to significant reduction in weight gain. The decrease in food consumption may indicate that there may be a scientific basis for the assertion that kolanut consumption reduces hunger (Abdulkarim *et. al*, 2005) by responders in epidemiological studies. There is at present no scientific data from experimental controlled studies to back this up and therefore more investigations should be done in order to fully define the effect of kolanut on food consumption. However a possible explanation may be the presence of polyphenolic compounds which have been shown to reduce the rate of absorption of nutrients from the gastrointestinal tract (Thompson *et. al.*, 1983) and inhibit digestive enzymes such as trypsin and amylase (Griffiths and Moseley, 1980).

The observed decrease in water consumption in the DEK treated rats may have been due to the reduced fluid loss on removal of the diuretic effect of caffeine. Conservation of fluid would then lead to dampening of the thirst reflex thereby reducing water consumption.

Effect of Caffeine, EEK, and DEK on oral glucose tolerance

In the present study, acute administration of caffeine increased the AUC (glucose) and glucose intolerance in the rat as seen in the delayed glucose disposal of the administered oral glucose load. This finding is consistent with the reports of Kolnes *et. al*, (2010) in which caffeine administration reduced glucose uptake by skeletal muscles. Kolnes *et. al*., (2010) in an experiment in which rat epitrochlcatis and soleus muscles were incubated with insulin and caffeine or theophylline found that both methylxnnthines completely blocked insulin-stimulated glucose uptake in the muscles. Furthermore, caffeine and theophylline reduced contraction-stimulated glucose uptake by about 50%, yet contraction-stimulated glycogen breakdown remained normal. The effect of caffeine in inducing glucose intolerance has been attributed to two main mechanisms. Firstly, stimulation of adrenaline secretion which in turn causes glucose intolerance by stimulating gluconeogenesis and glycogenolysis and inhibiting insulin action (Survit *et. al.*, 1993). To give further credence to this hypothesis, Battram *et. al.*, (2006) found that acute caffeine ingestion did not impair glucose tolerance in tetraplegic subject who could not secrete adrenaline. Thong and Graham, (2002) also found that beta-adrenergic receptor blockade abolished caffeine induced impairment of glucose tolerance in humans. Secondly, antagonism of adenosine receptors (Vergauwen *et. al.*, 1994). Evidence linking caffeine antagonism of adenosine receptor action was provided by Faulhaber-Walter *et. al.*, (2011) using A1AR deficient (knockout) mice compared to wild type mice fed standard or high fat diet for 8-12 weeks. They found that the *A1AR* deficient mice had significantly higher fat mass, fasting plasma glucose and insulin, along with a decrease in net glucose uptake in muscle and adipose tissue. They therefore concluded that adenosine/A1AR signaling contributes to insulin-controlled glucose homeostasis and insulin sensitivity in mice and is involved in the metabolic regulation of adipose tissue. However it is quite conceivable that the two mechanisms may be operating simultaneously in whole body (in-vivo situations).

Similar to the effect of caffeine, on glucose tolerance acute administration of kolanut (EEK) also lead to glucose intolerance as seen with greater area under the curve for glucose during the oral glucose tolerance test. There is currently no study documenting the acute effect of kolanut consumption on glucose metabolism in the rat or any other laboratory animal. The present study

appears to be the first to document the effect of acute administration of kolanut extract on glucose metabolism in the rat. The effect of acute administration of kolanut extract was by and large similar to that of the pure caffeine, that is, causing decreased glucose disposal. Although there are no reports on acute administration of kolanut extracts in humans and animals, reports from some other caffeine containing food substances such as coffee may be of interest. There are lots of human controlled studies on acute ingestion of coffee (Feinberg *et. al.*, 1968; Battram *et. al.*, 2006; Louie *et. al.*, 2008; Moisey *et. al.*, 2008; Moisey *et. al.*, 2010; Greenberg *et. al.*, 2010), which have demoostmted transient decrease in glucose disposal. Furthermore, a similar effect has been recorded with acute ingestion of coffee causing increased insulin concentrations in human subjects (van Dam *et. al.*, 2004). The similarity of effects of caffeine and kolanut is probably due to the high concentration of caffeine (17.5%) in the extract used in this study. The relatively high caffeine content found in the extract raises the question of whether the observed actions of kolanut could be largely or solely attributed to its caffeine content. Indeed other investigators notably Salahdeen and Alada (2009), and Salahdeen *et. al.*, (2014) have documented similarities between the effects of kolanut and pure caffeine. In an experiment on the effect of caffeine on glucose uptake in the canine hind limb, Salahdeen and Alada, (2009) found that the increase in hind limb glucose uptake caused by caffeine was essentially similar to that caused by infusion of kolanut. More recently, Salahdeen *et. al.*, (2014) found that ACh-induced relaxation of aortic rings of rats treated with either kolanut extract or caffeine had similar characteristics between the two groups suggesting that the action of kolanut extract was due to its caffeine content. They further proposed that the similarity in effect of kolanut and caffeine could have been due to the high caffeine content in the kolanut extract which in their study was up to 51%.

In view of the similarity between the effect of kolanut and caffeine, there is every reason to believe that the kolanut action is mediated through its caffeine content. Although it is agreed that caffeine is not the only active substance in the kolanut extract, the absence of glucose intolerance as caused by kolanut extract when decaffeinated kolanut was administered strengthens the view that kolanut extract action is most possibly carried out through the action of its caffeine component. The possibility of the other components of kolanut contributing to whatever action as observed cannot however be totally ruled out.

The effect of caffeine on glucose tolerance after chronic administration for eight weeks remained the same as in acute administration with delayed glucose disposal as seen in increased AUC

(glucose) after oral glucose load. This finding is in line with that of Shearer *et al.*, (2007), who experimented with male Sprague-Dawley rats fed a high-fat diet for 4 weeks while being given. placebo, decaffeinated coffee, and alkaloid caffeine added to decaffeinated coffee. Hyperinsulinemic-euglycemic clamps showed that glucose infusion rates and measures of whole-body metabolic clearance were greater in decaffeinated than in placebo or alkaloid caffeine+ decaffeinated coffee, indicating increased whole-body insulin sensitivity in decaffeinated coffee treated rats. Since the only difference was the addition of alkaloid caffeine to the decaffeinated coffee, they concluded that caffeine antagonizes the beneficial effects of decaffeinated coffee. Therefore chronic administrations of caffeine lead to reduced insulin sensitivity. Keijzers *et al.*, (2002) also reported caffeine can decrease insulin sensitivity in healthy humans, and attributed the effect to elevated plasma epinephrine levels since dipyridamole did not affect glucose uptake. Other investigators (Conde *et al.*, 2012; Tsch *et al.*, 2014; Rustenbek *et al.*, 2014) have however found that chronic caffeine administration lead to improved insulin sensitivity and in some cases even lend to reversal of insulin resistance in high sucrose fed rats. In a study by Conde *et al.*, 2012, reported that Caffeine reversed insulin resistance and hypertension induced by both the high fat (HF) and high sucrose (Hsu) diets. In the HF-fed animals caffeine treatment restored fasting insulin levels to control values and reversed increased weight gain and visceral fat mass. In the HSu group, caffeine reversed fasting hyperglycaemia and restored NEFA to control values. These effects were shown to be due to a decrease in circulating catecholamines.

The chronic administration of kolanut extract lead to improvement of glucose tolerance as seen in improved glucose disposal as well as reduced AUC (glucose). This finding of improved glucose tolerance on chronic consumption of kolanut extract is similar to the effect of caffeine containing coffee on glucose metabolism in which there is glucose intolerance on acute ingestion (Louie *et al.*, 2008; Moisey *et al.*, 2008) but improved glucose tolerance on chronic ingestion. This improvement in glucose tolerance on chronic coffeeconsumption has been documented in a large number of epidemiological studies (van Dam and Feskens, 2004; Salazar-Martinez *et al.*, 2007) but not in animal models under experimental conditions. So far, only one animal study on chronic coffee consumption in the rat by Morakinyo *et al.*, (2014) has corroborated the findings of those epidemiological studies on glucose tolerance in the rat. It is clear from this study that while caffeine produced the same effect on glucose tolerance under acute and chronic conditions, kolanut seemed to exhibit different effects on the glucose tolerance under acute and chronic conditions. Under

chronic conditioos the results show that caffeine and kolanut have antagonistic effect on glucose tolerance. While caffeine exacerbates glucose intolerance kolanut seemed to enhance glucose disposal. A similar observation to that observed in this study, has been reported in chronic coffee consumers in whom chronic coffee drinking bas improved glucose tolerance and reduced incidence of type 2 diabetes. It has beeo speculated that other components of coffee (Shearer *et al.*, 2003) could be responsible for the observed effect. Flavonoids are major components of kolanut which are known to enhance glucose disposal (Thompson *et. al.*, 1983) in many tissues. The possibility of the effect of kolanut being due to antagonism, synergism or mutualism between caffeine and its other components cannot be ruled out. The lack of effect of chronic administration of decaffeinated kolanut on glucose tolerance in the present study would seem to suggest that the effect of other components of kolanut rather than being antagonistic as in coffee are probably synergistic. That is to say that the caffeine on its own does improve glucose tolerance but only does so in the presence of certain substances in the kolanut extract. More studies involving the isolates of the different components and their effects on glucose metabolism will be needed to throw more light on how kolanut affects glucose tolerance

The effect of caffeine EEK and DEK on Insulin resistance

Acute administration of caffeine caused insulin resistance with elevation of insulin levels in the blood in the face of raised blood glucose. These findings are in agreement with previous studies which bave demonstrated stimulation of secretion of insulin on administration of caffeine. Bruton *et. al.*, (2002) showed that increased secretion of insulin on administration of glucose occurred in the presence of 11.2mM of glucose and was probably mediated by its ability to mobilize intracellular calcium. In this study, plasma insulin increased following ingestion of glucose load which invariably suggests that it is the plasma glucose that stimulates insulin secretion thereby showing insulin sensitivity. It is well established that blood glucose is a major stimulant of the β cells of the pancreas. Sacramento *et al.* (2015) found that acute administration of caffeine lead to insulin resistance and that the effect was mediated by A_1 and A_{2B} adenosine receptors. They also found that acute caffeine administration significantly decreased Glut 4, but not AMPK expression and therefore suggested that the insulin resistance induced by acute caffeine administration was due to blockage of A_1 and A_{2B} adenosine receptors and reduced glucose uptake by reduction in Glut 4 translocation.

The acute administration of EEK also caused insulin resistance as shown by increased insulin response and increased AUC (insulin). Since this is the first study to address the effect of acute administration of kolanut on glucose metabolism in the rat, this study is similar to the observations in which caffeine containing coffee was administered acutely. Our findings are similar to that of Johnston *et. al.*, (2003) investigating the effect of administration of caffeinated and decaffeinated coffee found that glucose and insulin concentrations tended to be higher in the first 30 min after caffeinated coffee consumption than aftercoasumption of decaffeinated coffee or the control.

The acute administration of DEK did not cause insulin resistance thereby suggesting that the caffeine content of our kolanut extract was the major factor responsible for the insulin resistance which occurred on administration of kolanut extract.

The finding of insulin resistance on chronic administration of caffeine would also seem to indicate that there is little or no tolerance to the effects of caffeine on insulin resistance. Dekker *et. al.*, (2007) also reported that carbohydrate metabolism remained disrupted after 14 days of caffeine consumption by previously caffeine-naive subjects.

The observed insulin resistance on chronic administration of caffeine is different from what has been observed in similar animal experiments by Yeh *et. al.*, (2014) who found that caffeine consumption reduced serum fasting glucose, insulin, homeostatic model assessment-insulin resistance, and triglyceride levels and increased the serum direct high-density lipoprotein level in fructose-fed mts. Their results suggested that caffeine may enhance insulin receptor substrate 1-phosphatidylinositol 3-kinase/Akt-neuronal nitric oxide synthase signaling to decrease blood pressure by abolishing superoxide production in the NTS. Similarly, Kim *et. al.*, (2015) investigated the clinical changes induced by a high fat diet (HFD) and caffeine consumption in a rat model. They found that mean body weight of the HFD with caffeine (HFDC)-fed rat decreased compared to that of the HFD-fed rat without caffeine. The levels of cholesterol, triglycerides (TGs), and free fatty acid, as well as the size of adipose tissue altered by HFD, were improved by caffeine suggesting that consumption of caffeine might potentially inhibit HFD-induced obesity. The insulin-secretory response to glucose is known to be influenced by different stimulatory and inhibitory factors (Zywert *et. al.* 2007). Dietary compounds such as amino acids (Newsholme *et al.*, 2005), and fatty acids (Nolan *et al.*, 2008) stimulate insulin secretion while the flavonoids tend to inhibit it by reducing the rate of glucose absorption by the gut (Johnston *et al.*, 2003).

The observed improvement in insulin sensitivity as shown in reduced AUC of insulin on chronic administration of kolanut extract for eight weeks is different from findings by Onyeanusi and Ikpe, (2003) which seemed to indicate reduced insulin sensitivity. In their study, administration of Cola acuminata by mixing with the feeds introduced to rats for six weeks lead to increase in basal blood glucose levels and elevated glycosylated haemoglobin. Apart from the difference in the specie of kolanut used (C acuminata as opposed to C nitida), the method of administration of the kolanut may account for the difference in findings.

The effect of caffeine EEK and DEK on liver glycogen content.

Liver glycogen content depletion observed on acute caffeine treatment despite glucose challenge was not unexpected given the reduced glucose disposal and insulin resistance. Under normal circumstances when plasma glucose levels rise (as has occurred with glucose loading of the rats in this experiment), the liver clears glucose and stores it as glycogen. The opposite process, degradation of glycogen to glucose occurs when blood glucose level falls (Greenberg *et. al.*, 2006). The majority of postprandial glucose is stored in skeletal muscle by virtue of its greater proportionate mass accounting for 50-80% of glucose clearance after the ingestion of a carbohydrate load (DeFronzo *et. al.*, 1982; Dent *et. al.*, 1990). Glucose transport is a key element in insulin sensitivity, and skeletal muscle largely accounts for the decreased insulin sensitivity observed in obese individuals and those with type 2 diabetes (Bonadonna *et. al.*, 1993). The observed liver glycogen depletion in the face of high blood glucose levels could have been the result of reduced glycogenesis a process which is essential for glucose disposal in the postprandial state or increased breakdown of glycogen. The caffeine administered seems to have prevented glycogenesis by inhibiting the uptake of glucose in the liver and possibly also in the skeletal muscles. The uptake of glucose and its subsequent incorporation into the glycogen molecule are processes regulated by insulin. Conde *et. al.*, (2012) reported that acute caffeine administration decreased insulin sensitivity in a dose-dependent manner, the effect probably being mediated by A1 and A2B receptors with a probable decrease in Glut4 expression in skeletal muscle. Furthermore, acute administration of the different adenosine receptors antagonist did not modify arterial pressure, fasting insulin and glucose levels. Caffeine has been shown to directly stimulate insulin secretion in the presence of glucose (Bruton *et. al.*, 2002), by releasing Ca^{2+} from

intracellular stores. The possibility of caffeine having similar action on liver glucose uptake and glycogen deposition cannot be ruled out.

On chronic administration of EEK, the liver glycogen deposition increased proportionately to the insulin response and increased glucose disposal. There is evidence of increased glucogenesis as distinct from glycogen depletion that seems to occur in the acute administration Gonzalez-Bermicz *et. al.*, (2002) identified the adenosine receptor subtype and events involved in the regulation of hepatic glycogen metabolism by measuring glycogenolysis, gluconeogenesis, cAMP, and cytosolic Ca2+ in isolated hepatocytes challenged with adenosine A1, A2A, and A3 receptor-selective agonists. They found that in isolated rat hepatocytes activation of the adenosine A1 receptor triggered Ca2+-mediated glycogenolysis, activation of the adenosine A2A receptor stimulated cAMP-mediated gluconeogenesis. Given that caffeine's major mechanism of action is by non-specific adenosine receptor antagonism we would have expected that administration of caffeine could result in direct inhibition of glycogenolysis and gluconeogenesis leading to and preservation of glycogen stores and reduction in blood glucose levels. The question would then arise as to why this process did not occur during the acute administration of caffeine. It can only be surmised that the changes leading to improved insulin sensitivity are gradual and may involve subtle changes in the genetic machinery of the hepatocytes and probably involve certain compounds in the kolanut extract working in synergy with the caffeine content of kolanut. The decaffeinated kolnnut treated rats showed no comparable improvement in insulin sensitivity probably because the residual caffeine was insufficient to manifest the effects.

The effects of Caffeine EEK and DEK on liver glycogen synthase activity

Acute caffeine administration resulted in reduction of glycogen synthase activity which is in line with the findings of reduced liver glycogen concentration. However, it is well known that an increase in blood glucose triggers the release of insulin from the pancreas which combines with the blood glucose to commence accumulation of glycogen by activating glycogen synthase in the liver and skeletal muscles (Villar-Palasi and Guinovart, 1997). Glycogen synthase activity is allosterically stimulated by glucose 6-phosphate which is in turn phosphorylated from glucose by the action of the enzyme hexokinase (Salvert *et. al.*, 1979). It is possible that the prevention of glucose uptake by the liver is responsible for the reduced activityof the glycogen synthase. Acute administration of EEK followed the same pattern showing a reduction in glycogen synthase activity.

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The effects of Caffeine EEK and DEK on liver glycogen phosphorylase activity

Acute administration of caffeine had no effect on glycogen phosphorylase activity. Kavinsky *et al.*, (1978) described a synergistic effect of caffeine on glucose inhibition of glycogen phosphorylase activity. Given the fact that the rats were challenged with a glucose load, synergism of the caffeine should have depressed the phosphorylase activity in comparison to the control. Rather interestingly the glycogen phosphorylase activity was greatly reduced by administration of EEK. This may be due to enhancement of caffeine actions by some other components of the kolanut.

Conclusion

In conclusion the results of this study has shown that the effects of caffeine and kolanut extract on glucose metabolism in the rat are essentially similar following acute administration while both exhibit different characteristics following chronic administration. Following acute administration of caffeine and ethanol extract of kolanut, there was a significant reduction in glucose tolerance in the rat, a decrease in insulin sensitivity, decrease in liver glycogen content and a decrease in both glycogen synthase and glycogen phosphorylase activity. However, decaffeinated ethanol extract of kolanut had no effect on these parameters. Following chronic administration of kjolanut, caffeine and decaffeinated kolanut, the glucose tolerance decreased for caffeine treated rats, increased for rats treated with the ethanol extract of kolanut, but was not affected by decaffeinated kolanut. On the other hand, insulin sensitivity was decreased by caffeine, increased by ethanol extract of kolanut but also not affected by decaffeinated kolanut. Again while liver glycogen was decreased by caffeine, but increased in response to kolanut extract the decaffeinated kolanut had no effect. Chronic administration of kolanut extract may have improved insulin sensitivity through the action of caffeine in synergy with as yet unidentified components of kolanut.

Contributions to Knowledge

1. Caffeine content of the kolanut extract in this study was significantly higher than previous reports.
2. This is the first study to report the effects of decaffeinated kolanut extract on glucose metabolism in the rat.
3. This study showed that chronic consumption of kolanut promotes glucose tolerance and insulin sensitivity

4. The study showed that chronic kolanut consumption inceased liver glycogen content by increasing liver glycogen synthase activity and decreasing liver glycogen phosphorylase activity
5. The study showed that decaffeinated kolanut had no significant effect on glucose tolerance and insulin sensitivity.

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OGTT following acute administration of caffeine and extracts (mg/dL)

	0 min	30 mins	60 mins	90 mins	120 mins
Control	85.9±3.4	132.9±4.0	144.4±3.6	128.9±2.1	97.1±4.4
CAF	107.0±2.1	139.6±4.3	148.0±1.8	132.3±1.8	121.1±1.9
EEK	89.6±5.7	123.3±3.0	134.4±2.4	117.4±3.0	95.0±2.3
DEEK	99.5±1.8	129.6±4.0	120.4±4.7	121.8±5.4	110.8±3.6

OGTT following chronic administration of caffeine and extracts (mg/dL)

	0 mins	30 mins	60 mins	90 mins	120 mins
Control	87.6±2.4	141.1±2.2	133.4±2.1	127.6±1.0	89.2±1.5
CAF	107.0±2.1	139.6±4.3	148.0±1.8	132.3±1.8	121.1±1.9
DEK	87.3±1.2	137.3±1.4	125.0±2.0	118.8±1.9	89.3±1.5
DEEK	99.5±1.8	129.6±4.0	120.4±4.7	121.8±5.4	110.8±3.6

Appendix I: AUC Glucose during OGTT following acute treatment (mg.min/dL)

	Control	CAF	EEK	DEEK
1	11790	13065	12615	11840
2	11215	14260	13090	11820
3	12390	13605	13480	11520
4	11575	13830	12645	11130
5	11535	14700	13870	11310
6	11570	13935	12610	11085
Mean	11679.2±143.7	13899.2±201.3***	13051.7±199.4**	11450.8±166.5

Appendix 2: AUC Glucose during OGTT following chronic treatment (mg.min/dL)

	Control	CAF	EEK	DEEK
1	15070	15995	13820	14030
2	13755	15570	14415	14480
3	15810	15665	13980	13700
4	15580	16620	14535	13940
5	15075	15725	13635	15605
6	14280	16590	13845	14085
Mean	14928.3±325.4	16027.5±188.1**	14038.3±228.1*	14306.7±326.9

Plasma insulin concentration during OGTT following acute administration of caffeine and extracts. n =6 (ng/mL). Values are Mean±S.E.M

	0 min	30 mins	60 mins	90 mins	120 mins
Control	0.7±0.02	1.78±0.06	1.19±0.04	0.89±0.02	0.62±0.01
CAF	1.08±0.06	3.12±0.22	2.04±0.15	1.23±0.06	0.87±0.04
EEK	0.89±0.03	2.23±0.17	1.14±0.04	0.92±0.02	0.67±0.17
DEEK	0.8±0.02	1.88±0.04	1.33±0.04	0.96±0.04	0.74±0.03

Plasma insulin concentration during OGTT following chronic administration of caffeine and extracts, n =6 (ng/mL). Values are Mean±S.E.M

	0 min	30 mins	60 mins	90 mins	120 mins
Control	0.93±0.04	1.51±0.06	1.13±0.06	0.89±0.05	0.76±0.05
CAF	1.14±0.06	1.75±0.1	1.35±0.06	1.14±0.05	0.99±0.04
EEK	0.71±0.05	1.34±0.06	0.99±0.06	0.85±0.03	0.69±0.04
DEEK	0.83±0.05	1.31±0.03	1.06±0.04	0.89±0.03	0.79±0.04

AUC Insulin following OGTT in acute treated rats n = 6 (ng.min/mL)

	Control	CAF	EEK	DEEK
	138.2	210.4	165.2	145.5
	135.0	243.4	157.0	159.7
	132.2	209.5	153.8	140.2
	132.3	221.4	173.5	143.9
	126.6	227.9	178.6	148.1
	139.2	225.8	148.4	151.4
Mean	133.9±1.9	223.0±5.1***	162.7±4.8*	148.1±2.8

AUC Insulin following OGTT in chronic treated rats n = 6 (ng min/mL

	Control	CAF	EEK	DEEK
1	110.5	159.0	118.5	114.0
2	127.5	168.0	110.5	132.0
3	140.5	157.5	101.5	134.5
4	130.0	143.0	105.0	138.5
5	141.5	177.0	129.0	151.0
6	139.5	138.0	122.5	131.0
Mean	131.6±4.8	157.1±6.0	114.5±4.3*	133.5±4.9

Liver glycogen content (mg/100g) in Acute treated glucose challenged rats n =6 (**** p < 0.0001)

	Control	CAF	EEK	DEEK
1	2567	994	1204	2846
2	2142	1272	1374	2308
3	2490	1071	1366	2131
4	2644	1254	1251	3106
5	2272	1019	1021	3016
6	1997	1159	1256	2816
Mean	2352.0±104.4	1128.2±48.5****	1245.3±56.7****	2703.8±160.9

Liver glycogen content (mg/100g) in chronic treated glucose challenged rats n =6 (*** p < 0.001)

.	Control	CAF	EEK	DEEK
1	1258	1368	2267	1884
2	1490	1225	1767	1296
3	1176	1170	2150	1396
4	1234	1305	1637	1606
5	1666	1198	1866	1679
6	1290	1015	2237	1617
Mean	1352.3±76.4	1213.5±49.6	1987.3±108.5***	1579.7±85.4

Glycogen synthase activity ng/mg protein in chronic treated glucose challenged rats n = 6, (*** = p<0.001, **** = p<0.0001)

	NA	CAFFEINE	KOLANUT	DECAFF
1	2711.28	1051.69	1439.88	2193.2
2	2341.19	787.06	871.71	1978.04
3	1783.45	689.76	1261.58	2624.8
4	2501.68	816.6	1081.68	1845.08
5	2278.87	1188.43	974.34	2506.8
6	2118.34	1345.79	1264.8	2951.86
Mean	2289.1±135.5	979.9±105.0****	1149.0±86.1***	2350.0±171.3

Glycogen synthase activity ng/mg protein in chronic treated glucose challenged rats n=6, (** = p<0.01)

	Control	CAF	EEK	DEK
1	1800	1500	2400	1800
2	1800	1800	3000	1400
3	800	2100	2000	2700
4	900	1500	2000	1500
5	1100	1100	1900	1700
6	1200	1600	3500	1600
Mean	1270±154.4	1600±118.3	2470±229.5**	1780±190.0

Glycogen phosphorylase activity pg/mg protein in chronic treated glucose challenged rats n = 6. (** = p<0.01)

	Control	CAFFEINE	KOLANUT	DECAFF
1	20.18	21.6	20.1	19
2	23.1	21.2	16.08	22
3	23.14	17.3	19.06	20
4	19.08	18.5	15.03	20
5	19.2	23.4	18.02	22
6	23.14	20.4	18.07	21
Mean	21.3±1.1	20.4±1.3	17.6±0.9**	20.8±0.7

LAUTECH HERBARIUM,
LADOKE AKINTOLA UNIVERSITY OF TECHNOLOGY,
OGBOMOSO, NIGERIA

CONFIRMATION OF PLANT IDENTITY

This is to Certify that the Identity of the Plant Material with Details of Collection and Voucher Information as stated hereunder, was confirmed at
LAUTECH Herbarium, Ogbomoso, Nigeria

Current Plant Name: *Cola nilida* (Vent.) Schott & Endl.

Synonym(s): *Cola vera* K. Schum., *Bichea nilida* (Vent.) Farw,
Cola acuminata (P. Beauv.) Schott & Endl. var. latifolia K.

Common Names: Kolanut, bitter kola. (Yor. Obi goro)

Family: Malvaceae
Order: Malvales
Part(s) Collected: Fruits
Date of Collection: 15/05/2010
Name of Collector & Collection number: Dr. Oladele A. Afolabi
Place of Collection: Purchased from Ilobu market, Osun state
Short Description of the Plant/ Habitat:
Voucher Number: LHO 442
Earlier Collections: LHO 431and 416

This Testimonial is hereby issued under my Authority in favour of

..... Dr. Oladele A. Afolabi .....
(Researcher/Research student/ Other)

of

..... Dept. of Physiology, LAUTECH, Ogbomoso .....
(Address)

Date..... September 4th 2015

ATJ Ogunkunle, *Ph.D.*
(Angiosperm Taxonomist)
Determinavit