EFFECTS OF ADMINISTRATION OF *HIBISCUS SABDARIFFA* AQUEOUS CALYX EXTRACTS ON NEONATAL PROGRAMMING OF METABOLIC DYSFUNCTION
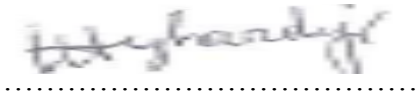
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A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science in Medicine

Johannesburg, 2015

DECLARATION

I, Kasimu Ghandi Ibrahim hereby declare that this dissertation is my own work, with the assistance of the acknowledged persons. It is being submitted for the degree of Master of Science in Medicine in the Faculty of Health Sciences in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University. All procedures used in this dissertation were approved by the Animal Ethics Screening Committee of the University of the Witwatersrand (AESC number 2013/46/05).

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Kasimu Ghandi Ibrahim

Signed on this 10th day of April, 2015

DEDICATION

To the loving memory of my father and sister

-Late Ibrahim Ghandi Dogondaji

(1935-2001)

-Late Amina Bello Dogondaji

(1961-2001)

both of whom did not live long enough to call me 'doctor'.

May you continue to rest in perfect peace in Jannatul Firdaus, amin.

CONFERENCE PRESENTATION

The following poster was presented at the 42nd congress of the Physiology Society of Southern Africa hosted by the University of Kwazulu- Natal at the Gateway hotel, Umhlanga, Durban, from the 14th- 17th September, 2014.

1. **Ibrahim, K.G., Chivandi, E., Mojiminiyi, F.B.O. and Erlwanger, K.H.** Effects of *Hibiscus sabdariffa* aqueous calyx extracts on the gastrointestinal tract and clinical biochemistry of suckling rats (poster A20 in the conference book of abstracts).

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ABSTRACT

Hibiscus sabdariffa (HS) is a plant of the Malvaceae family that has anti-obesity, antihypertensive, hypocholesterolaemic, anti-oxidant and anti-cancer properties. Dietary manipulations during the suckling period cause precocious maturation of the gastrointestinal tract (GIT). Events in early life affect the individual's metabolic health in later life. This study investigated the effects of early administration of the aqueous calyx extracts of HS on the growth performance, general health and the GIT of neonatal rats and whether it conferred protection or predisposition to the development of metabolic dysfunction in adolescence.

The study was carried out in two phases. The first phase was to determine the effects of HS aqueous calyx extracts on the growth performance, metabolic substrates and the development of the neonatal rat GIT. In the second phase, the effects of the early administration of the HSE on the response of the pups to diet induced metabolic dysfunction were investigated.

In phase one, forty two 4-day old Sprague Dawley pups of both sexes were randomly assigned to three treatment groups. Each group consisted of 7 males and 7 females. The control group received distilled water at 10ml.kg^{-1} b.w while the other two groups received either a low (50mg.kg^{-1}) or high dose (500mg.kg^{-1}) of the HS aqueous calyx extracts via oral gavage daily for 9 consecutive days. The rats were euthanased and their tissues harvested and analyzed. Pups that were administered with the high dose HSE had significantly heavier small intestines relative to the body mass when compared to those on the low dose HSE (P<0.01) and the control group (p<0.001). Pups in the high dose HSE group had significantly heavier caeca (p<0.05) than those in the low dose HSE group.

In the second phase, eighty five 4-day old Sprague Dawley rat pups were used. They were initially divided randomly into three groups and received similar treatments as in phase one up to postnatal day (PND) 14. There was no intervention from PND14 to PND 21 when the pups were weaned. The rats in each of the treatment groups were further divided into a control group that continued on their normal rat chow diet and a test group that received high fructose (20% w/v) in their drinking water for 30 days in order to induce metabolic dysfunction. Each of the six study groups had at least 5 male and 5 female rats. The male rats in each of the treatment groups gained more body mass than their corresponding female counterparts in the control and treatment groups ($p<0.001$). Female rats that received high dose HSE in the neonatal period had significantly greater visceral fat pad ($p \leq 0.05$) than the males in the groups. There were no negative effects on the rats' general health. At the end of the study, features of metabolic syndrome did not manifest in the control or any of the treatment groups. *Hibiscus sabdariffa* aqueous calyx extracts did not exhibit any long term effects and therefore may be considered safe for consumption in the neonatal age group.

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ABBREVIATIONS

α: Alpha

β: Beta

γ: Gamma

%:   Percent

AACE: American Association of Clinical Endocrinologists

AESC: Animal Ethics Screening Committee

ALT: Alanine aminotransferase

ALP: Alkaline phosphatase

AMPK: Adenosine monophosphate-activated protein kinase

ANOVA: Analysis of variance

AST: Aspartate aminotransferase

ATP III: Adult treatment panel three

BM: Body mass

BUN: Blood urea nitrogen

CAS: Central animal services

CCl$_4$:   Carbon tetrachloride

Creat: Creatinine

CTRL: Control

DNA: Deoxyribonucleic acid

DW: Distilled water

EGSIR: European group for the study of insulin resistance

ELISA: Enzyme-linked immunosorbent assay

FBG: Fasting blood glucose

FW: Fructose water

GFR: Glomerular filtration rate

GIT: Gastrointestinal tract

HCA: Hydroxycitric acid

HDL: High density lipoprotein

HD HSE: High dose *Hibiscus sabdariffa* extract

HHS: High dose *Hibiscus sabdariffa*

HOMA-IR: Homeostatic model of insulin resistance

HS: *Hibiscus sabdariffa*

IDF: International Diabetes Federation

IGF-1:   Insulin growth-like factor 1

IL-1:   Interleukin 1

LDH: Lactate dehydrogenase

LD HSE: Low dose *Hibiscus sabdariffa* extract

LDL: Low density lipoprotein

LI: Large intestines

LHS: Low dose *Hibiscus sabdariffa*

MDA: Malonyldialdehyde

mRNA: Messenger ribonucleic acid

MS: Metabolic syndrome

NCEP: National cholesterol education program

OGTT: Oral glucose tolerance test

PPAR: Peroxisome proliferator activated receptor

SD: Standard deviation

SHRs: Spontaneously hypertensive rats

SI: Small intestines

SREBP: Sterol regulatory element binding protein

STZ: Streptozocin

TAG: Triacylglyceride

TBARS: Thiobarbituric acid reactive substances

Tbil: Total biluribin

TG: Triglycerides

TNF: Tumour necrosis factor

TW: Tap water

USA: United States of America

VLDL: Very low density lipoprotein

WHO: World health organisation

w/v: weight/volume

CHAPTER ONE: INTRODUCTION AND JUSTIFICATION

1.0 Preface

Modernization has brought with it several issues amongst which are those that affect the health of mankind. Sedentary lifestyles combined with an increase in the consumption of energy-packed foods result in under-expenditure of daily caloric intake.

Consequently, the incidence of metabolic dysfunction such as obesity, dyslipidaemia and hyperglycaemia are on the increase worldwide. The aggregation of these metabolic problems in an individual is called metabolic syndrome, a condition which predisposes to the development of cardiovascular disease and type 2 diabetes mellitus. The incidence of childhood obesity and its associated medical and social complications is on the increase.

Events in the foetal and early neonatal environment have been linked to the development of metabolic syndrome in the adolescent and adult life. In the current management of metabolic syndrome, its risk factors are targeted with drugs such as fenofibrate, antihypertensives and hypoglycaemic agents. However, in many communities, medicinal plants are used because of their affordability and availability as opposed to the conventional drugs. While some of these plants have been fully characterized and their uses documented, there are many others whose potential beneficial or harmful effects have not been explored.

Hibiscus sabdariffa (HS) is one of such plants that have been exploited by researchers due its many scientifically proven activities which include antihypertensive, antidiabetic,

hypocholesterolaemic and anticancer among others. Extracts from its calyces contain a battery of phytochemicals including anthocyanins. The latter have antioxidant properties and protect the body against peroxidative damage. A detailed discussion on HS will follow in this chapter regarding its (HS) many uses and potential.

Hibiscus sabdariffa extracts are usually prepared into decoctions which are ingested orally thus coming into contact directly with the gastrointestinal tract (GIT). Research has shown that some plant extracts when administered during the suckling period can cause precocious maturation of the GIT (Linderoth et al., 2005).

This study therefore looked at the effects of HS calyx extracts on the growth performance, clinical biochemistry, and neonatal GIT morphometry; and also investigated whether its consumption in early life conferred some form of protection or predisposition to the development of metabolic dysfunction in the growing period.

1.1 Structure of the dissertation

This dissertation was written in five chapters. In the first chapter a general introduction was provided which included a discussion on the definitions and prevalence of metabolic syndrome. The botanical classification, traditional uses, phytochemistry, pharmacology and toxicology of *Hibiscus sabdariffa* plant were provided. The chapter also covered the justification, aims and objectives and the hypotheses of the study.

In the second chapter, the first phase of the study was reported. It covered discussions on the GIT, phytochemicals and their effects on health and the effects of HS on the GIT. The first experiment was described under the headings: materials and methods, results, discussion and conclusion.

The second phase of the study was presented in the third chapter. An introductory discourse was given on neonatal programming, childhood obesity and fructose-induced metabolic dysfunction. The materials and methods, results, discussion and conclusions of the second experiment were also presented in this chapter.

The fourth chapter covered the conclusions and recommendations that arose from the study while in the fifth chapter a list of all the references cited in the study was provided. Some appendices are also included.

1.2 Introduction

Metabolic syndrome, a condition which predisposes those affected to the development of type 2 diabetes mellitus and cardiovascular disease (Ford and Li, 2008), is a collection of risk factors that consist of several risk correlates of metabolic origin (Grundy, 2008). It is a cluster of anthropometric, physiological and biochemical abnormalities linked together by poorly understood underlying mechanisms (Ford and Li, 2008).

A clustering of several metabolic risk factors was first described by Reaven, (1997) a condition he called 'syndrome x' which he also recognized as a multiplex factor for cardiovascular disease. Other researchers postulated that insulin resistance underlies syndrome X (Grundy et al., 2004), hence the commonly used term 'insulin resistance syndrome". Syndrome x has also been referred to as the 'plurimetabolic syndrome' (Reaven, 1997) or simply 'metabolic syndrome'.

The World Health Organization (WHO) was the first to define the clinical criteria for the diagnosis of metabolic syndrome (Reaven, 2006; Zimmet et al., 2007; Grundy, 2008). The WHO report identified insulin resistance as the principal cause, making it necessary to identify its clinical indicators such as impaired glucose tolerance, impaired fasting glucose or insulin resistance for diagnosis (Grundy, 2008).

The National Cholesterol Education Program (NCEP) Adult Treatment Panel III (ATP III) recognized the critical role of abdominal obesity in the pathogenesis of metabolic syndrome and replaced the need to demonstrate insulin resistance with an increased waist circumference (NCEP, 2002). There are many other definitions of metabolic syndrome such as that by the American Association of Clinical Endocrinologists (AACE), the International Diabetes Federation (IDF) and European Group for study of Insulin Resistance (EGSIR).

Of all these definitions, the NCEP ATP III is simpler to implement in research and clinical practice (Ford and Li, 2008; Parikh and Mohan, 2012). It also considers the proinflammatory and prothrombotic states as components of metabolic syndrome though they have not been included among the criteria necessary for its definition and diagnosis (Parikh and Mohan, 2012).

Based on the NCEP's findings the primary clinical outcome of metabolic syndrome is cardiovascular disease. The presence of any three of the following: central/abdominal obesity, high fasting triglycerides, high fasting blood glucose, low fasting HDL cholesterol and high blood pressure qualifies as metabolic syndrome (NCEP, 2002).

There is an increasing incidence of metabolic dysfunction associated with metabolic syndrome.

1.3 Prevalence of metabolic syndrome

Two major factors account for an increase in the prevalence of metabolic syndrome (MS) worldwide; plentiful supply of inexpensive energy-packed foods and sedentary lifestyles.

Park et al., (2007) reported a prevalence of 20% for MS among the US adult population emphasizing that it varies with ethnicity and is usually associated with modifiable risk factors. In a study involving 8608 participants aged less than 20 years, Ford and Giles, (2003) reported an age-adjusted prevalence of 23.9% using the ATP III criteria and 25.1% using the WHO criteria. A low prevalence of 1.2% was reported among young Jamaican adults in a cross-sectional study with a 16% prevalence for abdominal obesity and 47% for low HDL cholesterol (Ferguson et al., 2010). Among North Indian adolescents, a prevalence of 3.5% was reported using the ATP III criteria and 1.5% using the IDF criteria (Bhat et al., 2014), while in Korea an increase in age-adjusted prevalence of metabolic syndrome was noticed from 24.9% in 1998 to 31.3% in 2007 (Lim et al., 2011).

In Africa, a high prevalence of MS has been observed. An urban population based study found a prevalence of 34.6% in Kenya (Kaduka et al., 2012), while Gyakobo et al., (2012) in Ghana, reported a prevalence of 15% using the ATP III criteria and 35.9% according to the IDF criteria. A prevalence of 12.1 % was reported for a rural (Adegoke et al., 2010) and 18% for a semi-urban community (Ulasi et al., 2010) in Nigeria. In the Republic of South Africa, an age-adjusted prevalence of 22.1% was reported for a rural population with a higher prevalence in females (25.0%) than in males (10.5%) Motala et al., (2011).

1.4 Current treatment of metabolic syndrome

The current treatment modalities for MS are directed at the management of the individual risk factors with a view to preventing the development of cardiovascular disease and type 2 diabetes mellitus. While lifestyle and dietary modifications resulting in a modest weight loss could improve all the components of MS (Wagh and Stone, 2004), some patients may require drug therapy (Staels et al., 1998; Wagh and Stone, 2004). Some of the drugs used to manage the risk factors of metabolic syndrome include lipid lowering agents such as fibrates (fenofibrate and bezafibrate), statins and niacin (Jialal and Smith, 2012). Hypoglycaemic agents such as metformin, sulfonylureas and thiazolidinedion are used to control hyperglycaemia (Florez et al., 2014). These conventional drugs are not readily available and affordable to most communities in the developing world (Ayyanar et al., 2008). Medicinal plants which have already gained folkloric prominence in the management of many ailments including chronic non-communicable diseases therefore become an alternative (Fyhrquist et al., 2006).

Consequently, there is increasing global interest in research with the aim of finding and documenting scientific evidence to support the folkloric claims associated with these plants (Dillard and German, 2000). *Hibiscus sabdariffa* is one of such plants that have received much research interest and is the plant of interest in this study.

1.5 *Hibiscus sabdariffa*

Hibiscus sabdariffa (HS) is an annual shrub which belongs to the Malvaceae family (Mahadevan et al., 2009; Maganha et al., 2010). It is called Roselle in English speaking countries while in North Africa, it is called *karkade* or carcade (Morton, 1987). It can be grown as a mono crop or mixed with other crops (Da-Costa-Rocha et al., 2014). It is thought to be native to Asia or tropical Africa where it is grown as a garden crop. In Sudan HS is a major export crop (Leung and Foster, 1996; Gautam, 2004).

Figure 1.1: *Hibiscus sabdariffa* plant in Sokoto, North West Nigeria (Photo taken by KG Ibrahim on 10/19/2014)

1.5.1 Botanical description

HS belongs to the kingdom Plantae, division Angiosperms, class Eudicots, order Malvales, family Malvaceae, genus *Hibiscus* and species *sabdariffa* (Pfeil and Crisp, 2005). It can grow to 2.4 meters or more in height(Alarcon-Aguilar et al., 2007), and has a deep penetrating tap root (Mahadevan et al., 2009). It has a smooth cylindrical, typically dark green to red stems (Leung and Foster, 1996; Maganha et al., 2010). The leaves are alternate, 7.5-12.5cm long, green with reddish veins and long petioles. The flowers are hermaphrodite and are insect pollinated (Mahadevan et al., 2009). It has a red calyx that consists of 5 large sepals with an epicalyx and bracteoles around the base (Da-Costa-Rocha et al., 2014). The capsule turns brown and splits open when it is matured and dry. The extracts of the calyx, stems and leaves are acidic (Julia, 1987; Ross, 2003; Da-Costa-Rocha et al., 2014).

1.5.2 Traditional and economic uses of HS

The different parts of the plant have many traditional uses in different communities across the world.

1.5.2.1 Uses of HS calyces

Of the different parts of the plant, the calyces are the most extensively used for both traditional and medicinal purposes. Fresh and dried HS calyces are used in the preparation of hot or cold beverages (Babalola et al., 2001; Herrera- Arellano et al., 2004), herbal drinks, fermented drinks, wine, jam, confectionaries, ice cream, chocolates, flavouring agents, puddings and cakes (Gaya et al., 2009; Ojeda et al., 2010; Da-Costa-Rocha et al., 2014). The calyces are boiled and processed into a local soft drink known as "Sobo" in Nigeria (Mojiminiyi et al., 2012) or "agua de Jamaica" in Mexico (Alarcon-Aguilar et al., 2007).

Hibiscus sabdariffa calyces are used to treat cardiac and nerve ailments and also to induce diuresis (Da-Costa-Rocha et al., 2014; Patel, 2014). An infusion of the calyces has also been used to lower body temperature (Leung and Foster, 1996) and to treat drunkenness (Morton, 1987). Extracts of the calyces are also used in North Africa as a remedy for sore throats and cough as well as genital problems (Da-Costa-Rocha et al., 2014; Patel, 2014).

Figure 1.2: Dried calyces of *Hibiscus sabdariffa* (Photo taken by KG Ibrahim on 08/20/2014)

1.5.2.2 *Hibiscus sabdariffa* leaves

The leaves of HS are consumed raw or cooked as a sour-flavoured vegetable or condiment (Ismail et al., 2008b). In Sudan and Malaysia they are eaten green or dried cooked with onions and ground nuts (Ismail et al., 2008b).

1.5.2.3 *Hibiscus sabdariffa* seeds

The seeds of HS are eaten roasted or ground into meals (Wilson and Menzel, 1964). As powders, they are used in meals such as oily soups and sauces or as a substitute for coffee (Morton, 1987; Atta and Imaizumi, 2002). *Hibiscus sabdariffa* seeds are also used in the treatment of indigestion and dysuria (Morton, 1987), and for inducing or enhancing lactation (Gaya et al., 2009).

1.5.3 Phytochemistry of HS calyces

Hibiscus sabdariffa phytochemistry has been extensively studied and documented. For the purpose of this study, only the phytochemical composition of the calyces will be reviewed.

1.5.3.1 Nutritional value of HS calyces

The reported nutritional value of HS calyces differs between studies (see table 1.1). These differences could result from the variety of HS, soil type, harvesting and processing practices employed (Da-Costa-Rocha et al., 2014).

Table 1.1: Nutritional composition of HS calyces

Nutrients	(Mahadevan et al., 2009)	(Ismail et al., 2008a)
Protein	1.145g/100g	1.9g/100g
Fat	2.61g/100g	0.1g/100g
Fibre	12.0g/100g	2.3g/100g
Ash	6.90g/100g	*
Calcium	12.63mg/100g	1.72mg/100g
Phosphorus	273.2mg/100g	*
Iron	8.98mg/100g	57mg/100g
Carotene	0.029mg/100g	300µg/100g
Thiamine	0.117mg/100g	*
Riboflavin	0.277mg/100g	*
Niacin	3.765mg/100g	*
Ascorbic acid	6.7mg/100g	14mg/100g
Carbohydrates	*	12.3g/100g

*= not reported

1.5.3.2 Organic acid content of HS calyces

Hibiscus sabdariffa calyces contain a high percentage of organic acids including citric acid, hydroxycitric acid, hibiscus acid, malic and tartaric acids as major organic acids and oxalic and ascorbic acid as minor organic acids (Mahadevan et al., 2009). The ascorbic acid content in the calyces varies between fresh (6.7-1.4mg/100g) (Morton, 1987; Ismail et al., 2008b) and dried calyces (260-280mg/100g) (Ismail et al., 2008b).

Hydroxycitric acid, hibiscus acid and its derivatives are the principal organic acids found in the calyces and leaves (Hida et al., 2007; Peng et al., 2011; Ramirez-Rodrigues et al., 2011b; Herranz-López et al., 2012). The hydroxycitric acid in HS calyces is the (+)- hydroxycitric acid also known as (+)-HCA (Yamada et al., 2007). Its Isomer (-)-HCA, is an inhibitor of citrate lyase (Sullivan et al., 1974b), and has been proposed as an anti-obesity agent (Sullivan et al., 1974a; Sullivan et al., 1974b; Hayamizu et al., 2003). In rats, hydroxycitric acid in HS calyx extract inhibits fat production from carbohydrates (Tee et al., 2002). It has been suggested that racemization of (+)-HCA to (-)-HCA by the intestinal flora may be an explanation to warrant the significant decrease in triacylglycerols in the experiment carried out on rats supplemented with HS calyx extracts (Carvajal-Zarrabal et al., 2009).

1.5.3.3 Anthocyanin content of HS

The anthocyanins are a group of flavonoid derivatives and natural pigments present in the dried flowers of HS whose (anthocyanins) colour varies with pH (Da-Costa-Rocha et al., 2014). These pigments have been researched extensively because of their recognized antioxidant activity (Alarcon-Alonso et al., 2012; Herranz-López et al., 2012).

Yamamoto and Oshima, (1932) identified the first anthocyanin from the calyces of HS "hibiscin" or "hiviscin" to which they assigned the structure cyanidin-3-glucoside and then later renamed it to delphinidin-pentoside-glucoside (Yamamoto and Oshima, 1936). Other anthocyanins including delphinidin-3-glucoside, cyanidin-3-glucoside (Shibata and Furukawa, 1969; Du and Francis, 1973) and cyanidin-3-sambubioside (gossipicyanin) (Shibata and Furukawa, 1969) were also identified. Subramanian and Nair, (1972) reported the presence of cyanidin-3, 5-diglucoside and cyanidin-3-(2G-glucosylrutinoside) in the flower pigments of HS.

Several studies identified delphinidin-3-sambubioside and cyanidin-3-sambubioside as the major anthocyanins in HS calyx extracts (Alarcon-Aguilar et al., 2007; Beltrán-Debón et al., 2010; Peng et al., 2011; Alarcon-Alonso et al., 2012; Herranz-López et al., 2012).

1.5.3.4 Flavonoid content of HS

Hibiscus sabdariffa contains simple and polymerised forms of polyphenolic compounds (Da-Costa-Rocha et al., 2014). Hibiscitrin, sabdaritrin, gossypitrin, gossytrin, quarcetin, luteolin, chlorogenic acid, procatechuic acid, pelagonidic acid and eugenol have been described in HS extracts (Mckay, 2009). Alarcon-Alonso et al., (2012) reported that the amount of quarcetin present in the aqueous extracts of HS calyces was 3.2mg/g while rutin was 2.1mg/g. Quarcetin and its conjugated glycosides as well as rutin were also identified in aqueous extracts of HS calyces together with kaempferol (Beltrán-Debón et al., 2010; Ramirez-Rodrigues et al., 2011a; Herranz-López et al., 2012).

Aqueous calyx extracts of HS showed the presence of procatechuic acid (24.24%), catechin (2.67%), gallocatechin (2.44%), caffeic acid (19.85%) and gallocatechin gallate (27.98%) (Huang et al., 2009; Yang et al., 2009).

Procatechuic acid (PCA), an important phenolic acid in the calyces of HS (Lee et al., 2002; Mckay, 2009), has been found to inhibit skin tumour promotion in mice (Tseng et al., 1998). *Hibiscus sabdariffa* calyces and leaves contain chlorogenic acid ; a phenolic (Salah et al., 2002; Beltrán-Debón et al., 2010; Peng et al., 2011; Ramirez-Rodrigues et al., 2011a) which belongs to a family of esters formed between certain tran-cinnamic and quinic acids (Clifford

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et al., 2003). The amount of chlorogenic acid in HS extract was reported to be 2.7mg/g (Alarcon-Alonso et al., 2012).

1.5.3.5 Polysaccharide content of HS

Polysaccharides are present in large quantities in HS. Müller and Franz, (1992) reported a yield of 10% reddish polysaccharides from ethanol-precipitated water extracts of HS calyces. Arabinose, galactose, glucose, rhamnose and smaller amounts of mannose and xylose were identified in two different fractions (Müller and Franz, 1992; Brunold et al., 2004). The petals of HS yielded 65% (dry weight) of mucilage which yielded galactose, and rhamnose on hydrolysis (El-Hamidi et al., 1967).

1.6 Pharmacology and biological activity of HS

Hibiscus sabdariffa has been used in folk medicine in the treatment of many conditions and most of these folkloric claims have been verified scientifically.

1.6.1 Anti-obesity and hypolipidaemic effects

The persistent increase in the global incidence of obesity is worrisome especially as it increases the risk of developing metabolic dysfunction (Villalpando-Arteaga et al., 2013b). *Hibiscus sabdariffa* has been proved to hold prospects in the prevention and treatment of obesity.

Alarcon-Aguilar et al., (2007) induced obesity in mice using monosodium glutamate (MSG) and then administered HS aqueous extracts for 60 days (33.64mg of total anthocyanins per 120mg of extract) to healthy and obese mice. They observed significantly reduced body mass gain in obese mice and increased liquid intake in obese and healthy mice. While investigating the effects of HS ethanol extracts on fat-absorption-excretion and body weight in rats, Carvajal-Zarrabal et al., (2009) concluded that components of HS at intermediate and greater concentrations could serve as anti-obesity agents. These anti-obesity effects could be as a result of the modification of the P13-K/Akt and ERK pathway which plays a vital role in adipogenesis (Kim et al., 2007).

Lin et al., (2007) investigated the effects of HS extract on serum cholesterol in human subjects and found that serum cholesterol was significantly reduced in subjects that took 2 capsules (1g) of HS extract for a month suggesting that HS extracts may be effective in hypercholesterolaemic patients. *Hibiscus sabdariffa* extracts (HSE) was also found to inhibit

serum lipids and to show anti-atherosclerotic activity (Chen et al., 2003). On histology, they found HSE to reduce foam cells formation and inhibit smooth muscle cell migration and calcification in the blood vessels of rabbits. *Hibiscus sabdariffa* extract (HSE) powder significantly reduced glucose and total cholesterol levels, increased TAG/HDL-c ratio, a marker of insulin resistance (Gurrola-Daiz et al., 2010).

A reduction was observed in the levels of LDL and the ratio of LDL-c to HDL-c when HS extracts were administered to high fructose-fed and high cholesterol-fed rats; suggesting that it may be used to inhibit LDL oxidation and to prevent various types of hyperlipidaemia in high fructose-fed and high cholesterol- fed rats (Chen et al., 2004). Aqueous calyx extracts of HS inhibit the accumulation of triglycerides better when the fibre and polysaccharide components have been removed (Herranz-López et al., 2012).

1.6.2 Antihypertensive and cardioprotective effects

Hypertension contributes significantly to the global burden of non-communicable diseases and is responsible for a lot of morbidities and mortalities (Wahabi et al., 2010). Annually hypertension accounts for about 13% of deaths globally (Brown, 1997). *Hibiscus sabdariffa* has been shown to have anti-hypertensive effects in animals (Onyenekwe et al., 1999; Odigie et al., 2003; Mojiminiyi et al., 2007; Inuwa et al., 2012; Mojiminiyi et al., 2012) and humans (Haji-Faraji and Haji-Tarkhani, 1999; Herrera- Arellano et al., 2004; Mckay, 2009;

Mozaffari-Khosravi et al., 2009). Its antihypertensive effect is thought to be mediated via a direct vaso-relaxant effect (Adegunloye et al., 1996; Onyenekwe et al., 1999; Ali et al., 2005), diuretic(Cáceres et al., 1987; Onyenekwe et al., 1999; Mojiminiyi et al., 2000), calcium ion channels modulation (Owolabi et al., 1995) and the inhibition of angiotensin converting enzyme by the anthocyanins (Ojeda et al., 2010).

Hibiscus sabdariffa has been found to exhibit cardio-protective activity by enhancing myocardial capillarization in spontaneously hypertensive rats that were orally fed its aqueous calyx extracts (Inuwa et al., 2012), and by reversing cardiac hypertrophy in 2K-1C (2Kidneys, 1 clip) hypertensive rats (Odigie et al., 2003).

1.6.3 Anti-diabetic effects

Diabetes mellitus is an endocrine disorder associated with hyperglycaemia, dyslipidaemia and results from defects in insulin secretion and/or action (Alberti and Zimmet, 1998; Da-Costa-Rocha et al., 2014). Globally, diabetes mellitus is a highly prevalent disease responsible for high morbidity and mortality (Peng et al., 2011). When HS polyphenolic extract was administered to a type 2 diabetic rat model at a dose of $200mg.kg^{-1}$ it reduced hyperglycaemia, hyperinsulinaemia, advanced glycation end product and lipid peroxidation (Peng et al., 2011). *Hibiscus sabdariffa* was shown to be a potent inhibitor of pancreatic α-amylase (Adisakwattana et al., 2012). The inhibition of pancreatic α-amylase and intestinal α-

glucosidase slows down the digestion of carbohydrates to more absorbable monosaccharides; a therapeutic strategy currently in vogue for the control of post prandial hyperglycaemia (Adisakwattana et al., 2012). The hypolipidaemic and antioxidant properties of HS ethanolic extract were confirmed in alloxan-induced diabetic rats (Farombi and Ige, 2007) where they showed therapeutic potentials in preventing the development of atherosclerosis and other cardiovascular complications associated with diabetes. Similarly, Huang et al., (2009) reported that a polyphenolic extract of HS suppressed the high-glucose stimulated cell proliferation and migration in a dose and time dependent manner.

1.6.4 Anti-oxidant effects

Antioxidants protect the tissues from peroxidative damage (Halliwell, 1997; Olaleye, 2007). Several studies have reported the antioxidant activity of HS (Farombi and Fakoya, 2005; Hirunpanich et al., 2005; Usoh et al., 2005; Christian, 2006; Olaleye and Rocha, 2007; Mohd-Esa et al., 2010; Mossalam et al., 2011). This antioxidant activity is expressed in different ways and by both aqueous and ethanolic extract of the calyces, seeds and leaves (Mohd-Esa et al., 2010). The extract can exert their antioxidant action by scavenging free radicals and reactive oxygen species (Tseng et al., 1998; Farombi and Fakoya, 2005; Usoh et al., 2005), inhibition of xanthine oxidase activity (Tseng et al., 1998) and prevention of cell damage via lipid peroxidation (Farombi and Fakoya, 2005). *Hibiscus sabdariffa* extracts also inhibit the formation of malonyldialdehyde (Farombi and Fakoya, 2005; Usoh et al., 2005) and

oxidation of low density lipoprotein and formation of thiobarbituric reactive substances (TBARS) by Cu^{2+} (Hirunpanich et al., 2005). *Hibiscus sabdariffa* could reduce glutathione depletion and also alter the activities of superoxide dismutase and catalase in the liver and blood (Usoh et al., 2005).

1.6.5 Hepatoprotective effects

Lee et al., (2012) found that pre-treatment with a polyphenolic extract of HS protected the liver from acetaminophen-induced liver injury in BALB/c mice. The extracts increased the level of glutathione, decreased the level of peroxidation and increased catalase activity in the liver.

Hibiscus sabdariffa also reduced liver steatosis and fibrosis, decreased the elevation of aspartate aminotransferase (AST) and alanine aminotransferase (Liu et al., 2006). During Carbon tetrachloride (CCl_4) treatment, HS extracts restored the decrease in glutathione content and inhibited lipid peroxidation (Liu et al., 2006).

Hibiscus sabdariffa aqueous extract was found to reduce body mass gain and protect the liver by reducing fat accumulation, attenuating steatosis, down-regulating sterol regulatory element binding protein (SREBP-1c) and peroxisome proliferator- activated receptor gamma (PPAR-

γ), blocking the increase of interleukin one (IL-1), tumour necrosis factor alpha messenger ribonucleic acid (TNF-α mRNA), lipoperoxidation and increasing catalase mRNA in obese C57BL/6NHsd mice (Villalpando-Arteaga et al., 2013b).

1.6.6 Nephroprotective effects

Most often diabetic nephropathy progresses to end stage renal disease and this is thought to be mediated by oxidative stress (Lee et al., 2009). Phenolic extracts of HS reduced the kidney mass and improved the hyperglycaemia-induced osmotic diuresis in the proximal tubules while significantly lowering the serum triglyceride, total cholesterol, LDL and increasing the activity of catalase, glutathione and reducing lipid peroxidation in streptozotocin-induced diabetic rats (Lee et al., 2009). These findings were in agreement with those of Wang et al., (2011) . The latter proposed that the observed HS extracts nephroprotective effects might involve the up-regulation of Akt/Bad/14-3-3γ and nuclear factor-kappa B (NF-κB)-mediated transcription. In a study on the effects of HS calyces on creatinine and serum electrolytes, no significant harmful changes were observed in blood urea nitrogen (BUN), serum creatinine, sodium and potassium levels (Mohagheghi et al., 2010).

1.6.7 Anti-cancer effects

Hibiscus sabdariffa protocatechuic acid was found to inhibit the 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced promotion in skin tumours of female CD-1 mice (Tseng et al., 1998). The topical application of protocatechuic acid twice weekly prior to TPA treatment inhibited the incidence of tumours in mice significantly while all the mice that were not treated developed the tumour.

In an *in vitro* study, HS anthocyanins were found to induce apoptosis in cancer cells especially in human promyelocytic leukaemia (HL-60) cells using promyelocytic leaukemic cells (Chang et al., 2005). A polyphenol-rich extract of HS was also found to induce apoptosis in human gastric carcinoma cells by causing DNA fragmentation with an increase in the distribution of the hypodiploid phase (Lin et al., 2005). This action is thought to be mediated via tumour protein 53 (p53) phosphorylation and p38 mitogen-activated protein kinase/ Fas Ligand (MAPK/FasL) cascade pathway (Lin et al., 2005).

1.6.8 Anti-pyretic effects

Hibiscus sabdariffa calyx aqueous and ethanolic extracts were investigated for their nociceptive, anti-inflammatory and anti-pyretic effects using the writhing, hot plate and formalin tests in mice; yeast-induced fever and carrageenaan-induced paw oedema in rats respectively (Reanmongkol and Itharat, 2007). The ethanol and vacuum dried extracts of HS ($200\text{-}800\text{mg.kg}^{-1}$) reduced the yeast-induced fever but had no effect on the other tests suggesting that HS calyx extracts have anti-pyretic effects through mechanisms different from those of aspirin (Reanmongkol and Itharat, 2007).

1.6.9 Sexual maturity and lactogenic effects

Hibiscus sabdariffa aqueous extracts administered to pregnant Sprague Dawley rats resulted in post natal weight gain in the pups, delayed onset of puberty in the female offspring and increased body mass index through mechanisms that may be linked to glucocorticoid and leptin signalling (Iyare and Adegoke, 2008; Iyare and Nwagha, 2009). Gaya et al., (2009) in a lactogenic study on the seeds of HS found an elevation in the serum prolactin levels of Albino Wistar rats in a dose dependent manner with the maximum effect occurring at doses of 800mg.kg^{-1} and 1600mg.kg^{-1}.

1.6.10 Anti-bacterial effects

Methanolic extracts of HS were studied for their antimicrobial effects *in vitro* using a disc-diffusion method. The extracts exhibited activities comparable to that of streptomycin against various clinically important gram positive and gram negative bacteria such as *Staphylococcus aureus, Micrococcus luteus, Clostridium sporogens, Escherichia coli, Klebsiella pneumonaie, Bacillus cereus* and *Pseudomonas fluorescence* (Olaleye, 2007). Fullerton et al., (2011) investigated the antimicrobial effects of HS calyces against *Escherichia coli* O157:H7 found in food, veterinary and clinical samples. The calyx extracts of HS exhibited antimicrobial action against *Escherichia coli* at 2.5%, 5% and 10 % concentrations.

1.6.11 Effects on smooth muscles

An aqueous extract of the petals of HS was shown to have a vaso-relaxant effect on rat aortic rings through endothelium-dependent and independent mechanisms (Obiefuna et al., 1994). Fouda et al., (2007) reported an inhibition of rat bladder and uterine contractility by an aqueous calyx extract of HS through mechanisms unrelated to local or remote autonomic receptors or calcium channels as previously suggested in a study of the effects of HS on the GIT (Salah et al., 2002).

1.6.12 Haematenic effects

The dried fermented calyces of HS have a very low pH (3.2) which increases the bioavailability of other minerals such as iron and calcium (Falade et al., 2005). The high ascorbic content of HS calyces could also be responsible for an increase in the bioavailability of other minerals including iron which are essential for erythropoesis (Falade et al., 2005). There may therefore be a role for HS in iron supplementation in the prevention or treatment of anaemia. Adigun et al., (2006) studied the effects of HS extracts on some haematological parameters in Wistar albino rats. After 14 days of HS oral administration, a significant elevation of haematocrit and haemoglobin in the groups that received low doses of the extract (200mg.kg^{-1} body wt. and 400mg.kg^{-1} body wt.) was observed while the groups that received higher doses had significant reduction in haematocrit but not haemoglobin concentration.

1.6.13 Other effects

Hibiscus sabdariffa aqueous extracts were shown to possess diuretic and natriuretic properties via the compound quarcetin which causes the vascular endothelium to release nitric oxide and cause renal vasodilation and increased kidney filtration in rats (Alarcon-Alonso et al., 2012). *Hibiscus sabdariffa* extracts were also suggested as a valuable ethnomedicine in the management of chronic inflammatory diseases because of its ability to modulate the production of monocyte chemoattractant protein-1 (Beltrán-Debón et al., 2010). This

immunomodulatory action might be both cell and humoural mediated (Fakeye et al., 2008). There is also evidence for use of aqueous dried calyx extracts as an anti-diarrhoeal agent as it was found to increase transit time and reduce intestinal motility in rats (Owulade et al., 2004).

1.7 Toxicological aspects of HS plant

Hibiscus sabdariffa has been investigated extensively to determine its safety. An LD_{50} of 0.4-0.6ml (120-180mg) for a 30% aqueous extract of HS was reported by Sharaf, (1962) during an intraperitoneal administration in mice. In an acute toxicity test using the method of Lorke, Onyenekwe et al., (1999) observed no deaths in the experimental mice fourteen days after intraperitoneal administration of 1000-5000mg.kg^{-1} body weight of the HS calyx extract and calculated an LD 50 greater than 5000mg.$kg^{-1.}$ While investigating the antihypertensive effects of HS in spontaneously hypertensive rats, using the Wistar Kyoto rats as controls, sudden deaths of all the hypertensive rats by day 21 of continuous administration of the HS extracts at a dose of 1000mg.kg^{-1} body weight was observed (Onyenekwe et al., 1999). The authors concluded that the sudden deaths of the animals could have been due to the diuretic effect of the extracts. An LD50 of > 5000mg.kg^{-1} was reported in acute toxicity studies in albino mice for a methanolic dried flower extract (Ndu et al., 2011) and during a toxicological and lactogenic study in Albino Wistar rats (Gaya et al., 2009).

While investigating the toxicology of an aqueous-methanolic extract of HS calyces, Akindahunsi and Olaleye, (2003) administered 250mg.kg^{-1} body mass per day of the extract

to six groups of Wistar albino rats (0,1,3,5,10 and 15 doses per day). The rats in all the treatment groups showed increased activities of serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) but not alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) when compared to the control group that had received normal saline. They concluded that consumption of doses of HS between 150-180mg.kg^{-1} per day appeared safe while higher doses could affect the liver.

Feeding of pregnant Sprague Dawley rats with an aqueous calyx extract of HS resulted in a decrease in food and fluid intake, decrease in litter size, increased birth mass but had no effect on the length of gestation (Iyare and Adegoke, 2008; Iyare and Nwagha, 2009). However chronic administration of high doses of HS up to 4600mg.kg^{-1} have been associated with histopathological changes in the testes and increased serum creatinine levels (Orisakwe et al., 2004; Fakeye et al., 2009). Considering the above data, there is a need for further studies on the safety or otherwise of HS extracts.

1.8 Justification of the study

Hibiscus sabdariffa is a plant with multiple ethnomedicinal uses. The beverages made from its calyces are consumed by all age groups in Nigeria and most tropical countries where it is produced. The seeds of HS are also used to induce or enhance lactation (Gaya et al., 2009). Neonatal exposure can occur directly when the neonates are fed with the beverage or decoctions of HS for medicinal reasons or indirectly via the mother's milk.

As is the case for most other nutraceuticals, HS is consumed orally; consequently the GIT is its first point of contact with the body. Having in mind that the neonatal GIT is highly susceptible to structural and functional changes most especially during the suckling period which could be perpetuated throughout life (Pacha, 2000) and that certain plant extracts could cause accelerated structural and functional maturation of the neonatal GIT (Linderoth et al., 2005; Beya et al., 2012; Dangarembizi et al., 2014), it becomes imperative to study the effects of HS on the developing GIT.

Previous studies on the extracts of HS have been focused on adults and usually sought to determine its phytochemistry, pharmacology or toxicity but not its capacity to be used as a preventive intervention. There is an increasing incidence of metabolic syndrome and its complications worldwide (Armitage et al., 2004) with a horrifying increase in childhood obesity and its sequelae (Ebbeling et al., 2002). These tendencies are associated with altered lifestyle and dietary changes (Armitage et al., 2004). There is abundant evidence to suggest that the antenatal and early post natal environment can influence health outcomes later in life (Patel and Srinivasan, 2002; Armitage et al., 2004; Moura and Passos, 2005). This phenomenon is referred to as neonatal programming.

Several studies using the neonatal rat model (Linderoth et al., 2005; Erlwanger and Cooper, 2008; Beya et al., 2012; Dangarembizi et al., 2014) have speculated on the perpetuation of

the changes they observed through to adulthood and whether they would confer any benefit to the animals later in life.

In this present study, I aimed to provide data on the effects of HS on the morphometry of the neonatal GIT, growth performance, metabolic substrates and general health; and to explore whether the early administration of HS in the suckling period will have any effect in the response of the animals to a high fructose diet during the adolescent period.

The 4-day old neonatal Sprague Dawley rat pups were used to model the human neonate while the weanling rats were used to model the growing child in this study that was done in two phases.

1.9 Aims and objectives of the study

The main objective of this study was to determine the effect of administration of aqueous calyx extracts of HS on the gastrointestinal tract, growth performance, metabolic substrates and biochemical markers of health in the neonatal rat; and to investigate whether it would confer any protection or predisposition to the development of diet-induced metabolic dysfunction in the growing period.

The study aimed to specifically determine:

a.   the growth performance of the rats through the measurement of body weight gain, linear growth by determining the lengths and masses of the tibiae and femur.

b.   glucose tolerance in the rats (phase 2).

c.   the plasma concentration of insulin and determination of the homeostatic model of insulin resistance (HOMA-IR) in phase 2.

d.   circulating metabolic substrates (glucose and triglycerides).

e.   hepatic storage of lipids and glycogen.

f.   the morphology of the GIT and other viscera by gross measurement of their lengths and masses and histological assessment of the small intestines (phase1).

g.   biochemical markers of health by measuring in plasma.

- levels of alanine aminotransferase (ALT) and alkaline phosphatase (ALP) as surrogate markers of liver function.

- levels of Blood Urea Nitrogen (BUN) and creatinine as surrogate markers of renal function.

-total bilirubin, globulin, calcium, phosphorus, total protein, albumin, total cholesterol, and amylase.

h.   the plasma concentration of thiobarbituric acid reactive substances as a measure of lipid peroxidation (phase 2).

i.   the gender-based differences in all the above measured parameters in both the neonatal (phase 1) and the growing rats (phase 2).

1.10 Hypotheses

For phase 1

H_o: Aqueous calyx extracts of HS do not affect the gastrointestinal tract, growth performance, metabolic substrates and biochemical markers of health of suckling rat pups.

H_1: Aqueous calyx extracts of HS affect the gastrointestinal tract, growth performance, metabolic substrates and biochemical markers of health of suckling rat pups.

For phase 2

H_o: Aqueous calyx extracts of HS administered in the suckling period (preweaning) do not affect the response of Sprague Dawley rats to the inclusion of a high concentration of fructose in their diet post weaning.

H$_1$: Aqueous calyx extracts of HS administered in the suckling period affect the response of weanling Sprague Dawley rats to the inclusion of a high concentration of fructose in their diet post weaning.

CHAPTER TWO: EFFECTS OF *HIBISCUS SABDARIFFA* AQUEOUS CALYX EXTRACTS ON THE GROWTH PERFORMANCE, GASTROINTESTINAL TRACT MORPHOMETRY AND METABOLIC SUBSTRATES OF SUCKLING RATS

2.1 Introduction

As previously mentioned, the gastrointestinal tract serves as the first point of contact for nutraceuticals, being that they are mostly ingested orally. *Hibiscus sabdariffa* decoctions for the purpose of beverage or tea are also taken orally. In Nigeria and most tropical areas, HS is consumed by all age groups. The seeds of HS are used to induce lactation and improve milk let-down in some communities (Gaya et al., 2009). The neonatal GIT is susceptible to structural and functional changes when it is exposed to certain dietary constituents especially during the critical periods of gestation and lactation when there is relative plasticity of the tissues (Pacha, 2000). Plant extracts can cause precocious maturation of the gut (Linderoth et al., 2005). Artificial milk has also been shown to cause a similar effect (Dvorak et al., 2000).

Many peptides that regulate metabolic function in the body originate from the GIT (Sorensen et al., 2010). Therefore any insult that affects the GIT will also likely affect the availability of these peptides and consequently affect the growth performance and uptake of metabolic substrates in the neonate. Considering that there is exposure of the neonatal GIT to HS extracts either directly or indirectly through the breast milk, it becomes imperative to study and document its effects.

2.1.1 The gastrointestinal tract

The GIT is a specialised system that serves as a link between the body and the outside

environment, allowing for food to be digested and absorbed for the benefit of the individual

(Schneeman, 2002). At birth, the GIT undergoes changes to enable it cope with the shift from

the parenteral placental nutrition to enteral nutrition (Sangild et al., 2000). Most of the

development of the human GIT occurs *in utero* during the weeks leading to parturition but

full functions of some organs especially the intestines are not attained until the age of 2 years

(Sangild et al., 2000; Corpeleijn et al., 2008). The GIT performs very important functions in

the overall regulation of body homeostasis including among others: digestion and absorption

of nutrients, metabolism and immune response (Baltrop and Brueton, 1990). For effective

distribution of nutrients to various organs and also for control of food intake and utilisation,

the GIT is connected with other systems of the body such as the cardiovascular and central

nervous systems (Schneeman, 2002).

The neonatal GIT allows for selective pinocytosis of macromolecules during the suckling

period which permits passage of maternal antibodies to the pups for protection (Linderoth et

al., 2005). This ability is however lost as the enterocytes attain adult configuration and gut

closure occurs at 3 weeks under the influence of glucocorticoids (Henning, 1986). The

structure of the GIT is composed of four specialised layers: the mucosa, sub-mucosa,

muscularis and each of the layers adapted to particular functions (Xu, 1996). The mucosa

comes in direct contact with the external environment and its structural and functional integrity determines its absorptive capacity, nutrient availability and therefore health and wellbeing of the individual (Buddington and Kimura, 2003). In the small intestines, the mucosa is thrown into folds called villi which are covered by enterocytes that originate from the crypts and differentiate as they migrate to the villi tip (Baltrop and Brueton, 1990). The GIT mucosa is a major source of peptides such as ghrelin and cholecystokinin which regulate metabolic and digestive functions in the body (Sorensen et al., 2010). It therefore follows that interference with the integrity of the GIT mucosa affects the secretion of these peptides with consequences on the metabolism of the organism.

2.1.2 Phytochemicals and health

Phytochemicals are plant components that possess biological activities (Farombi., 2003). They affect the metabolism and physiology of the individual when ingested (Dillard and German, 2000; Liu, 2007). Most of these phytochemicals are incorporated into food and pharmaceutical products as nutraceuticals. Nutraceuticals are non-toxic food extract supplements that have been scientifically proved to have health benefits in both prevention and treatment of diseases (Dillard and German, 2000; Holst and Williamson, 2008). Phytochemicals are broadly classified into three groups: terpenoids, phenolics and alkaloids and other nitrogen-containing constituents (Harborne, 1999). Some phytochemicals such as anthocyanins have been shown to have antioxidant and anti-mutagenic properties (Dillard and

German, 2000). Phytochemicals affect the general health of the body in a number of ways which could either be beneficial or detrimental. They could act as substrates for biochemical reactions, co-factors of enzymatic reactions, enzyme inhibitors and as absorbents that bind to and help in eliminating unwanted materials from the GIT (Dillard and German, 2000; Bagchi et al., 2011). Phytochemicals could also act as agonists or antagonists of biological receptors, scavengers of toxic substances or as enhancers of absorption of essential nutrients (Dillard and German, 2000). *Hibiscus sabdariffa* has an abundance of phytochemicals (as discussed in chapter one) which could affect the general health of individuals consuming it either for medicinal or nutritional purposes.

2.1.3 *Hibiscus sabdariffa* and the GIT

Only two studies were found that investigated the effects of HS on the GIT. *Hibiscus sabdariffa* aqueous (Owulade et al., 2004) and methanolic (Salah et al., 2002) extracts were found to increase intestinal transit time and inhibit intestinal motility in adult rats. The flavonoids, quercetin and eugenol were postulated to be responsible for these effects via modulation of Ca^{2+} channels (Salah et al., 2002).

2.2 Materials and methods

2.2.1. Study setting

The study was conducted from 2013 to 2014 at the Central Animal Services facility and the School of Physiology, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, Republic of South Africa.

2.2.2. Ethics clearance

All the protocols used in the study were approved by the Animal Ethics Screening Committee of the University of the Witwatersrand, Johannesburg (AESC/2013/46/05) with subsequent modifications (appendices 1, 2 and 3).

2.3 Materials

2.3.1 Source of HS Calyces

Dried calyces of HS were purchased at the central market in Sokoto, North West Nigeria. They were authenticated by Mr. Halilu E. Mshelia of the department of Pharmacognosy and Ethnopharmacy, Faculty of Pharmaceutical Sciences, Usmanu Danfodiyo University, Sokoto

and a voucher specimen was deposited at the departmental herbarium

(PCG/UDUS/Malv/0001). The dried calyces were then imported into RSA to the University

of the Witwatersrand, Johannesburg where they were further processed and the animal studies

were conducted.

2.4 Methods

2.4.1 Preparation of the calyx extracts

The dried calyces were ground to a fine powder using a Waring blender (Waring[®], USA).

Two hundred and ten grammes of the calyx powder were extracted in 1400ml of distilled

water at 95ºC for 2 hours (Lin et al., 2007). The extracted solution was then filtered through

Whatman 1 filter paper and the filtrate was concentrated using a rotor evaporator (Labocon

(Pty) Ltd, Krugersdorp, South Africa). The concentrated suspension of the extracts was then

dried in an oven (Salvis®, Salvis Lab, Schweiz, Switzerland) at 40ºC (Dangarembizi et al.,

2014) and the residual powder extracts were recovered and stored in dark, tightly sealed glass

vials at 4ºC for future use (Ali et al., 2003; Lin et al., 2007). The recovered powder weighed

22% of the starting calyces.

2.4.2 Study animals

Forty two, 4-day old, suckling Sprague Dawley pups from four dams that were sourced from the Central Animal Services, University of the Witwatersrand, were used in the study.

2.4.3 Housing and care of the animals

Each dam and its litter were housed in Perspex cages lined with wood shavings. The dams were supplied with standard rat cubes (Epol[®], Johannesburg, South Africa) and tap water *ad libitum.* The room temperature was maintained at 26±2ºC and a 12 hr light cycle followed (lights on 07.00hrs- 19.00hrs). The pups were allowed to nurse freely with their dams throughout the duration of the study.

2.4.4 Study design

The birth day of the pups was designated day zero (Erlwanger and Cooper, 2008) and on day 4, the pups in each litter were randomly assigned to three treatment groups (in a split litter manner) which were distinctly identified using unique, non toxic colour-coded markings on their tails. The first group, the control group (n=14, 7 males and 7 females) received distilled water at 10ml.kg^{-1} BM, Group 2 (n = 14, 7 males and 7 females) received a low dose of HS extract (50mg.kg^{-1}) while the third group (n = 14, 7 males and 7 females) received a high dose

of the extracts ($500mg.kg^{-1}$). The treatments were administered once daily in the morning (08.00hrs-09.00hrs) via oral gavage using an orogastric tube mounted on a 1ml syringe (Erlwanger and Cooper, 2008; Beya et al., 2012; Dangarembizi et al., 2014) for 9 consecutive days till post natal day 14. This was to prevent the compounding effects on the HS treatment that could result due to exploratory feeding of the pups after they open their eyes on postnatal day 14. The doses of HS used in this study were within the ranges used by other researchers without recording adverse effects on health (Onyenekwe et al., 1999; Gaya et al., 2009; Ndu et al., 2011).

2.4.5 Body mass measurements

The pups were weighed daily on a balance (Snowrex Electronic Scale, Clover Scales, Johannesburg) in order to adjust the amount of aqueous HS calyces extract administered so as to maintain a constant dose and also to monitor their growth performance. The dams were weighed weekly as part of routine health monitoring.

2.4.6 Terminal procedures

2.4.6.1 Termination of pups

On post natal day 14, blood was obtained from the pups via a pin prick of the tail and used for determination of non-fasting glucose with a calibrated glucometer (Ascensia Elite[TM] Blood

glucose meter, Bayer Corporation, Mishawaka, USA). Thereafter, the pups were euthanased by intraperitoneal anaesthetic overdose of sodium pentobarbitone (Eutha-naze, Bayer, Johannesburg, South Africa) at 200mg.kg^{-1} body mass.

2.4.6.2 Collection of blood, plasma processing and storage

Following euthanasia, the thorax was opened and blood was collected via cardiac puncture with 21G needles and 5ml syringes. The blood was immediately transferred to lithium-heparin blood tubes (Becton Dickinson Vacutainer Systems Europe, Meylan Cedex, France) which were gently inverted to ensure proper mixing with the anticoagulant. The blood was then centrifuged at 4000 x G for 15 minutes at 20ºC in a centrifuge (SorvalIIRT®6000B, DuPont Instruments, New York, USA). Plasma was stored in microtubes (Eppendorf, Hamburg, Germany) at - 20ºC in a freezer (Bosch, Johannesburg, South Africa) until needed for assay of the biochemical markers.

2.4.6.3 Morphometry of the GIT and other viscera

Through a midline incision, the abdomen was opened. The GIT and liver were carefully dissected out. The contents of the stomach, small intestines, large intestines and caecum were gently emptied. Both the GIT and the above mentioned viscera were weighed with an electronic balance (Presica 310M, Presica Instruments AG, Switzerland) and recorded. As

described in similar studies (Erlwanger and Cooper, 2008; Beya et al., 2012; Dangarembizi et al., 2014) the small and large intestines were gently stretched on a cool dissecting board on which a ruler was mounted, and their lengths were recorded. The liver was preserved in a freezer (Haier Biomedical, China) at -20ºC for future determination of hepatic storage of lipids and glycogen.

2.4.6.4 Determination of long bone parameters

The right hind legs from the carcasses were carefully dissected and detached from the acetabulum using a pair of scissors and scalpel blade which were also used to meticulously clean the femur and tibia of all non-bony tissues. The bones were then dried in an oven (Salvis [®], Salvis Lab, Switzerland) at 50ºC for 5 days until uniform mass was achieved. This was to remove any moisture content from the bones since the carcasses were initially stored in a freezer (Haier Biomedical, China) before the bones were dissected out at a later date. This dry mass, together with the bone length determined using a vernier calliper (Hi-impact, Dejuca, South Africa) were used to calculate the bone density. The bone density was calculated using the formula:

Bone density= mass of the bone (mg)/length of the bone (mm) (Monteagudo et al., 1997)

2.4.6.5 Hepatic storage of metabolic substrates

2.4.6.5.1 Quantification of hepatic lipid stores

The hepatic storage of lipids was determined using a solvent extraction method as described by Bligh and Dyer, (1959) . Essentially, 300-500mg of liver sample was placed in 150ml of 2:1 chloroform: methanol solution and allowed to extract overnight at 4ºC. The solution was then filtered through filter paper (Whatmann®, No 1, size 185 mm, pore size 7-11µm, England) into separation funnels where 30ml of 0.9% saline was added to the filtrate from a dispensing bottle. The separation funnels containing the solutions were kept overnight at 4ºC to allow separation into two phases. The bottom phase was collected into round bottom flasks and evaporated to dryness under vacuum with a rotor evaporator (Labocon (pty) Ltd, Krugersdorp, South Africa) at 37ºC. 20ml of chloroform was then added to make up the stock solution from which an aliquot of 2ml was taken, placed in dried, pre-weighed 10ml scintillating bottles and dried in an oven (Salvis®, Salvis Lab, Schweiz, Switzerland) at 50ºC for 30 minutes. The scintillating bottles were then re-weighed and the percentage lipid content was then calculated as follows:

% liver lipids = total mass of the lipids/mass of the sample x 100.

2.4.6.5.2 Quantification of hepatic glycogen stores

The liver glycogen stores were determined indirectly by hydrolyzing the glycogen to glucose and then determining the concentration of glucose as described by Passonneau and Lauderdale, (1974). Briefly, 0.1g of the liver sample was placed in a test tube and homogenized (Ultra turrax® homogenizer, Janke and Kunkel, Germany) in 1ml of 0.03M HCl. Into the homogenized liver was added 1ml of 1M HCl. The sample was then thoroughly mixed using a vortex mixer (Vortex mixer WM-300, Cannic Inc. USA), sealed and then placed in a boiling water bath for 2 hours to hydrolyse the glycogen. Thereafter, 1ml of 1M NaOH was added to the sample and mixed with the vortex mixer to neutralize the acid. The concentration of glucose in the hydrosylate was then determined colorimetrically with a glucometer (Accu-Chek Active, Roche, Germany) and the result expressed as glucose equivalents in the homogenate.

2.4.6.6 Determination of clinical biochemical markers of health

A total of thirteen parameters were measured using a VetTest analyser (IDEXX VetTest® Clinical Chemistry Analyser, IDEXX Laboratories Inc., USA) to determine the effects of HS administration on the health profile of the study animals. The machine is a colorimetric chemistry analyser and was calibrated according to the manufacturer's instructions before the assay. Plasma samples that had previously been frozen were thawed, warmed to room

temperature and gently agitated for uniform mixing of the sample. A pipette that was attached to a probe from the analyser sucked up 150μl of the plasma and deposited it on the pre-loaded discs. The samples were then analysed and a print out of the results was retrieved from the machine.

2.5 Statistical analysis

All data from the study was expressed as mean ± standard deviation. Data was analysed using Graph pad Prism version 5 (Graph-pad Software Inc., San Diego, USA). The level of significance was set at $p \leq 0.05$. A one way analysis of variance was used to assess the effects of HS extracts on the parameters measured. This was followed by a Bonferroni *post hoc* test for comparison of the means.

2.6 Results

There were no gender differences observed in all the parameters assayed across all the treatment groups hence the presentation of combined (male and female) results.

2.6.1 Morbidity and mortality

All the pups remained healthy throughout the study with no incidental or iatrogenic morbidities or mortalities recorded.

2.6.2. Effects of HS aqueous calyx extract on growth performance

2.6.2.1 Body mass

Figure 2.1 shows the effects of orally administered HS aqueous calyx extracts on the terminal body masses of the rat pups. The induction and terminal masses of the rats were not significantly different ($p > 0.05$) across the treatment groups (Figure 2.1). The pups in all the treatment groups however significantly grew ($p < 0.001$) from induction to termination (Figure 2.1).

Figure 2.2 shows the percentage body mass gain of the rat pups following oral administration of HS aqueous calyx extracts. There was no significant difference ($p > 0.05$) in the percentage body mass gain of the rat pups during the treatment period.
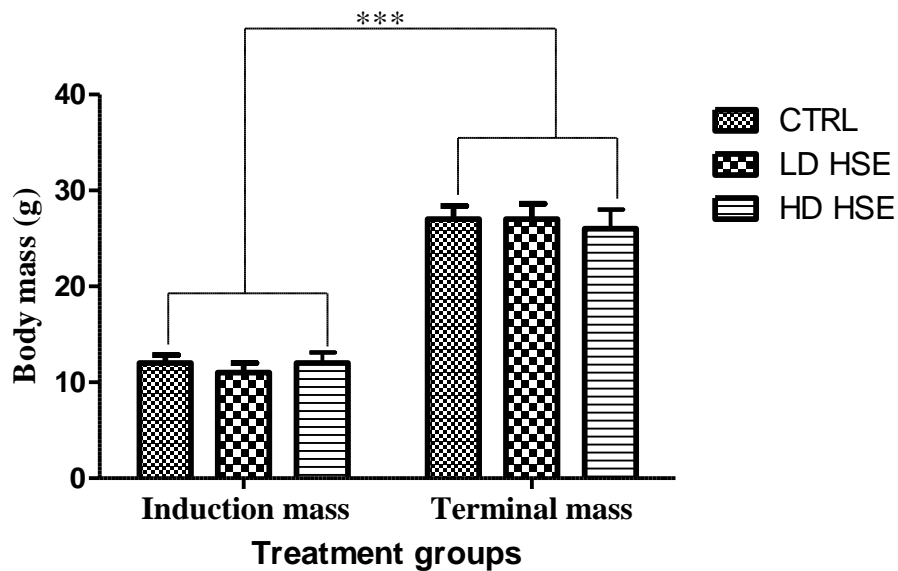
Figure 2.1: Effect of oral administration of aqueous HSE on the terminal body masses of rat pups. ***=p<0.001. CTRL= control (10ml.kg^{-1} distilled water), HD HSE= high dose of HS extract (500mg.kg^{-1}), LD HSE= low dose of HS extract (50mg.kg^{-1}). Data presented as mean± SD; n=14 in each group.
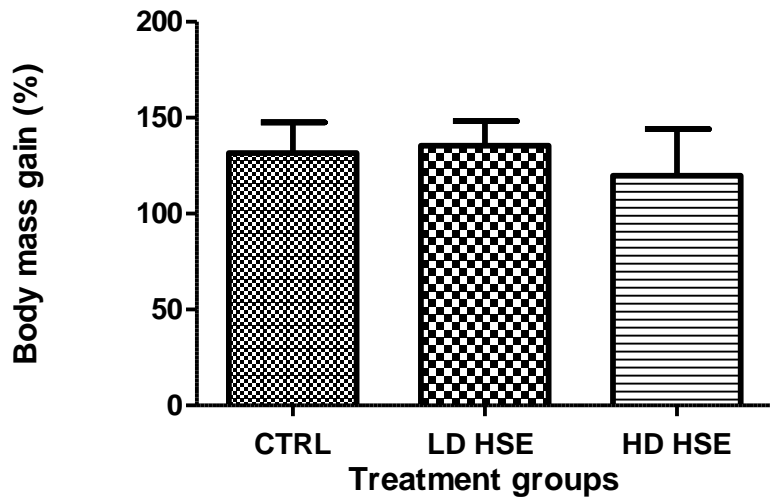
Figure 2.2: Effect of oral administration of HS aqueous calyx extract on Percentage change in body mass of pups. CTRL= control (10ml.kg^{-1} distilled water), HD HSE= high dose of HS extract (500mg.kg^{-1}), LD HSE= low dose of HS extract (50mg.kg^{-1}). Data presented as mean± SD; n=14 in each group.

2.6.2.2 Linear growth

Table 2.1 shows the effect of oral administration of HS aqueous calyx extracts on the masses, lengths and densities of tibiae and femora of suckling Sprague Dawley pups. Pups administered with the LD HSE had significantly heavier tibiae ($p<0.05$) compared to those of the control and HD HSE (Table 2.1). Pups in the LD HSE had denser tibiae than pups in the HD HSE and CTRL groups (Table 2.1).

Table 2.1 Effect of oral administration of HS aqueous calyx extracts on the masses, lengths and density of tibiae and femora of suckling Sprague Dawley pups

	CTRL	LD HSE	HD HSE
Tibia (mg)	32 ± 3.5^a	34 ± 3.5^b	30 ± 4.6^a
Tibia (mm)	16 ± 1.0^a	15 ± 1.2^a	15 ± 1.4^a
Tibia (mg.mm^{-1})	2.0 ± 0.2^a	2.9 ± 0.3^b	2.0 ± 0.2^a
Femur (mg)	32 ± 3.2^a	32 ± 3.4^a	30 ± 2.8^a
Femur (mm)	12 ± 0.7^a	12 ± 0.7^a	12 ± 0.4^a
Femur (mg.mm^{-1})	2.7 ± 0.2^a	2.7 ± 0.2^a	2.6 ± 0.2^a

[ab]Within rows, means with different superscripts are statistically different from each other. CTRL= control (10ml.kg^{-1} distilled water), HD HSE= high dose of HS extract (500mg.kg^{-1}), LD HSE= low dose of HS extract (50mg.kg^{-1}). Data expressed as mean$\pm$ SD, n=14 in each group.

2.6.3 Hepatic storage of metabolic substrates

Figures 2.3A and 2.3B show hepatic lipid and glycogen content respectively. There were no differences ($p>0.05$) in the liver lipid (Figure 2.3A) and glycogen content (expressed as glucose equivalents in liver hydrosylate) (Figure 2.3B) across the treatment groups.

2.6.3.1 Hepatic lipid content
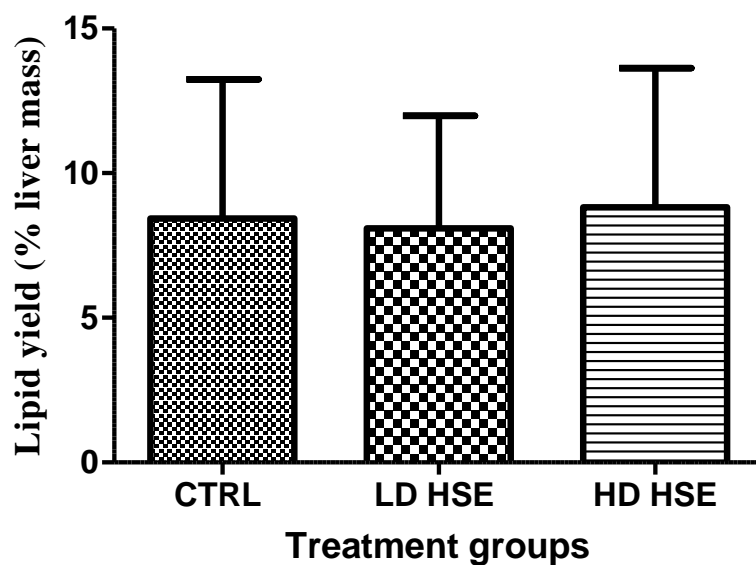
Figure 2.3A: Effect of administration of HS aqueous calyx extracts on liver lipid content in suckling Sprague Dawley rat pups

CTRL= control (10ml.kg^{-1} distilled water), HD HSE= high dose of HS extract (500mg.kg^{-1}), LD HSE= low dose of HS extract (50mg.kg^{-1}). Data expressed as mean± SD, n=14 in each group.

2.6.3.2 Hepatic glycogen content
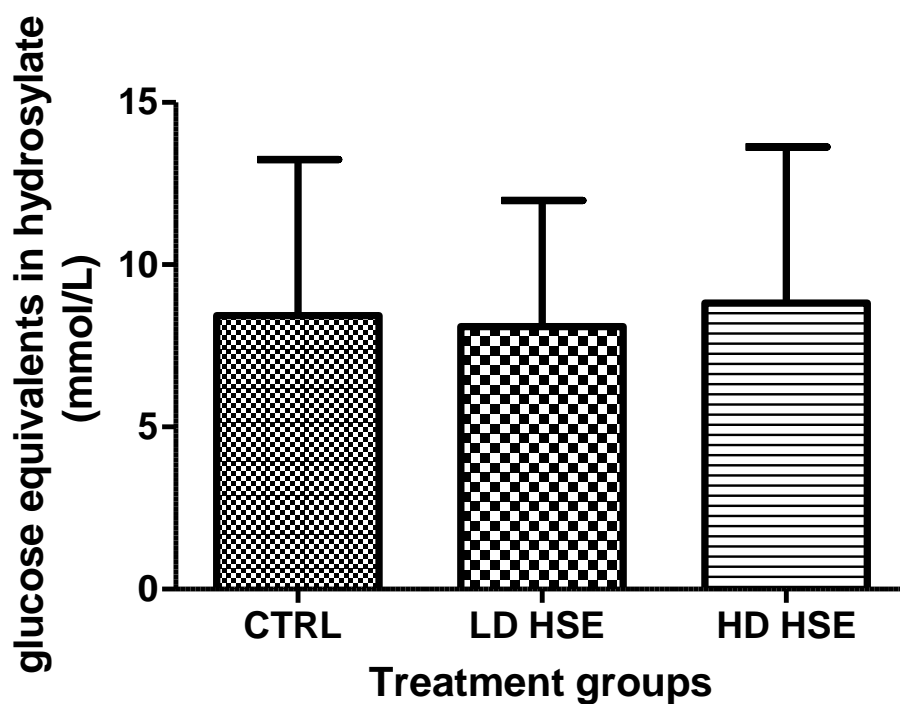
Figure 2.3B: Effects of administration of HS aqueous calyx extract on the liver glycogen stores of suckling Sprague Dawley rats

CTRL= control (10ml.kg^{-1} distilled water), HD HSE= high dose of HS extract (500mg.kg^{-1}), LD HSE= low dose of HS extract (50mg.kg^{-1}). Data expressed as mean± SD, n=14 in each group.

2.6.4 Morphology and morphometry of the GIT and other viscera

Table 2.2 shows the effect of administration of HS aqueous calyx extract on the morphometry of the GIT and liver of suckling Sprague Dawley pups. The small intestines of the pups administered with the HD HSE were significantly heavier relative to the body mass ($p < 0.05$) when compared to those on the LD HSE and the CTRL group ($p < 0.001$). The caeca of pups administered with the HD HSE were significantly heavier (absolute and relative to the body mass) compared to those on the LD HSE ($p < 0.05$). There were no differences observed ($p > 0.05$) in the lengths of the small and large intestines, absolute and relative masses of the large intestines, stomach and liver (Table 2.2).

Table 2.2: Effect of administration of HS aqueous calyx extract on the morphometry of the GIT and liver of suckling Sprague Dawley rats

	CTRL	LD HSE	HD HSE
S.I.(g)	0.71 ± 0.07	0.72 ± 0.06	0.77 ± 0.09
S.I. (% BM)	2.7 ± 0.30^{b}	2.7 ± 0.20^{b}	3.0 ± 0.20^{a}
S.I. (mm)	498 ± 27.00	517 ± 43.00	509 ± 30.00
L.I. (g)	0.10 ± 0.01	0.10 ± 0.01	0.11 ± 0.01
L.I. (% BM)	0.39 ± 0.05	0.40 ± 0.05	0.42 ± 0.05
L.I. (mm)	71 ± 4.80	72 ± 4.70	70 ± 5.30
Liver (g)	0.80 ± 0.06	0.79 ± 0.07	0.76 ± 0.06
Liver (%BM)	3.0 ± 0.17	2.9 ± 0.17	3.0 ± 0.14
Caecum (g)	0.056 ± 0.01^{a}	0.049 ± 0.01^{ac}	0.062 ± 0.02^{ab}
Caecum(%BM)	0.21 ± 0.05^{a}	0.19 ± 0.04^{ac}	0.24 ± 0.06^{ab}
Stomach (g)	0.17 ± 0.02	0.16 ± 0.02	0.17 ± 0.02
Stomach (%BM)	0.63 ± 0.08	0.59 ± 0.07	0.66 ± 0.07

[ab]Within row data with different superscripts indicate significant difference (p <0.05). S.I. = small intestines, L.I. = large intestines, BM = body mass, CTRL= control (10ml.kg^{-1} distilled water), HD HSE= high dose of HS extract (500mg.kg^{-1}), LD HSE= low dose of HS extract (50mg.kg^{-1}). Data expressed as mean± SD, n=14 in each group.

2.6.5 Biochemical health profile

Table 2.3 shows the effect of administration of HS aqueous calyx extract on the biochemical markers of health of suckling Sprague Dawley pups. There was no significant difference (P>0.05) in biochemical markers of health measured across the treatment groups (Table 2.3).

Table 2.3: Effects of administration of HS aqueous calyx extract on the biochemical markers of health of suckling Sprague Dawley rats

	CTRL	LD HSE	HD HSE
Glucose (mmol.L^{-1})	6.4 ± 1.30	6.2 ± 0.94	6.9 ±1.10
BUN (mmol.L$^{-1)}$)	3.8 ± 0.94	3.8 ± 0.67	4.0 ± 1.20
Creatinine (μmol.L^{-1})	18 ± 6.10	21 ± 12.00	19 ± 9.70
Phosphate (mmol.L^{-1})	2.9 ± 0.29	2.9 ± 0.21	2.7 ± 0.23
Calcium (mmol.L^{-1})	2.4 ± 0.53	2.3 ± 0.36	2.5 ± 0.24
Total Protein (g.L^{-1})	39 ± 4.80	40 ± 2.80	40 ± 2.90
Albumin (g.L^{-1})	16 ± 4.70	15 ± 5.90	16 ± 5.60
Globulin (g.L^{-1})	23 ± 1.10	25 ± 4.90	24 ± 3.40
ALT (U.L^{-1})	50± 36.00	59 ± 56.00	44 ± 24.00
ALP (U.L^{-1})	382 ± 67.00	425 ± 76.00	438 ± 65.00
Total Biluribin (μmol.L^{-1})	10 ± 3.60	10 ± 5.70	9.2 ± 3.40
Cholesterol (mmol.L^{-1})	4.1 ± 0.32	4.4 ± 0.28	4.4 ± 0.30
Amylase (U.L^{-1})	1272 ± 254.00	1349 ± 162.00	1347 ± 173.00

BUN= blood urea nitrogen, CTRL= Control (10ml.kg^{-1}), HD HSE= High dose of HS extracts (500mg.kg^{-1}), LD HSE= Low dose of HS extracts (50mg.kg^{-1}), Data expressed as mean± SD, n=14 in each group.

2.7 Discussion

In this phase of the study, the effects of administration of an aqueous calyx extract of HS on the growth performance, metabolic substrates, gastrointestinal tract and general health of suckling rat pups was investigated. The findings of this phase will be discussed in the subsequent paragraphs.

2.7.1 Effects of HS aqueous calyx extracts on growth performance

The pups in all the treatment groups had similar induction masses and grew significantly during the study period. The effects of sex hormones that may account for differences in growth rates only appear around postnatal day 25-36 in rats (Eden, 1979; Gabriel et al., 1992). The pups in this phase of the study were terminated at postnatal day 14 and this might explain why gender differences were not observed in the measured parameters. Body mass is usually affected by a range of factors such as genetic predisposition, the state of hydration of the individual, nutritional status and disease conditions (MacCracken and Stebbings, 2012). The morphometric characteristics of the tibiae and femora are more reliable indices of growth because they are related to growth hormone and IGF-1 activity in a dose dependent manner compared to other tissues (Baum et al., 1996; Eshet et al., 2004). The long bones are also not susceptible to daily fluctuations resulting from hydration status or gut fill (Baum et al., 1996).

The tibiae of the pups in the low dose HSE group were significantly longer and heavier than those of the control and high dose HS groups. The determination of the bone density was done indirectly by computation of the lengths and masses of the bones and not through radiographs and densitometry. Therefore the higher density of the tibiae might be due to proteins or other bone components and not necessarily due to mineralization. *Hibiscus sabdariffa* calyces have about 12.63mg/100g calcium (Mahadevan et al., 2009), which is in addition to the calcium content of the dam's milk and might contribute to the mineralization of the long bones. However, HS calyces were also reported to have a high phosphorus content of about 273.2mg/100g (Mahadevan et al., 2009) with a calcium/phosphorus ratio of 0.05. Calcium/phosphorus ratio range from 1.51 in neonatal to 1.69 in adult rats (Legros et al., 1987; Nieves, 2005). Excessive intake of phosphorus in relation to calcium has deleterious effects on bone mineralization (Nieves, 2005). This might explain why the pups that were administered with the high dose HSE had shorter and lighter tibiae than those given the high dose of HSE.

2.7.2 Effects of HS on hepatic storage of lipids and glycogen

Dietary interventions and starvation have been shown to affect the liver storage of lipids and glycogen (Barcellos et al., 2010). The liver is an important organ in the body performing a wide range of metabolic, synthetic and storage functions. Hepatic lipids serve as a concentrate of large energy stores to be used by the body in times of need (Sugden et al., 2002). *Hibiscus*

sabdariffa extracts have been reported to decrease lipid content of hepatocytes via the activation of adenosine monophosphate-activated protein kinase (AMPK) and reduction of sterol regulatory element- binding protein 1 (SREBP-1) consequently preventing the expression of the enzymes: fatty acid synthase and HMG-CoA reductase (Yang et al., 2009). In this study, there was no difference observed in the hepatic storage of lipids between the HSE treated and control groups suggesting that HSE might not have had an effect on the hepatic liver content of the pups.

Hibiscus sabdariffa has been reported to have hypoglycaemic effects (Peng et al., 2011; Adisakwattana et al., 2012; Villalpando-Arteaga et al., 2013a) which should have affected the liver glycogen stores. The hypoglycaemic effect should have caused glycogenolysis in the liver leading to a reduction in the hepatic glycogen stores. There was no significant difference observed in liver glycogen content between the HS treated groups and the control group in this study. This lack of difference in the glycogen stores across the treatment groups might be because the pups were not fasted prior to termination. Fasting of the pups would have required separating them from their dams and since the pups do not all suckle at the same time it would be difficult to determine the duration of fasting.

2.7.3 Effects of HS on the morphometry of the GIT

The GIT is the most widely exposed system to the effects of phytochemicals and responds to this exposure in diverse ways (Buddington and Kimura, 2003). Some vertebrates are able to increase the size of their GIT in response to the fibre content of the diet (Sorensen et al., 2010). Plant extracts have previously been shown to cause precocious maturation of the GIT in rats (Linderoth et al., 2005). In this study, HS extracts caused a significant increase ($p<0.001$) in the mass of the small intestines relative to the body mass in the high dose group when compared to the control group and low dose groups ($p<0.01$). This could probably point to trophic effects of high dose HSE on the small intestines in this period of relative plasticity. HS extracts might have interfered with the absorption of nutrients in the small intestines leading to their accumulation and subsequent increase in the mass of the intestines (Younes et al., 2001; Lembede, 2014).

The caecum of the rat is the main site of degradation of fermentable carbohydrates and other residual nutrients (Munro et al., 1998; Younes et al., 2001). In this study, the absolute (g) and relative (%BM) mass of the caecum differed significantly between the high dose HS ($500mg.kg^{-1}$) and the low dose HS ($50mg.kg^{-1}$) treatment groups but not with the control group. An increase in the caecal mass usually occurs following introduction of a variety of substances into the GIT and is thought to result from the osmotic activity of substances not absorbed by the small intestines or an alteration of the GIT flora by dietary changes (Ford et al., 1983; Munro et al., 1998; Erlwanger and Cooper, 2008). There may be positive or

negative associative factors responsible for this observed difference in caecal mass between the two different treatment groups (LD HSE and HD HSE) which are both similar to the control group. However, full explanation cannot be provided by this study.

 Hibiscus sabdariffa extracts did not have any grossly discernable effects on the remaining GIT and non-GIT viscera.

2.7.4 Effect of HS on markers of health

There were no adverse health outcomes observed in the pups following the administration of HS aqueous calyx extracts. Even though some researchers have found adverse effects in the liver enzymes following chronic administration of high doses of HS (Akindahunsi and Olaleye, 2003) others reported HS to be safe (Onyenekwe et al., 1999; Gaya et al., 2009; Ndu et al., 2011). Plasma activity of alkaline phosphatase (ALP) though not significantly different (p>0.05) was found to be elevated above the upper limit ($370U.L^{-1}$) for adult rats (Espandiari et al., 2008) in all the treatment groups. Alkaline phosphatase is a non- specific enzyme released from multiple sources which include the intestinal mucosa, osteoblasts and epithelial cells of biliary canaliculi (Alhassan et al., 2009). The increased activity of ALP might be due to increased osteoblastic activity that accompanies growth (Alhassan et al., 2009) and not as a result of damage to the liver. The particular ALP isozyme that was elevated was not

determined in this study due to technical limitations and would have shed more light on the cause of the increased plasma activity. Plasma activity of ALT and plasma total biluribin and total protein concentration, which are all markers of hepatic damage, were not different across the treatment groups. Plasma albumin concentrations were low (reference values given by the IDEXX machine) but not significantly different in all the treatment groups. However albumin concentrations have been reported to be low from 4 days post partum till about 20 days post partum due to protein synthesis limitation in the hepatoblasts (Papworth and Clubb, 1995).

Blood urea nitrogen (BUN) and creatinine were used as surrogate markers of renal function in this study. There were no adverse effects observed in these parameters following administration of HS aqueous calyx extracts. This finding is in agreement with those of Mohagheghi et al., (2010) who observed no significant harmful changes in blood urea nitrogen (BUN), serum creatinine, sodium and potassium concentrations following administration of HS tea twice daily for 2 weeks in patients with essential hypertension. Other researchers have also attributed reno-protective effects on the extracts of HS through the amelioration of diabetic nephropathy (Lee et al., 2009; Wang et al., 2011). Other markers of health assayed in the study were also not adversely affected by the administration of HS. These results tend to suggest that HS aqueous calyx extracts could be used in neonates without any adverse effects on their health.

2.11 Conclusion

In this phase of the study, aqueous HS calyx extract was administered to neonatal rat pups via oral gavage to determine its effects on their growth performance, metabolic substrates, gastrointestinal tract and general health. While the extracts had a trophic effect on the small intestines and caecum of the rat pups, it had no effect on the other parameters that were assayed in the study. These findings suggest that aqueous HS calyx extracts administered during the neonatal period could cause precocious structural maturation of the GIT which could affect the metabolism and physiology of the animal without any adverse effects on their health. Further research is needed in order to identify the specific phytochemicals and mechanisms of action involved in the causation of precocious maturation of GIT and its implications for long term health.

Previous studies in which neonatal rat pups were administered with plant extracts (Erlwanger and Cooper, 2008; Beya et al., 2012; Dangarembizi et al., 2014) had speculated on the possible perpetuation of the changes observed in the neonatal phase into adulthood and whether they conferred any advantage or otherwise in later life.

The second phase of this study which is described in the next chapter, investigated whether the trophic changes observed in the small intestines of the pups translated into any form of advantage or not in the response of weanling rats to a second intervention (20% fructose w/v

in the drinking water of the weanling rats). It thus also investigated whether the early

administration of the HSE had any programming effect on the manifestation of metabolic

dysfunction in the adolescent stage of the rats.

CHAPTER THREE: EFFECTS OF EARLYADMINISTRATION OF HS AQUEOUS CALYX EXTRACT ON THE RESPONSE TO A HIGH FRUCTOSE DIET

3.0 Introduction

In the previous chapter, HS aqueous calyx extracts were administered to suckling Sprague Dawley rat pups. As stated earlier, previous interventions in the neonatal rats had looked at the acute changes in the GIT morphology, growth performance and general health of the pups and speculated that the changes observed might have beneficial effects for the animals later in life.

This chapter reports on the second phase of the present study. In addition to the administration of the HS aqueous calyx extracts in the suckling period, the rats were subjected to a high fructose diet at 20% fructose (w/v) in their drinking water. Unlike glucose which activates phospho-fructokinase in the glycolytic pathway, fructose activates fructose-1-phosphatase leading to lipogenesis (Basciano et al., 2005). Fructose is also common additive in foods (Angelova and Boyadjiev, 2013). The aim was to investigate whether the initial intervention with HS had conferred any benefits or predisposition to the development of diet induced metabolic dysfunction in the growing period.

3.1 Neonatal programming of metabolic dysfunction

Even though the rising incidence of metabolic syndrome has been blamed on lifestyle and dietary factors, the exact mechanisms by which these factors affect the physiological systems

that regulate appetite, body mass and aetiology of metabolic diseases is still poorly understood (Moura and Passos, 2005). There is strong evidence that some metabolic disorders of adulthood actually are as a result of events that happened in early life (Osmond et al., 1993; Hales and Barker, 2001; Gluckman and Hanson, 2004).

An individual's growth and development is normally controlled in a programmed sequence dictated by the genome right from conception (Patel and Srinivasan, 2002). However, studies have shown that during the early periods of development, there is relative plasticity of the tissues and the individual responds to environmental stimuli by adapting to them at the cellular level (Patel and Srinivasan, 2002).

Neonatal programming has been defined as "an epigenetic phenomenon by which nutritional, hormonal, physical, psychological and other stressful events acting in a critical period of life such as gestation and lactation, modify in a prolonged way certain physiological functions"(Moura and Passos, 2005).

There is abundant evidence from epidemiological studies linking a nutrient deficient uterine environment either due to maternal factors or placental insufficiency to the development of cardiovascular and metabolic diseases (Armitage et al., 2004). Hales and Barker, (2001) stimulated a lot of research in this field when they proposed the 'foetal origins' hypothesis

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which is now used to describe any situation where an event during development causes a permanent physiological response. According to Hales and Barker, (2001) this foetal origin of metabolic diseases leads to the expression of a 'thrifty phenotype' which allows the foetus to maximise nutrient uptake and utilization in the nutrient-deficient *in utero* environment. A predictive adaptive advantage is conferred on the foetus that prepares it for similar nutrient deficient post natal environment (Armitage et al., 2004; Cottrell and Ozanne, 2008).

However, the availability of plentiful nutrition in the post natal environment which exceeds the range of predictive adaptive responses of the neonate will result in an increased risk of developing obesity and type two diabetes mellitus (Armitage et al., 2004; Cottrell and Ozanne, 2008).

Neonatal programming is an epigenetic phenomenon and occurs at the level of gene expression rather than gene sequence (Gallou-Kabani and Junien, 2005). Therefore nutritional insults during those critical stages of development could influence how certain genes are expressed or silenced (Gallou-Kabani and Junien, 2005) by interfering with the normal epigenetic processes.

These detrimental metabolic consequences of programming can be transmitted to subsequent generations without necessarily repeating the initial insult (Drake and Walker, 2004) probably due to permanent changes in the genome (Gallou-Kabani and Junien, 2005).

Previously, the thrifty hypothesis was based on the premise that only offsprings with low birth weight were at an increased risk of metabolic diseases in their adult life. Considering the rise in the incidence of obesity in the developed world where maternal malnutrition or under nutrition is rarely obtained, research now focused on the link between maternal over-nutrition and deleterious metabolic outcomes in later life (Cottrell and Ozanne, 2008). It has now been established that offspring of mothers with over-nutrition or those with gestational diabetes are large for age at birth with increased risk of obesity and diabetes later in life (Ehrenberg et al., 2004). This means that at both extremes of birth weight, there is an increased risk of obesity (Parsons et al., 2001).

Animal models of growth restriction or over-nutrition have been used to study the mechanisms and outcomes of neonatal programming and have provided valuable information (Armitage et al., 2004). The lactation period appears to be the most crucial time in mammals for the initiation of metabolic programming (Moura and Passos, 2005). Maternal dietary manipulation in the form of caloric or protein restriction during gestation have been frequently employed as models of metabolic programming with the effects observed in the offsprings (Patel and Srinivasan, 2002; Armitage et al., 2004; Moura and Passos, 2005; Cottrell and Ozanne, 2008). Unlike the effect of over nutrition in rat pups which has been

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studied by reducing litter size (McCance, 1962), the effect of altered nutrition during the suckling period has been difficult to study because it would involve feeding the pups with a modified formula away from their dams (Patel and Srinivasan, 2002).

Considering the alarming increase in the incidence of metabolic dysfunction and the fact that these impairments can be transmitted across generations, the goal of treatment should be to prevent this transmission. There is evidence that metabolic symptoms in those individuals with intrauterine growth restriction could be reversed with maternal dietary manipulation during lactation (Wyrwoll et al., 2006). Therefore, research in this area needs to be intensified with the aim of determining whether these early interventions would confer any advantages for long term health (Cottrell and Ozanne, 2008).

The incidence of metabolic syndrome is no longer the exclusive preserve of the adults as there is an alarming increase in the prevalence of childhood obesity globally (Stamatakis et al., 2009).

3.2 Childhood Obesity: prevalence and complications

Insulin resistance and type two diabetes mellitus are rapidly emerging as chronic disorders of childhood associated with an increase in the prevalence of obesity (Kohen-Avramoglu et al., 2003). There is a change in the prevalence of childhood obesity globally, attaining an

epidemic proportion (Ebbeling et al., 2002; Birch and Ventura, 2009; Stamatakis et al., 2009). This increase in prevalence is due to an interplay of genetic factors, sedentary life style and altered diets (Biro and Wien, 2010). Different epidemiological studies defined childhood obesity and over-weight in a different manner, making it difficult to make comparisons (Ebbeling et al., 2002).

The worldwide prevalence of childhood obesity which increased from 4.2% in 1990 to 6.7% in 2010 is expected to reach 9.1% by 2020 (De Onis et al., 2010). In Africa, the estimated prevalence of 8.5% in 2010 is expected to increase to 12.7% by 2020 (De Onis et al., 2010). The prevalence of childhood obesity in South Africa was found to be increasing with age, reaching 20-25% by late adolescence (Kimani-Murage et al., 2010). In Nigeria, a prevalence of 5.2% was reported among pre-school children (Senbanjo and Adejuyigbe, 2007) and 1.8% among children attending primary and secondary schools in a semi-urban community (Musa et al., 2012).

There are several complications associated with childhood obesity including hypertension, dyslipidaemia, hyperinsulinaemia, proinflammatory and prothrombotic tendencies (Ford, 2005). Other complications can be grouped as below:

-psychosocial characterised by poor self esteem, depression and eating disorders (Davison and Birch, 2001).

-neurological such as pseudo-tumour cerebri (Balcer et al., 1999).

-pulmonary which include sleep apnoea, asthma and exercise intolerance (Redline et al., 1999; Figueroa-Munoz et al., 2001).

- GIT such as gall stones and steatohepatitis (Strauss et al., 2000).

-renal complications like glomerulosclerosis (Adelman et al., 2001).

- musculoskeletal such as slipped capital femoral epiphysis, Blount's disease, forearm fracture and flat feet (Loder et al., 1993; Dowling et al., 2001).

-endocrine complications which include, type 2 diabetes, precocious puberty, polycystic ovarian disease in girls and hypogonadism in boys (Good et al., 1997; Kaplowitz et al., 2001; Ludwig and Ebbeling, 2001).

- cardiovascular characterized by dyslipidaemia, hypertension, coagulopathy, chronic inflammation and endothelial dysfunction (Freedman et al., 1999; Ford, 2005).

It is clear that the complications of childhood obesity are enormous and therefore there is a need to intensify research aimed at finding affordable and readily available remedies. Resveratrol (a natural phenolic produced in most plants), has been shown to reduce lipid peroxidation and increase sirtuin 1 expression in adult Wistar rats that were programmed by maternal protein restriction during lactation (Franco et al., 2010). Perhaps other phytochemicals in plants could be used to modify the epigenome in the early critical periods and prevent the development of obesity.

3.3 Fructose-induced metabolic dysfunction

The rise in incidence of metabolic syndrome has been linked to changes in dietary habits and reduced physical exercise (Armitage et al., 2004). Therefore it is appropriate, when studying the pathogenesis of the syndrome to use diet induced animal models for those methods not applicable to humans (Angelova and Boyadjiev, 2013). Some of the diet induced models used in rats include: 'high-fat diet, high-carbohydrate diet, combined high-fat high-carbohydrate diet, diet with a high content of sodium chloride and fructose, dietary regimens simulating dietary habits in people as well as prenatal and perinatal dietary manipulations'(Angelova and Boyadjiev, 2013).

Due to its sweetness and affordability, high fructose corn syrup is found in many processed foods (Gajda et al., 2007; Angelova and Boyadjiev, 2013). Studies have shown that a high fructose diet in rats resulted in insulin resistance and hypertriglyceridaemia within two weeks (Pagliassoti et al., 1996). Different amounts and dietary formulations for oral intake of fructose have been used with different features of metabolic syndrome manifesting (Abdulla et al., 2011). Fructose feeding in rats causes increased body mass and reduced glucose tolerance (Angelova and Boyadjiev, 2013). This is however dependent on the stage of maturity at which fructose is introduced (Abdulla et al., 2011). Younger animals tend to gain more mass than older ones (Huynh et al., 2008). Fructose suppresses leptin thereby inhibiting satiety and increasing caloric intake and hence weight gain (Teff et al., 2004).

Hyperinsulinaemia and hepatic insulin resistance in rats have been reported in fructose feeding (Tobey et al., 1982) and in humans (Wei et al., 2007). This insulin resistance in fructose-fed rats could be as a result of down regulation of insulin receptors in skeletal muscles (Abdulla et al., 2011). In rats, fructose feeding for ten weeks produced hyperinsulinaemia, hypertriglyceridaemia and hyperuricaemia (Nakagawa et al., 2006). In a study on the mechanisms of fructose-induced hypertriglyceridaemia, Zavaroni et al., (1982) found that fructose acts directly on the liver to increase VLDL-TG secretion, hyperinsulinaemia and insulin resistance in a rat model. High carbohydrate diets in the rat were observed to stimulate lipogenesis in the liver (Chen et al., 1995; Lee et al., 2008) with subsequent production of palmitoyl-CoA thereby increasing the fatty acid composition of structural lipids in the liver. This increase in structural lipids has been associated with reduced skeletal muscle insulin action and may be a mechanism of action of hepatic insulin resistance (Lee et al., 2008).

3.4 Materials and methods

3.4.1 Source and preparation of HS Calyces

Dried calyces of HS were sourced and processed as mentioned in Chapter two (2.3.1).

3.5 Methods

3.5.1 Study animals

A total of eighty five, 4-day old, suckling Sprague Dawley pups from four dams that were sourced from the Central Animal Services, University of the Witwatersrand, were used in this phase of the study.

3.5.2 Housing and care of the animals

Dams and their respective litter were housed as described in Chapter two (2.4.3) in the first part of the study. In the second part of the study, the dams were returned to stock and the pups were housed in individual Perspex cages and the environment controlled as described in chapter two (2.4.3).

3.5.3 Study design

This phase of the study was done in three stages. The first stage up to day 14 was as described in chapter two (2.4.4). Briefly, the pups were randomly assigned to three treatment

groups using a split litter pattern. The first group, the control group (n=27, 12males, 15 females) received 10ml.kg^{-1} of distilled water. The second group (n=30, 13 males, 17 females) received 50mg.kg^{-1} of aqueous HS calyx extracts while the third group (n=28, 11males, 17 females) received 500mg.kg^{-1} of aqueous HS calyx extract. All the treatments in this phase were administered via oral gavage for 9 consecutive days as described in chapter two (2.4.4). The interventions were stopped at 14 days of age which marked the second stage whence the pups continued to nurse with their dams till post natal day 21 when they were weaned. The dams were returned to stock and the pups were then housed individually as described in the preceding section (3.5.2).

In the third stage of this phase of the study, the pups in each of the three treatment groups were further sub-divided into a control group that continued on tap water as their drinking water and another that received 20% fructose (w/v) in their drinking tap water. The groups were as follows:

I.   DW (distilled water) + TW (tap water) = 10ml.kg^{-1} of distilled water in the first stage and tap water in the second and third stages.

II.   DW + FW (fructose water) = 10ml.kg^{-1} of distilled water in the first stage, tap water in the second stage and 20% fructose (w/v) in their drinking water in the third stage.

III.   LHS (low dose HS) + TW = 50mg.kg^{-1} HS aqueous calyx extract in the first stage and tap water in the second and third stages.

IV.   LHS + FW = 50mg.kg^{-1} HS aqueous calyx extract in the first stage, tap water in the second stage and 20% fructose (w/v) in their drinking water in the third stage.

V.   HHS (high dose HS) + TW = 500mg.kg^{-1} of HS aqueous calyx extract in the first stage and tap water in the second and third stages.

VI.   HHS + FW = 500mg.kg^{-1} of HS aqueous calyx extract in the first stage, tap water in the second stage and 20% fructose (w/v) in the third stage.

The above treatments were continued for four weeks till post natal day 49, when the animals were subjected to an oral glucose tolerance test (OGTT), returned to their treatment groups and then terminated two days later.

3.5.4 Body mass measurements

The pups were weighed daily in the first stage with a balance (Snowrex Electronic Scale, Clover Scales, Johannesburg) in order to adjust the amount of HSE administered so as to maintain a constant dose and also to monitor their growth performance. The rats were weighed twice weekly in the second phase using the same balance to monitor their growth and general health.

3.5.5 Oral glucose tolerance test (OGTT)

Following four weeks of fructose treatment, the rats were subjected to an overnight fast for 12 hours (rats in all the treatment groups had access to tap water during this period) and then subjected to an oral glucose tolerance test to assess their ability to handle a glucose load. On the day of the test, the rats were restrained manually, placed in perspex rat restrainers and allowed to adjust for an hour (in the week preceding the test, the rats had been placed in the restrainers for two hours daily to habituate them). The fasting blood glucose concentration was determined for all the rats (0 minute) before they were orally gavaged with a 50% glucose solution (D-(+)-Glucose (Sigma-Aldrich, France) at $0.2ml.100g^{-1}$ body weight. Serial blood glucose concentrations were determined at 15, 30, 60 and 120 minutes (Loxham et al., 2007) using a calibrated glucometer (Ascensia Elite^{TM}, Bayer Corporation, Mishawaka, USA). The tails of the rats were cleaned with alcohol impregnated swabs and blood obtained via a pin prick to the tails. The rats were then placed back into their treatment groups for 48 hours before study termination.

3.6 Terminal procedures

3.6.1 Termination

The rats were euthanased on post natal day 51, by anaesthetic overdose using intraperitoneal sodium pentobarbitone (Eutha-naze, Bayer, Johannesburg, South Africa) at $200mg.kg^{-1}$ body mass following a 12-hour overnight fast.

3.6.2 Collection of blood, plasma processing and storage

This was done as described in chapter two (2.4.6.2). The modification was that blood was collected via cardiac puncture using 20G needles and 10ml syringes (in place of the 21G needles and 5 ml syringes used in phase one) to collect about 8ml of blood. Plasma was processed as previously described and transferred into three microtubes (Eppendorf, Hamburg, Germany) for each animal. Two of the microtubes (for each rat) were stored at -20ºC in a freezer (Bosh, Johannesburg, South Africa) for clinical biochemistry and assay of hormones while the third microtube was stored at -80ºC in a freezer
(SS engineering, Johannesburg, South Africa) until needed for thiobarbituric acid reactive substances (TBARS) assay.

3.6.3 Morphometry of the GIT and other viscera

This was carried out as described in chapter two (2.4.6.3).

3.6.4 Determination of indices of linear growth

The morphometric characteristics of the anti-gravity bones were determined as described in chapter two (2.4.6.4).

3.6.5 Determination of hepatic storage of metabolic substrates

3.6.5.1 Quantification of hepatic lipid stores

The liver lipid storage was quantified using the method described by Bligh and Dyer, (1959) as was done in chapter two (2.4.6.5.1). About 4-6g of liver sample was used.

3.6.5.2 Quantification of hepatic glycogen stores

Hepatic glycogen stores were quantified as previously described in chapter two (2.4.6.5.2).

3.6.6 Determination of biochemical health markers

Biochemical markers were determined as previously described in chapter two (2.4.6.6). Additionally, a drop of the plasma was used to determine the plasma levels of triglycerides using a calibrated TG-meter (Accutrend® Plus, Roche, Mannheim, Germany).

3.6.7 Determination of thiobarbituric acid reactive substances in the plasma

Thiobarbituric acid reactive substances in the plasma were determined using a TBARS kit (R and D Systems, USA) according to the manufacturer's instructions. It is based on the principle that lipid peroxidation leads to the formation of lipid peroxides which result in the formation of malondialdehyde (MDA). The MDA reacts in the presence of heat with thiobarbituric acid to yield a coloured product that absorbs light which can then be measured as TBARS. The samples were thawed and acid treated with TBARS acid reagent to precipitate interfering proteins and other substances. The samples were then incubated at room temperature for 15 minutes and then centrifuged in an eppendorf centrifuge (Eppendorf, Hamburg, Germany) at 12,000 x G for 4 minutes. The supernatant was then removed and used for the analysis. The optical density was pre-read at 532nm using a microplate reader (Bio-Tek Instruments, Vermont, USA). The samples were then incubated at 48ºC in an oven (Labcon, South Africa) for two and half hours and the optical densities were read again. The

initial optical densities were then subtracted from the final readings. A standard curve was generated from the test standards and used to determine the TBARS concentrations of the test samples.

3.6.8 Plasma insulin concentration determination and computation of HOMA-IR index

Plasma insulin concentration was determined using the sandwich enzyme linked immunosorbent assay kit (DRG®, Rat Insulin High Range, USA) which uses monoclonal antibodies that are specific for rat insulin. Optical densities were read at an absorbance of 450nm using a microplate reader (Multiskan Ascent, Lab system, model nº 354, Helsinki, Finland). The concentration of insulin was determined in relation to a standard curve that was generated using the test standards.

The homeostatic model assessment of insulin resistance (HOMA-IR) was computed by using the equation provided by Matthews et al., (1985) :

HOMA-IR = [Fasting plasma glucose (mg.dl^{-1}) × fasting plasma insulin (μU.mL^{-1})] / 40

3.6.9 Statistical analysis

All data from the study was expressed as mean ± standard deviation. Data was analysed using GraphPad Prism version 5 (Graph-pad Software Inc., San Diego, USA). The level of significance was set at $p \leq 0.05$. With the exception of OGTT, data in this phase was analysed using a one way analysis of variance to assess the effects of HS extracts on the parameters measured. Data from the OGTT was analysed using the repeated measures analysis of variance (ANOVA). This was followed by a Bonferroni *post hoc* test.

3.7 Results

3.7.1 Growth performance

3.7.1.1 Body mass changes

The growth performance of male and female Sprague Dawley rats is presented in Figures 3.1A and 3.1B.

The rats in all the treatment groups gained weight significantly in both sexes ($p<0.001$) however there was no significant difference ($p>0.05$) in the body masses of the rats in the different treatment groups at induction, weaning and termination. The male rats gained more mass ($p<0.001$) than the female rats across the treatment groups.
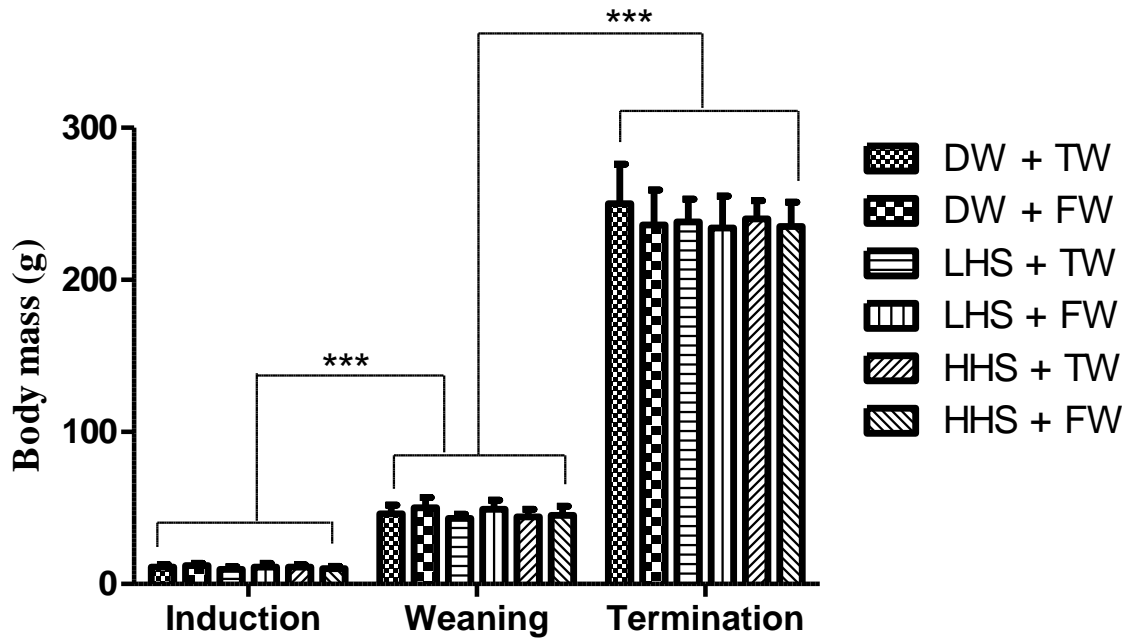
Figure 3.1A: Effect of fructose administration on the growth pattern of male experimental rats across the treatment groups

*** =$p<0.001$. DW + TW= 10ml.kg^{-1} distilled water + tap water in the growing period (n=6),

DW+ FW= 10ml.kg^{-1} distilled water + 20% fructose (w/v) in the drinking water (n=6),

LHS + TW= 50mgkg^{-1} HS extract + tap water (n=6), LHS + FW= 50mg.kg^{-1} HS extract + 20% fructose (w/v) in the drinking water (n=7), HHS + TW= 500mgkg^{-1} HS + tap water (n=6), HHS + FW= 500mg.kg^{-1} HS extract + 20% fructose (w/v) in the drinking water (n=5).

Data expressed as mean ± SD.

Figure 3.1B: Effects of fructose administration on the growth pattern of female experimental rats across the treatment groups.

***= $p < 0.001$. DW + TW= 10ml.kg^{-1} distilled water + tap water in the growing period (n= 8),

DW+ FW= 10ml.kg^{-1} distilled water + 20% fructose (w/v) in the drinking water (n=7),

LHS + TW= 50mgkg^{-1} HS extract + tap water (n=9), LHS + FW= 50mg.kg^{-1} HS extract + 20% fructose (w/v) in the drinking water (n=8), HHS + TW= 500mgkg^{-1} HS + tap water (n=8), HHS + FW= 500mg.kg^{-1} HS extract + 20% fructose (w/v) in the drinking water (n=9). Data expressed as mean ± SD.

3.7.1.2 Linear growth

Table 3.1 shows the effect of fructose administration on the masses, lengths and densities of tibiae and femora of male and female Sprague Dawley rats. The masses, lengths and densities of both the tibiae and femora were similar (p>0.05) in the male rats across the treatment groups. Similarly, the masses lengths and densities of the tibiae and femora were not different (p>0.05) in the female rats across the treatment groups. The male rats in all the treatment groups tended to have heavier and longer tibiae than the corresponding females in the groups, though not statistically significant (p>0.05). With the exception of the HHS + FW group, the male rats across the treatment groups had heavier and longer femora than the corresponding females in the groups.

Table 3.1: Effect of fructose administration on the masses, lengths and densities of tibiae and femora of male and female rats

Treatments	Gender	Tibia			Femur		
		Mass (mg)	Length (mm)	Density (mg.mm^{-1})	Mass (mg)	Length (mm)	Density (mg.mm^{-1})
DW + TW	Males	344 ± 34	35 ±1.6	9.9 ± 0.6	375 ± 65	28 ±2.0	13 ± 1.4
	Females	294 ± 29	33 ±1.3	8.9 ± 0.6	369 ± 19	27 ±0.9	14 ± 0.4
DW + FW	Males	327± 39	34± 2.1	9.7± 0.9	380 ± 61	28± 1.5	14± 0.4
	Females	311± 26	33± 1.1	9.6± 0.7	348 ± 37	26± 1.7	13± 0.8
LHS + TW	Males	334± 18	34 ±1.4	9.7± 0.7	390± 21	28± 1.0	14± 0.6
	Females	295 ± 40	33± 1.8	8.9 ± 0.9	357± 29	27± 0.6	13± 0.9
LHS + FW	Males	339 ± 36	34± 1.5	9.9 ± 0.7	370 ± 67	27± 1.7	14± 1.8
	Females	297± 32	32± 1.9	9.3± 0.8	348 ± 47	26± 1.7	13± 1.2
HHS + TW	Males	348± 32	34± 2.3	10.0 ± 1.1	395± 21	29± 0.7	14± 1.8
	Females	297± 30	32 ±1.2	9.2 ± 0.7	365± 13	26± 1.1	14± 0.4
HHS + FW	Males	343 ± 9.5	35± 1.0	9.9 ± 0.5	347± 47	27± 1.7	13± 1.0
	Females	306± 42	33± 2.0	9.3± 0.8	355± 50	27± 1.4	13± 1.4

DW + TW= 10ml.kg^{-1} distilled water + tap water, DW+ FW= 10ml.kg^{-1} distilled water + 20%

fructose (w/v) in the drinking water, LHS + TW= 50mgkg^{-1} HS extract + tap water, LHS +

FW= 50mg.kg^{-1} HS extract + 20% fructose (w/v) in the drinking water, HHS + TW=

500mgkg^{-1} HS + tap water, HHS + FW= 500mg.kg^{-1} HS extract + 20% fructose (w/v) in the

drinking water. Data expressed as mean ± SD.

3.7.2 Glucose tolerance

Figure 3.2A and 3.2B show the different blood glucose concentrations of male and female Sprague Dawley rats at 0, 15, 30, 60 and 120 minutes following oral gavage of 2g.kg^{-1} of glucose solution. Figure 3.3A and 3.3B show the total area under the curve of the oral glucose tolerance test in male and female Sprague Dawley rats.

At 15 minutes post gavage, the following trend was observed: blood glucose concentration of rats in DW + TW was significantly higher ($p<0.01$) than that of HHS + TW group while that of DW + FW was significantly higher ($p<0.01$) than that of LHS + TW (Figure 3.2A). The glucose concentration of rats in the LHS + TW was significantly higher than that of HHS + TW ($p< 001$) and HHS + FW ($p<0.05$) (Figure 3.2A). There was no difference in blood glucose concentration ($p>0.05$) at all the other time intervals across the treatment groups (Figure 3.4A). There were no differences in blood glucose concentrations in the female rats across the treatment groups at the different time intervals except at 15 minutes when blood glucose concentration in the DW + FW was significantly higher ($p<0.01$) than that of HHS + FW group (Figure 3.2B). The total area under the curve of the oral glucose tolerance test were not significantly different ($p>0.05$) across the treatment groups in both male and female rats (Figures 3.3A and 3.3B).
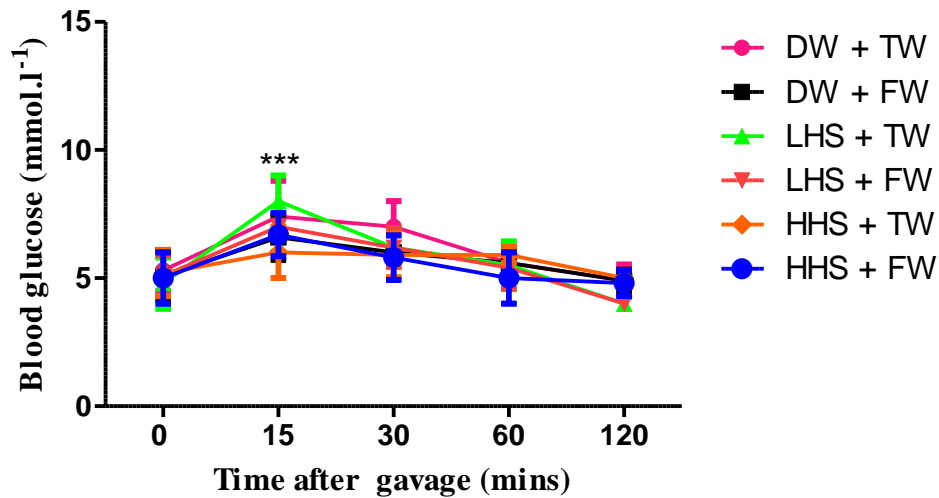
Figure 3.2A: Effects of fructose administration on glucose tolerance in male rats

***p=<0.001. Blood glucose concentration in all the treatment groups peaked at 15 minutes and returned to the basal concentration by 120 minutes post gavage. Blood glucose concentration at 15 minutes in all the groups differed (p<0.001) from the basal concentration and from that at 120 minutes (p<0.001). DW + TW= 10ml.kg^{-1} distilled water + tap water in the growing period (n=6), DW+ FW= 10ml.kg^{-1} distilled water + 20% fructose (w/v) in the drinking water (n=6), LHS + TW= 50mgkg^{-1} HS extract + tap water (n=6), LHS + FW= 50mg.kg^{-1} HS extract + 20% fructose (w/v) in the drinking water (n=7), HHS + TW= 500mgkg^{-1} HS + tap water (n=6), HHS + FW= 500mg.kg^{-1} HS extract + 20% fructose (w/v) in the drinking water (n=5). Data expressed as mean± SD.
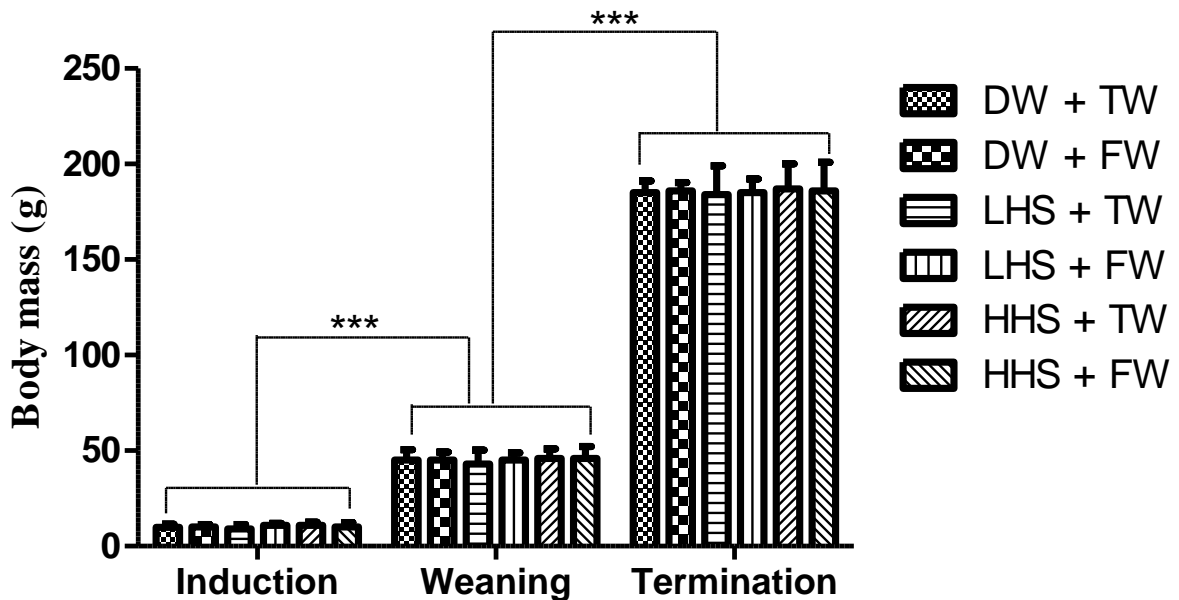
Figure 3.2B: Effects of fructose administration on glucose tolerance in female rats

***=p<0.001. Blood glucose concentration of female rats in all the groups peaked at 15 minutes and returned to basal level by 120 minutes post gavage. Blood glucose concentration at 15 minutes in all the groups differed (p<0.001) from the basal concentration and from that at 120 minutes (p<0.001). DW + TW= 10ml.kg^{-1} distilled water + tap water in the growing period (n= 8),DW+ FW= 10ml.kg^{-1} distilled water + 20% fructose (w/v) in the drinking water (n=7), LHS + TW= 50mgkg^{-1} HS extract + tap water (n=9), LHS + FW= 50mg.kg^{-1} HS extract + 20% fructose (w/v) in the drinking water (n=8), HHS + TW= 500mg.kg^{-1} HS + tap water (n=8), HHS + FW= 500mg.kg^{-1} HS extract + 20% fructose (w/v) in the drinking water (n=9). Data expressed as mean ± SD.

Figure 3.3A: Effects of fructose administration on the total area under the curve of oral glucose tolerance test in male rats

DW + TW= 10ml.kg^{-1} distilled water + tap water in the growing period (n=6), DW+ FW= 10ml.kg^{-1} distilled water + 20% fructose (w/v) in the drinking water (n=6), LHS + TW= 50mgkg^{-1} HS extract + tap water (n=6), LHS + FW= 50mg.kg^{-1} HS extract + 20% fructose (w/v) in the drinking water (n=7), HHS + TW= 500mgkg^{-1} HS + tap water (n=6), HHS + FW= 500mg.kg^{-1} HS extract + 20% fructose (w/v) in the drinking water (n=5), AUC= area under the curve. Data expressed as mean± SD.
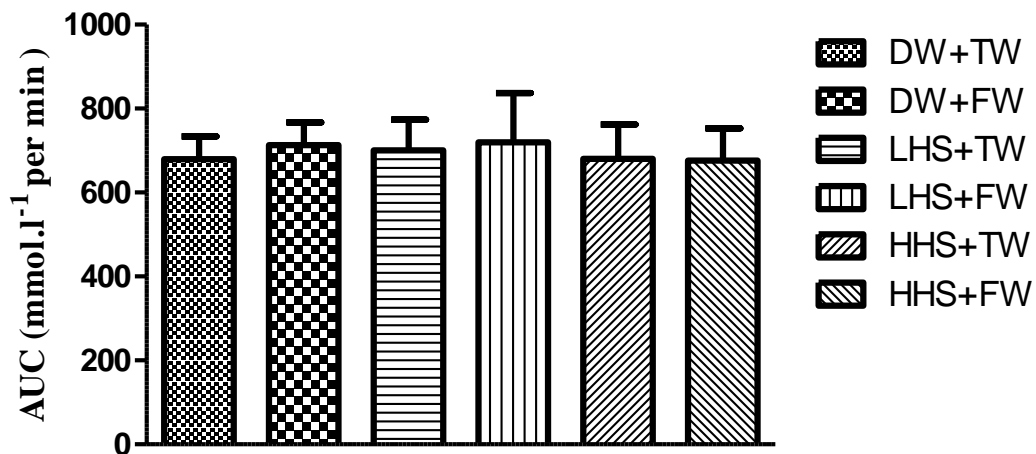
Figure3.3B: Effect of fructose administration on the total area under the curve of oral glucose tolerance test in female rats

DW + TW= 10ml.kg^{-1} distilled water + tap water in the growing period (n= 8),DW+ FW= 10ml.kg^{-1} distilled water + 20% fructose (w/v) in the drinking water (n=7), LHS + TW= 50mgkg^{-1} HS extract + tap water (n=9), LHS + FW= 50mg.kg^{-1} HS extract + 20% fructose (w/v) in the drinking water (n=8), HHS + TW= 500mgkg^{-1} HS + tap water (n=8), HHS + FW= 500mg.kg^{-1} HS extract + 20% fructose (w/v) in the drinking water (n=9), AUC= area under the curve. Data expressed as mean ± SD.

3.7.3: Effects of fructose administration on circulating metabolic substrates, TBARS, insulin and HOMA-IR

Table 3.2 shows the effect of fructose administration on circulating metabolic substrates, TBARS, insulin and HOMA-IR of male and female Sprague Dawley rats. There were no statistical differences observed ($p>0.05$) in the plasma fasting blood glucose concentration and triglycerides in rats of both sexes across the treatment groups (Table 3.2). The plasma concentration of TBARS, insulin, as well as the HOMA-IR were also similar ($p>0.05$) across the treatment groups (Table 3.2).

Table 3.2: Effect of fructose administration on metabolic substrates, TBARS, Insulin and HOMA-IR of male and female Sprague Dawley rats

Treatments	Gender	TBARS (μM)	TGs (mmol.L^{-1})	FBG (mmol.L^{-1})	Insulin μU.ml^{-1}	HOMA- IR
DW + TW	Males	0.16±0.03	2.5± 1.30	5.3± 0.81	15± 6.30	3.6± 1.80
	Females	0.15±0.03	1.6±0.36	4.6± 0.70	11± 7.30	2.3± 1.50
DW + FW	Males	0.15±0.03	2.4± 0.73	5.1± 0.94	11± 4.70	2.6± 1.50
	Females	0.16±0.03	2.3±0.81$^{\beta}$	4.9± 0.67	17± 7.20	4.0± 1.60
LHS + TW	Males	0.15±0.03	1.8± 0.29	4.8± 1.00	12± 8.40	2.6± 1.80
	Females	0.16±0.03	1.7±0.31	5.0± 0.76	9.4± 4.50	2.2± 1.10
LHS + FW	Males	0.15±0.03	2.2± 0.81	5.1± 0.79	15± 8.00	3.6± 2.10
	Females	0.15±0.03	2.8±0.51	4.7± 0.83	15± 9.20	3.3± 2.30
HHS +TW	Males	0.15±0.03	2.0± 0.60	5.2± 0.91	13± 7.10	2.9± 1.70
	Females	0.15±0.03	1.8±0.49	4.8± 0.60	12± 4.50	2.5± 1.00
HHS + FW	Males	0.15±0.03	2.4± 0.41	5.0± 1.00	11± 5.20	2.5± 1.30
	Females	0.16±0.03	2.0±0.50	4.8± 0.79	13± 8.30	2.9± 2.00

β = significantly different from the corresponding males in the group.

TBARS= Thiobarbituric reactive substances, TGs= Triglycerides, FBG= Fasting blood glucose, HOMA-IR= Homeostatic model of Insulin resistance, DW + TW= 10ml.kg^{-1} distilled water + tap water,   DW+ FW= 10ml.kg^{-1} distilled water + 20% fructose (w/v) in the drinking water, LHS + TW= 50mgkg^{-1} HS extract + tap water, LHS + FW= 50mg.kg^{-1} HS extract + 20% fructose (w/v) in the drinking water, HHS + TW= 500mgkg^{-1} HS + tap water, HHS + FW= 500mg.kg^{-1} HS extract + 20% fructose (w/v) in the drinking water. Data expressed as mean ± SD.

3.7.4 Hepatic storage of metabolic substrates

Table 3.3 shows the liver lipids and glycogen content in male and female rats. There was no significant difference ($p > 0.05$) in the liver lipid and glycogen content across the treatment groups (Table 3.3). No gender differences were also observed (Table 3.3).

Table 3.3: Liver lipids and glycogen (expressed as glucose equivalents) in male and female rats.

Treatment	Gender	Liver lipids (% liver mass)	Liver glycogen ($mmol.L^{-1}$)
DW + TW	Male	2.8 ± 1.00	2.6 ± 0.86
	Females	2.9 ± 1.10	2.0 ± 0.57
DW + FW	Males	3.9 ± 0.85	2.3 ± 1.00
	Females	4.4 ± 1.30	1.4 ± 0.87
LHS + TW	Males	3.2 ± 1.00	2.1 ± 0.28
	Females	4.1 ± 0.93	1.8 ± 0.66
LHS + FW	Males	3.3 ± 0.74	2.4 ± 0.69
	Females	3.5 ± 0.54	1.4 ± 0.87
HHS + TW	Males	3.4 ± 0.90	2.2 ± 0.46
	Females	3.7 ± 0.48	1.8 ± 0.99
HHS + FW	Males	3.8 ± 0.93	2.0 ± 1.10
	Females	3.8 ± 0.52	1.8 ± 0.69

DW + TW= 10ml.kg^{-1} distilled water + tap water,   DW+ FW= 10ml.kg^{-1} distilled water + 20% fructose (w/v) in the drinking water, LHS + TW= 50mgkg^{-1} HS extract + tap water, LHS + FW= 50mg.kg^{-1} HS extract + 20% fructose (w/v) in the drinking water, HHS + TW= 500mgkg^{-1} HS + tap water, HHS + FW= 500mg.kg^{-1} HS extract + 20% fructose (w/v) in the drinking water. Data expressed as mean ± SD.
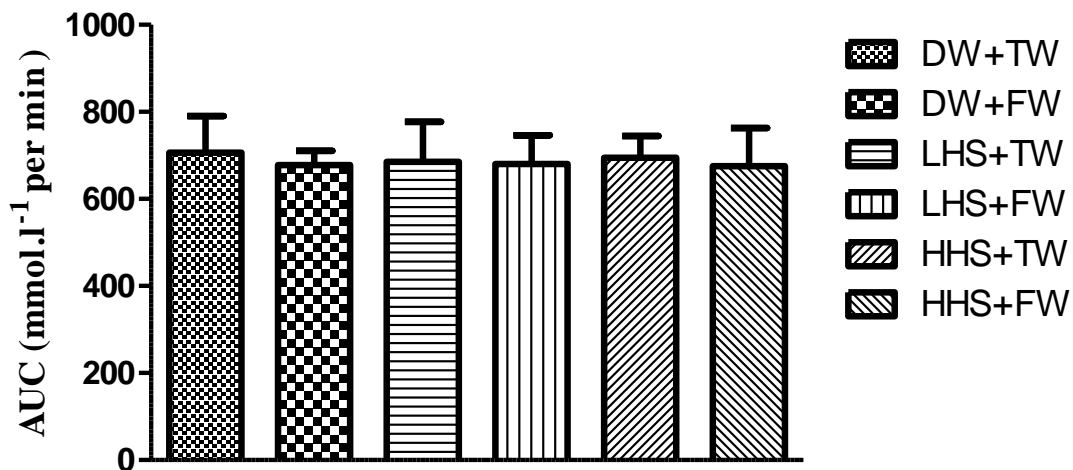
3.7.5 Morphology of the GIT and other viscera

Table 3.4 shows the effect of fructose administration on the absolute (g), relative masses (%BM) and lengths of small and large intestines of male and female rats. Table 3.5 shows the effects of fructose administration on the absolute (g) and relative (%BM) masses of some abdominal viscera of male and female rats. The absolute mass of the caeca of male rats in the LHS + TW was only significantly greater ($p<0.01$) than those of HHS + FW group (Table 3.4). However, when compared relative to the body mass, the caeca of the male rats in the LHS +TW were significantly greater than those of their male counterparts in DW + FW ($p<0.05$), LHS + FW ($p<0.05$) and HHS + FW ($p< 0.05$) treatment groups (Table 3.5). The male rats had significantly heavier (absolute) small intestines (Table 3.4) and liver (Table 3.5) when compared to the corresponding females in all the treatment groups. The only gender difference observed was in the absolute masses of the large intestines (Table 3.4) in the DW + TW group ($p<0.01$) where those of the male rats were significantly heavier. The absolute masses of the stomach were heavier in the male rats (Table 3.5) than the corresponding females in the DW + TW ($p<0.01$) and the HHS + FW ($p<0.05$) groups. In the absolute masses of the caecum (Table 3.5), gender differences were observed in the LHS + TW ($p<0.001$) and HHS + TW ($P<0.01$) groups with those of the male rats being heavier. There was no significant difference ($p>0.05$) in the masses of the GIT and other viscera relative to the terminal body masses (%BM) (Tables 3.4 and 3.5) except for the visceral fat where the female rats in the HHS + TW ($p<0.01$) and HHS + FW ($p<0.01$) groups had significantly higher amounts of visceral fat than their corresponding male counterparts. The

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male rats in the DW + TW (p<0.05), DW + FW (p<0.05) and LHS + TW (p<0.001) treatment groups (Table 3.4) had significantly longer small intestines than their female counterparts. There were no differences observed in the lengths of large intestines in the rats (Table 3.4) across the treatment groups (P>0.05).

Table 3.4 Effect of fructose administration on the absolute (g), relative masses (%BM) and lengths of the small and large intestines of male and female rats

	Gender	S.I (g)	S.I (%BM)	S.I (mm)	L.I (g)	L.I (%BM)	L.I (mm)
DW + TW	Males	7.9 ± 1.10	3.2 ± 0.31	1393 ±78.00	1.9 ± 0.28	0.75 ± 0.07	230 ± 21.00
	Females	6.1 ± 0.59$^\beta$	3.3 ± 0.39	1253 ±47.00$^\beta$	1.5 ± 0.08$^\beta$	0.80 ± 0.05	203 ± 15.00
DW + FW	Males	8.1 ± 0.67	3.4 ± 0.33	1392 ± 64.00	1.7 ± 0.20	0.72 ± 0.09	214 ± 4.90
	Females	6.2 ± 0.31$^\beta$	3.3 ± 0.20	1249 ±48.00$^\beta$	1.4 ± 0.15	0.76 ± 0.08	199 ± 14.00
LHS + TW	Males	8.0 ± 0.96	3.3 ± 0.21	1433 ± 82.00	1.7± 0.17	0.73 ± 0.05	227 ± 12.00
	Females	6.3 ± 0.54$^\beta$	3.4 ± 0.28	1252 ±97.00$^\beta$	1.5 ± 0.19	0.82 ± 0.05	211 ± 15.00
LHS + FW	Males	7.9 ± 0.83	3.4 ± 0.35	1379 ± 51.00	1.7± 0.23	0.74 ± 0.05	222 ± 18.00
	Females	6.4 ± 0.30$^\beta$	3.5 ± 0.12	1298 ± 58.00	1.4 ± 0.11	0.77 ± 0.06	210 ± 8.00
HHS + TW	Males	7.8 ± 0.61	3.3 ± 0.20	1379 ± 51.00	1.7 ± 0.11	0.72 ± 0.04	224 ± 9.20
	Females	6.4 ± 0.59$^\beta$	3.4 ± 0.20	1275 ± 81.00	1.6 ± 0.24	0.81 ± 0.08	204 ± 15.00
HHS + FW	Males	7.8 ± 0.72	3.3 ± 0.41	1346 ± 24.00	1.7 ± 0.18	0.72 ± 0.06	220 ± 26.00
	Females	6.1 ± 0.44$^\beta$	3.3 ± 0.16	1247 ± 38.00	1.4 ± 0.10	0.74 ± 0.09	199 ± 15.00

$^\beta$ = significantly different from the corresponding males in the group. S.I= small intestines,

L.I= large intestines, BM= body mass, DW + TW= 10ml.kg^{-1} distilled water + tap water,

DW+ FW= 10ml.kg^{-1} distilled water + 20% fructose (w/v) in the drinking water, LHS + TW=

50mgkg^{-1} HS extract + tap water, LHS + FW= 50mg.kg^{-1} HS extract + 20% fructose (w/v) in

the drinking water, HHS + TW= 500mgkg^{-1} HS + tap water, HHS + FW= 500mg.kg^{-1} HS

extract + 20% fructose (w/v) in the drinking water. Data expressed as mean ± SD

Table 3.5: Effect of fructose administration on the absolute (g) and relative (%BM) masses of some abdominal viscera of male and female rats

Treatments	Gender	Liver (g)	Liver (%BM)	Stomach (g)	Stomach (%BM)	Caecum (g)	Caecum (%BM)	Visceral fat (g)	Visceral fat(%BM)
DW + TW	Males	10± 1.40	4.0± 0.25	1.6± 0.18	0.64±0.06	1.3±0.18^{ab}	0.51±0.06^{ab}	2.9 ± 0.81	1.2 ± 0.24
	Females	7.0±0.38$^{\beta}$	3.8± 0.20	1.3±0.11$^{\beta}$	0.68±0.06	1.0±0.10^{ab}	0.55±0.05^{ab}	3.4 ± 0.73	1.9 ± 0.42
DW + FW	Males	9.9± 1.10	4.2± 0.27	1.6± 0.12	0.67±0.04	1.1±0.21^{ab}	0.48±0.08^{b}	3.9 ± 1.20	1.6 ± 0.35
	Females	7.5± 0.44$^{\beta}$	4.0± 0.28	1.3± 0.11	0.72±0.06	0.9±0.10^{ab}	0.49±0.06^{ab}	4.3 ± 0.81	2.3 ± 0.42
LHS + TW	Males	9.5± 1.70	4.0± 0.55	1.5± 0.17	0.63±0.05	1.5±0.18^{b}	0.62±0.07^{a}	3.0 ± 0.81	1.3 ± 0.32
	Females	7.4± 0.89$^{\beta}$	4.0± 0.34	1.4 ± 0.16	0.74±0.08	1.0±0.14$^{ab\beta}$	0.53±0.07^{ab}	3.7 ± 0.99	2.0 ± 0.47
LHS + FW	Males	9.4± 0.83	4.0± 0.26	1.6± 0.14	0.67±0.05	1.2±0.17^{ab}	0.49±0.04^{b}	3.4 ± 0.72	1.4 ± 0.25
	Females	7.2± 0.54$^{\beta}$	3.9± 0.31	1.4± 0.08	0.73±0.02	1.0±0.14^{ab}	0.52±0.07^{ab}	4.1 ± 1.10	2.2 ± 0.55
HHS + TW	Males	9.5± 0.61	4.0± 0.22	1.6± 0.14	0.66±0.07	1.4±0.17^{ab}	0.57±0.07^{ab}	2.7 ± 0.40	1.1 ± 0.13
	Females	7.3± 1.00$^{\beta}$	3.9± 0.35	1.3± 0.18	0.71±0.06	1.0±0.21$^{ab\beta}$	0.55±0.08^{ab}	3.9 ± 0.92	2.1 ± 0.43$^{\beta}$
HHS + FW	Males	9.7± 0.89	4.1 ±0.31	1.6± 0.17	0.69±0.10	1.1±0.13^{a}	0.47±0.07^{b}	3.4 ± 0.54	1.4 ± 0.23
	Females	7.5± 0.87$^{\beta}$	4.0± 0.21	1.3±0.11$^{\beta}$	0.71±0.04	0.9±0.11^{ab}	0.48±0.07^{ab}	4.7 ± 1.70	2.5 ± 0.77$^{\beta}$

^{ab} within columns means with different superscripts are significantly different at p $\leq$0.05.

$^{\beta}$ = significantly different from the corresponding males in the group. DW + TW= 10ml.kg^{-1} distilled water + tap water,   DW+ FW= 10ml.kg^{-1} distilled water + 20% fructose (w/v) in the drinking water, LHS + TW= 50mgkg^{-1} HS extract + tap water, LHS + FW= 50mg.kg^{-1} HS extract + 20% fructose (w/v) in the drinking water, HHS + TW= 500mgkg^{-1} HS + tap water, HHS + FW= 500mg.kg^{-1} HS extract + 20% fructose (w/v) in the drinking water. Data expressed as mean ± SD.

3.7.6 Markers of hepatic and renal health

Table 3.6 shows the effect of fructose administration on surrogate markers of renal and hepatic health in male and female Sprague Dawley rats. The concentration of alanine transaminase (ALT) in the male rats in the DW + FW was significantly higher ($p<0.01$) than their male counterparts in the HHS + TW (Table 3.6). The plasma concentration of ALT was significantly higher in the male rats than their corresponding females in the HHS + TW ($p<0.05$) treatment groups (Table 3.6). Similarly, the plasma concentrations of alkaline phosphatase (ALP) was higher in the male rats in the DW + TW ($p<0.01$), LHS + FW ($p<0.01$), HHS + TW ($p<0.05$) and HHS + FW ($p<0.05$) treatment groups than the corresponding females in the groups (Table 3.6).

There was no significant difference observed in the plasma concentration of total biluribin, creatinine and blood urea nitrogen ($p>0.05$) across the treatment groups (Table 3.6).

Table 3.6: Effect of fructose administration on surrogate markers of hepatic and renal health in male and female Sprague Dawley rats

Treatments	Gender	ALT (U.L^{-1})	ALP (U.L^{-1})	Tbil (μmol.L^{-1})	BUN (mmol.L^{-1})	Creat (mmol.L^{-1})
DW + TW	Males	87±19.00^{ab}	380± 61	2.0± 0.00	5.5± 1.40	28± 3.30
	Females	85±20.00^{ab}	243±32^{β}	2.6± 1.10	4.3± 1.20	29± 5.90
DW + FW	Males	67±16.00^{a}	335± 47	2.2± 0.41	4.5± 1.40	28± 6.30
	Females	74± 14.00^{ab}	253± 58	3.1± 2.60	4.6± 1.10	34± 5.80
LHS + TW	Males	98± 23.00^{ab}	356± 45	2.0± 0.00	4.8± 1.90	24± 4.60
	Females	91± 18.00^{ab}	273± 82	2.1± 0.33	5.6± 0.99	30± 9.60
LHS + FW	Males	78± 13.00^{ab}	366± 75	2.1± 0.38	5.0± 0.40	29± 6.40
	Females	69± 12.00^{ab}	229±41^{β}	2.1± 0.35	5.3± 1.00	30± 4.10
HHS +TW	Males	107±28.00^{b}	374± 87	3.5± 2.70	6.1± 1.50	27± 7.60
	Females	74± 6.10^{abβ}	252±47^{β}	2.0± 0.00	5.4± 0.79	31± 7.90
HHS + FW	Males	78± 15.00^{ab}	359± 61	2.6± 1.30	5.0± 1.20	28± 7.10
	Females	70± 10.00^{ab}	241±53^{β}	2.0± 0.00	4.4± 0.81	29± 5.60

Means with different superscripts in a column are significantly different (p ≤0.05), [β] = significantly different from the corresponding males in the groups. ALT= Alanine transaminase, ALP= Alkaline phosphatase, BUN= Blood Urea Nitrogen, Tbil= Total Biluribin, Creat= Creatinine , DW + TW= $10ml.kg^{-1}$ distilled water + tap water, DW+ FW= $10ml.kg^{-1}$ distilled water + 20% fructose (w/v) in the drinking water, LHS + TW= $50mgkg^{-1}$ HS extract + tap water, LHS + FW= $50mg.kg^{-1}$ HS extract + 20% fructose (w/v) in the drinking water, HHS + TW= $500mgkg^{-1}$ HS + tap water, HHS + FW= $500mg.kg^{-1}$ HS extract + 20% fructose (w/v) in the drinking water. Data expressed as means ± SD.

3.7.7 Markers of general health

Table 3.7 shows the effect of fructose administration on the general health markers in the plasma of male and female Sprague Dawley rats. There was no significant difference ($p>0.05$) in all the general health markers assayed between the male rats in all the treatment groups. There was also no significant difference ($p>0.05$) observed between the female rats across the treatment groups in all the general health markers assayed. The plasma concentration of calcium was significantly higher in male rats than their corresponding females ($p<0.05$) in the LHS + FW ($p<0.05$) treatment group (Table 3.7). Plasma concentrations of amylase was higher in the male rats compared to the corresponding females in the DW + TW ($p<0.001$), LHS + TW ($p<0.05$), LHS + TW ($p<0.05$), LHS + FW ($p<0.001$) and HHS + FW ($p<0.05$) treatment groups (Table 3.7). However, the plasma concentration of cholesterol was higher in the female rats compared to the male rats in the HHS + FW ($p<0.05$) group. There were no significant differences ($p>0.05$) in the other health markers measured across all the treatment groups (Table 3.7).

Table 3.7: Effect of fructose administration on the general health markers in the plasma of male and female Sprague Dawley rats

Treatments	Gender	Phosphate (mmol.L^{-1})	Calcium (mmol.L^{-1})	Total protein (g.L^{-1})	Albumin (g.L^{-1})	Globulin (g.L^{-1})	Amylase (U.L^{-1})	Cholesterol (mmol.L^{-1})
DW + TW	Males	2.6± 0.37	2.9± 0.17	55± 2.90	28± 2.10	27± 1.20	1833± 211	1.8± 0.21
	Females	2.2± 0.53	2.8± 0.04	54± 2.30	28± 1.30	26± 0.89	1147± 244$^{\beta}$	1.9± 0.25
DW + FW	Males	2.4± 0.27	2.8± 0.05	56± 1.90	29± 1.30	27± 1.50	1950±123	2.1± 0.12
	Females	2.5± 0.20	2.8± 0.04	55± 2.30	29± 2.40	25± 1.50	1552±418	2.2± 0.19
LHS + TW	Males	2.7± 0.29	2.8± 0.08	54± 2.30	27± 0.98	26± 1.80	1746±86	1.7± 0.08
	Females	2.4± 0.31	2.8± 0.08	55± 3.40	30± 2.50	25± 2.40	1263±259$^{\beta}$	2.0± 0.20
LHS + FW	Males	2.7± 0.41	3.0± 0.04	54± 1.90	29± 1.90	25± 1.10	1997± 254	1.9± 0.31
	Females	2.3± 0.24	2.8± 0.05$^{\beta}$	55± 2.00	29± 1.90	26± 1.30	1375± 207$^{\beta}$	2.2± 0.42

Table 3.7: continued

HHS + TW	Males	2.5± 0.35	2.8± 0.06	55± 2.20	29± 1.70	26± 1.20	1735± 201	1.8± 0.14
	Females	2.2± 0.16	2.8± 0.06	56± 1.80	29± 1.20	27± 1.10	1295±2241	2.2± 0.27
HHS + FW	Males	2.7± 0.28	2.9± 0.10	54± 3.10	29± 2.40	25± 1.10	2084± 218	1.7± 0.18
	Females	2.2± 0.38	2.8± 0.07	55± 1.40	29± 1.20	26± 0.88	1540± 303$^{\beta}$	2.2± 0.34$^{\beta}$

$^\beta$ = significantly different from the corresponding males in the groups. DW + TW= 10ml.kg^{-1} distilled water + tap water,   DW+ FW= 10ml.kg^{-1} distilled water + 20% fructose (w/v) in the drinking water, LHS + TW= 50mgkg^{-1} HS extract + tap water, LHS + FW= 50mg.kg^{-1} HS extract + 20% fructose (w/v) in the drinking water, HHS + TW= 500mgkg^{-1} HS + tap water, HHS + FW= 500mg.kg^{-1} HS extract + 20% fructose (w/v) in the drinking water. Data expressed as means ± SD.

3.8 Discussion

In this phase of the study, the rat pups were initially administered with HS extracts and then at weaning were given 20% fructose (w/v) in their drinking water to investigate whether the earlier administration of HS had any effect on their response to an insult by a high fructose diet. The discussion will focus on the observed differences in response to fructose challenge among the treatment groups taking into consideration any gender differences that may have occurred.

3.8.1 Growth performance of male and female rats

Sex differences in rates of growth of rats usually begin to manifest between post natal day 25 to 33 in favour of the males and are usually preceded by an increase in testosterone levels (Eden, 1979; Gabriel et al., 1992). This could explain why the males in all the experimental groups in this study gained more weight than their corresponding female counterparts.
As discussed in chapter two, body mass is usually affected by a number of factors such as hydration status and GIT filling (Ellis et al., 2002; MacCracken and Stebbings, 2012) and may therefore not be the best indicator of growth performance. The lengths of the long bones are better markers of growth as they correlate with growth hormone secretion in a dose-dependent manner (Baum et al., 1996; Eshet et al., 2004). In this study, there was no significant difference in the lengths, masses and densities of the tibiae and femora across the different

groups although the male rats had longer and heavier tibiae when compared to their corresponding females. With the exception of the HHS + FW group, the male rats in all the other groups also had longer and heavier femora than the corresponding females in the groups. These findings tend to suggest that neither the earlier administration of HS extracts nor that of 20% fructose had any effect on the growth performance of the male and female rats.

3.8.2 Glucose tolerance, fasting blood glucose, Insulin and HOMA-IR

Fructose administration in rats is known to produce features of metabolic syndrome including impaired glucose tolerance. It has previously been shown that 20% fructose in drinking water for 5 weeks (Ramadan et al., 2012) and 8 weeks (Mamikutty et al., 2014) is sufficient to cause metabolic derangements in Wistar Albino rats. When 25% fructose was used in the drinking water, there was a reduction in fluid intake of the rats probably due the increased sweetness of the fluid (Mamikutty et al., 2014). Hence, 20% fructose in drinking water was used. In this study, both males and female rats in all the treatment groups showed normal ability to handle an oral glucose load (Figures 3.3A and 3.3B). The blood glucose concentrations peaked at 15 minutes following the administration of the glucose load and returned to basal levels by 120 minutes post gavage. The differences observed in blood glucose concentration at 15 minutes post gavage across the treatment groups, especially in the male rats may indicate early manifestation of glucose handling problems. However, further analysis with area under the

curve of the OGTT revealed no differences. In this study, there was no significant difference in the fasting blood glucose, insulin concentration and the computed HOMA-IR across the treatment groups. The fasting plasma triglycerides concentrations were also not significantly different except between the males and females in the low dose HSE + FW group .This similarity in the glucose tolerance, fasting plasma glucose, fasting triglycerides, cholesterol, insulin and the HOMA-IR suggests that the administration of 20% fructose (w/v) in drinking water of the rats did not induce metabolic dysfunction. Fructose administration is known to cause reduced glucose tolerance and body mass gain (Angelova and Boyadjiev, 2013), hyperinsulinaemia and insulin resistance in rats (Tobey et al., 1982). Fructose feeding for ten weeks in adolescent rats (150-200g) had previously been shown to produce hyperinsulinaemia, hypertriglyceridaemia and hyperuricaemia (Nakagawa et al., 2006). The findings in this study were also at variance with those of Pagliassoti et al., (1996) that fructose feeding for two weeks is enough to produce hypertriglyceridaemia and insulin resistance. With the exception of the triglycerides where there was a gender difference in the LHS + FW group, there was no gender difference in the response of the rats to fructose administration for glucose handling, fasting plasma glucose, insulin and HOMA-IR. The age of the rats at termination (51 days) and the mode of fructose administration could have been factors responsible for the absence of markers of metabolic syndrome. Wistar rats between the ages of 2 to 6 months were found to have lower levels of serum glucose, TGs, total cholesterol, HDL and LDL when compared to their 12 month old counterparts (Ghezzi et al., 2012). High fructose intake has been shown to be more effective in inducing metabolic syndrome in adults than in young rats (de Moura et al., 2009). Fructose in the feed rather than

in drinking water is also a more effective means of producing metabolic syndrome (de Moura et al., 2009) probably because the rats would eat more food (and hence more fructose) than water . Unfortunately, even though the rats drank the fructose water, I was unable to take record of their actual fluid (fructose) intake in this study.

3.8.3 Lipid peroxidation

Fructose feeding in rats activates stress pathways especially in the liver due to burden of fructose metabolism (Kelley et al., 2004). High fructose feeding produces hypertriglyceridemia, increased serum glucose and total cholesterol which increase lipid peroxidation due to the altered lipid metabolism and consequent increase in tissue and plasma lipid peroxides (Anurag and Anuradha, 2002; Kelley et al., 2004). Insulin resistance and hyperinsulineamia have been associated with increased plasma lipid peroxide levels in patients with impaired glucose tolerance (Niskanen et al., 1995) and with the stress pathway (Kelley et al., 2004). *Hibiscus sabdariffa* has an abundance of antioxidants (Farombi and Fakoya, 2005; Usoh et al., 2005; Hirunpanich et al., 2006) which have been found to decrease the increased levels of TBARS usually associated with high fructose feeding (Miatello et al., 2005). In this study, the TBARS assay was used to determine lipid peroxidation in the plasma as a measure of oxidative damage. The TBARS values obtained showed no significant difference across all the treatment groups. This is probably due to non-development of the symptoms of metabolic syndrome which would have triggered the peroxidative damage. However, from the available data in this study, the earlier administration of HS in the neonatal period had no effect on the TBARS measured.

3.8.4 Hepatic metabolic substrates storage

The liver is a key organ in the body performing numerous functions that maintain homeostasis(Michalopoulos, 2007). These functions include maintaining circulating metabolic substrates, detoxification, hormone inactivation and storage functions among many others (Sawchenko and Mark, 1979; Sallie et al., 1991; Michalopoulos, 2007; Seyama and Kokudo, 2009). High fructose feeding causes disturbances in carbohydrate and lipid metabolism, consequently affecting the homeostatic functions of the liver (Kelley et al., 2004). Conventional drugs and plant extracts are partly metabolised in the liver and can also alter the functions of the liver. Measurement of surrogate markers of liver function is therefore quite important.

High fructose diet causes hypertriglycerideamia which provide substrates for hepatic lipogenesis, lipid overload and insulin resistance (Stanhope and Havel, 2008). A 7 day administration of a high fructose diet increased ectopic lipid deposition in the liver and muscles of healthy non-smoking males (Lê et al., 2009).

High fructose diet causes an increase in hepatic glycogen stores due to increased conversion of fructose to glycogen via gluconeogenesis (Conlee et al., 1987; Koo et al., 2008). Rats fed 63% fructose (w/w) 4 hours per day for 2 weeks showed increased hepatic glycogen stores (Koo et al., 2008).

In this study, there was no significant difference (p>0.05) in both hepatic lipids and glycogen content across all the treatment groups when compared to the control group. This similarity in hepatic lipid and glycogen content could be due to the fact that the animals were fasted overnight prior to their termination and sample collection.

3.8.5 The GIT and other viscera

Measurement of organ masses is very important in toxicological studies as it helps in identifying effects of xenobiotics on specific organs (Baltrop and Brueton, 1990; Bailey et al., 2004). The absolute mass of any organ correlates with the body mass of the animal (Bailey et al., 2004). Relative mass which compare the ratio of the organ mass to the body mass are better for comparisons of organ masses in different treatment groups because they account for the differences in body mass (Bailey et al., 2004). As described earlier, the terminal masses of the male rats differed significantly with those of the corresponding females across all the treatment groups. It is therefore understandable that the absolute masses of the organs differed between male and female rats in the treatment groups. The absolute masses of the small intestines and liver differed between the male and female rats in all the treatment groups. However, the absolute masses of some organs such as the large intestines, stomach and caecum differed in some but not all the groups.

Except in the visceral fat pad where the female rats in the HHS+ TW (p<0.01) and HHS+ FW (p<0.01) had significantly greater visceral fat pad than their corresponding male rats, the relative masses of all the organs weighed were similar. *Hibiscus sabdariffa* aqueous extracts were shown to increase body mass index and delay onset of puberty in female rats when consumed in the post weaning period (Iyare and Adegoke, 2008). Even though the authors also speculated that HS may predispose the female rats to the development of obesity, they did not quantify the visceral fats of the animals. The increased mass of the visceral fat pad in the female rats that received high dose of HS in the pre-weaning period needs to be further investigated to establish the long term consequences. Hence, neither the administration of HS in the neonatal period nor the subsequent fructose feeding might have had a long term effect on the morphology of the GIT and non-GIT viscera.

3.8.6 Markers of renal and hepatic health

The glomerular filtration rate is the gold standard for determining renal function but because it is difficult to measure clinically, it is usually estimated from serum creatinine concentrations (Levey et al., 2009). Serum creatinine and urea concentrations are usually elevated in renal disease (Brigden et al., 1982; Haase-Fielitz et al., 2009). In this study, plasma creatinine and BUN were used as surrogate markers of renal health. Renal diseases are increasingly associated with metabolic syndrome (Wahba and Mak, 2007). Feeding of 60% fructose (w/w) for 6 weeks pre- and 11 weeks post-nephrectomy to male Sprague Dawley rats

accelerated the progression of chronic kidney disease in the remnant kidney (Gersch et al., 2007). There were no differences (p>0.05) observed between the treatment groups in both plasma creatinine and BUN. It is probably safe to say that the administration of high fructose did not result in adverse effects in the kidneys. Because there were no differences observed even in the control group (DW + FW), it is difficult to conclude that HS aqueous extracts were responsible for preventing the adverse effects of fructose administration in the kidneys.

Alanine transaminase (ALT) is present within the cytosol of the hepatocytes and its elevation in the plasma is specifically indicative of damage to the hepatocytes (Thapa and Walia, 2007; Thulin et al., 2008; Rajesh et al., 2009). Alkaline phosphatase (ALP) on the other hand, arises from multiple sources and elevation of its levels could be as a result of liver damage, osteoblastic, placental, intestinal or tumour sources (Pratt and Kaplan, 2000; Thulin et al., 2008). It is therefore not very specific to the liver. A rise in total plasma biluribin is indicative of decreased hepatic clearance or haemolysis (Pratt and Kaplan, 2000; Thapa and Walia, 2007). Albumin, an important plasma protein is produced by the liver and its level in the plasma is a reflection of the synthetic functions of the liver (Alhassan et al., 2009). Plasma albumin, total biluribin and total proteins are a measure of hepato-synthetic functions (Thapa and Walia, 2007). The plasma concentration of ALT, ALP and total biluribin were used as surrogate markers of liver health in this study.

High fructose diet has been shown to cause non-alcoholic liver diseases in rats and this has been associated with an increase in the levels of ALT (de Castro et al., 2013). There was no

significant difference (p>0.05) in the plasma levels of ALT, ALP and total bilirubin across the treatment groups. Gender differences were however found in the level of ALT between the males and corresponding females in the HHS + TW ($500mg.kg^{-1}$ HS + tap water) group (p<0.05). There was also a significant difference (p<0.01) between the male rats in DW + FW ($10ml.kg^{-1}$ distilled water + 20% fructose in drinking water) and HHS + TW groups ($500mg.kg^{-1}$ HS + tap water). These differences, though statistically significant may not be of any biological significance. Gender differences were found in the plasma ALP concentrations in all the treatment groups except DW + FW ($10ml.kg^{-1}$ distilled water + 20% fructose in drinking water) and LHS + TW ($50mg.kg^{-1}$ HS + tap water). Since the male rats in all the treatment groups had a significantly higher body mass, the gender differences observed in plasma ALP levels might be from the increased osteoblastic activity associated with growth. However, further studies are needed to further evaluate the cause(s) of these gender differences following administration of a high fructose diet.

3.8.7 Markers of general health

Plasma concentration of calcium, phosphorus, cholesterol and activity of amylase were used as surrogate markers of general health. Calcium and phosphorus are involved in bone formation and mineralization and their metabolism is regulated by calcitonin and parathyroid hormone (De Paula and Rosen, 2010). Because both calcium and phosphorus are reabsorbed in the GIT and kidneys, an increase in their plasma concentrations correlates with an

increased osteoblastic activity and increased GIT or kidney absorption. Though fructose feeding has been associated with an increased urinary calcium excretion (Taylor and Curhan, 2007) and other risks of kidney diseases, there was no significant finding in that regard in this study. With the exception of a gender difference ($p<0.05$) observed in the LHS + FW (50mg.kg^{-1} HS extract + 20% fructose in drinking water), there was no significant difference observed ($p>0.05$) in the plasma concentrations of both calcium and phosphorus.

Amylases are enzymes which catalyse starch, dextrins and glycogen hydrolysis into glucose (Janowitz and Dreiling, 1959). They are produced mainly in the salivary glands, pancreas and liver with contributions from fallopian tubes, striated muscles and adipose tissues (Janowitz and Dreiling, 1959; Simpson et al., 1991). An increase in the plasma levels of amylase is an important guide in the diagnosis of acute pancreatitis or of exercabation of chronic pancreatitis (Janowitz and Dreiling, 1959). There was no significant difference ($p>0.05$) in the plasma amylase concentration across the treatment groups suggesting that the administration of fructose did not have negative effects in the salivary glands and exocrine pancreata of the rats. Feeding of 7 week old rats with 60% fructose (w/w) for 8 weeks did not produce any adverse effects on serum amylase (Huang et al., 2004). However, significant differences were found between the males and the corresponding females in the following groups: DW + TW (10ml.kg^{-1} distilled water + tap water), LHS + TW (50mg.kg^{-1} HS + tap water), LHS + FW (50mg.kg^{-1} HS + 20% fructose in drinking water) and HHS + FW (500mg.kg^{-1} HS + 20% fructose in drinking water). There is a higher content of α-amylase in the fallopian tubes

than in the serum of humans, cows, rabbits and sheep but not in rats, dog, pigs, monkeys and cats (Janowitz and Dreiling, 1959). Still, I would have expected the plasma amylase activity in the females to be higher than those in the males due to the extra secretion from the fallopian tubes.

Though elevated levels of serum total cholesterol have been reported in 8 week old rats following oral fructose administration for 8 weeks (Yadav et al., 2007), no significant differences were found across the treatment groups in this study . However, the female rats in the HHS + FW (500mg.kg^{-1} HS + 20% fructose in drinking water) had a significantly higher (p<0.05) plasma cholesterol concentration than their male counterparts. Previous studies in humans (Seidell et al., 1991) and mice (Bruell et al., 1962) have reported higher serum cholesterol values for males compared to females.

3.8.8 Conclusion

In this phase of the study, male and female Sprague Dawley rats that had been administered with HS extracts in the neonatal period were subjected to a high fructose in their drinking water (20% fructose w/v). The aim was to investigate whether the initial administration of HS extracts would have any effect in the development of metabolic dysfunction in the rats. The rats did not develop features of metabolic dysfunction (hyperglycaemia, hypertriglycerideamia, insulin resistance, impaired glucose tolerance, hypercholesterolemia) after four weeks of fructose administration. The female rats that were administered with a

high dose HSE had a significantly greater amount of visceral fat pad compared to the male

rats in the group. The gender differences that were observed in some of the measured

parameters though statistically significant might not be of any biological significance.

CHAPTER FOUR: CONCLUSIONS AND RECOMMENDATIONS

4.0 Conclusion

The study was done in two phases. In the first phase, the effects of HS aqueous calyx extracts on the growth performance, gastrointestinal tract morphometry and metabolic substrates of suckling rats was investigated. This phase of the study is very important because there is a high chance of exposure of the neonates to this widely consumed plant. Moreover, no studies were found in which the HS extracts were administered to suckling rats directly. Findings from this phase suggest that HS when consumed in the suckling period could cause precocious maturation of the small intestines. *Hibiscus sabdariffa* aqueous calyx extracts however had no adverse effects on the growth performance, metabolic substrates and general health of the pups.

In the second experiment, metabolic dysfunction was not induced despite administration of a 20% fructose (w/v) in the drinking water of the rats for four weeks. The rats did not show the hypertriglyceridaemia and insulin resistance associated with fructose administration. As expected, in this phase, the male rats gained more weight than the female rats. The female rats that had received the high dose HSE in the neonatal period had greater amount of visceral fat compared to the corresponding males in their groups. There was no difference in the response of the rats to the fructose diet between the groups that had received HS in the suckling period and those that did not. There were no adverse effects observed either due to the administration of HS or the fructose diet across all the treatment groups. The rats in the fructose groups drank fructose water as their only source of water though actual intake was

not recorded. It might be safe to conclude that the administration of the HS extracts in the suckling period did not have any influence on how the growing rats responded to the 20% fructose diet. The short term administration of 20% fructose (w/v) in the drinking water of the growing rats did not also induce metabolic dysfunction as suggested by literature.

4.1 Limitations of the study

The small amount of plasma obtained in phase one did not allow for the determination of hormones associated with growth such as IGF-1 and insulin. Histological samples of the small intestines were prepared but unfortunately were not well oriented on the slides. Histological findings would have revealed if there were changes in the villi height, crypt depth or the villi height/crypt depth ratio which would explain the increase in the masses of the small intestines relative to the body mass in the HS groups. The pups were also not fasted prior to termination in phase one because it would have required separating them from their dams and since the pups do not all suckle at the same time it would have been very difficult to standardise the duration of fasting between the different pups. Functional tests such as perfused gut loops, immunohistochemistry and cell cultures were not performed on the GIT to assess whether functional maturation also occurred in the small intestines. A higher concentration of fructose and a more prolonged period of its administration might have induced metabolic dysfunction as anticipated.

4.2 Recommendations

For future studies, neonatal programming should be induced by manipulating the maternal environment through diet restriction or some other stressor events before interventions are instituted in the pups. A more prolonged period of fructose feeding preferably up to adulthood should be used to determine the effects of the programming on adult metabolic health. The fructose should also be administered in the diet rather than drinking water of the rats as this has been shown to be more potent in inducing metabolic dysfunction.

CHAPTER FIVE: REFERENCES

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APPENDICES

APPENDIX 1: ETHICS CLEARANCE CERTIFICATE

STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO.   2013/46/05

APPLICANT: Mr GK Ibrahim

SCHOOL: Medical School
DEPARTMENT: Physiology
LOCATION: Faculty of Health Sciences

PROJECT TITLE: *The effects of administration of Aqueous Calyx Extracts of hibiscus sabdariffa on neonatal Programming of diet induced metabolic dysfunction in Sprague Dawley rats*
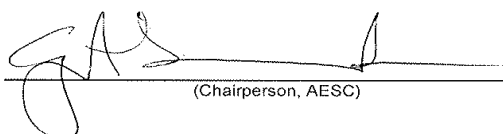
<u>**Number and Species**</u>

99 Rats

Approval was given for to the use of animals for the project described above at an AESC meeting held on 17 September 2013. This approval remains valid until 16 September 2015.

The use of these animals is subject to AESC guidelines for the use and care of animals, is limited to the procedures described in the application form and is subject to any additional conditions listed below:

   (i) For purposes of monitoring health of rats, and reduction in body mass must be calculated in comparison to the equivalent body mass of a growing pup.
   (ii) Trails should start with tests at the low dosage.

Signed: _____   Date: 30 / 9 / 2013
             (Chairperson, AESC)

I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23 (1) (c) of the Veterinary and Para-Veterinary Professions Act (19 of 1982)

Signed: _____   Date: _____
             (Registered Veterinarian)

cc: Supervisor: Prof K Erlwanger
    Director: CAS

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APPENDIX 2: FIRST MODIFICATION TO THE ETHICS CLEARANCE

Please note that <u>only typewritten applications</u> will be accepted.

UNIVERSITY OF THE WITWATERSRAND
ANIMAL ETHICS SCREENING COMMITTEE
MODIFICATIONS AND EXTENSIONS TO EXPERIMENTS

a.   Name: Ibrahim, Kasimu Ghandi

b.   Department: Physiology

c.   Experiment to be modified / extended   **AESC NO**

Original AESC number	2013	46	05
Other M&Es :			

d.   Project Title: Effects of administration of *Hibiscus sabdariffa* aqueous calyx extracts on the gastrointestinal tract and neonatal programming of metabolic dysfunction.

		No.	**Species**
e.	Number and species of animals originally approved:	9 dams and 90 pups	*SD rats*
f.	Number of additional animals previously allocated on M&Es:	0	0
g.	Total number of animals allocated to the experiment to date:	5 dams and 55 Pups	SD rats
h.	Number of animals used to date:	5 dams and 55 pups	SD rats

Specific modification / extension requested:
I. An additional four dams and thirty eight pups.
II. To use 2ml of the blood that will be collected terminally from rats in phase 2 of the study for osmotic fragility tests.

Motivation for modification / extension: Following a review of my protocol by the faculty postgraduate committee it was recommended that for my study I should investigate the effects of gender as previous studies have shown differences in the susceptibility to developing metabolic syndrome.

 Thus far, I have received a total of five dams and fifty five pups of which I allocated forty four pups to the first phase of the study (three treatment groups with a minimum of 7 males and 7 females per group).
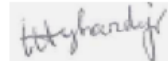
 In the second phase of the study, in order to investigate the effects of gender a total of eighty four rat pups are required. As described in the original protocol, they will be divided into six treatment groups (7 males and 7 females per group). Thus the total number of rat pups required will be 128. To achieve this I require a further 38 pups (and four dams).

I would like to investigate the effects of administering the plant extract on osmotic fragility of the rats' red blood cells to further determine whether it has an effect on the general health of the pups. Standard protocols using serially diluted concentrations of phosphate buffered saline will be used for the osmotic fragility test (Moyo et al., 2012).

All other procedures will remain as originally approved by the AESC.

Date: 20th February, 2014   Signature:

RECOMMENDATIONS. Approved:
i. Additional 38 pups and 4 dams.
ii. Use of 2ml of blood collected terminally for osmotic fragility tests.

Date: 21 February 2014   Signature:

Chairman, AESC

Reference

Moyo, D., Erlwanger, K. H. & Chivandi, E. 2012. Effect of Graded Dietary Substitution of Soyabean Meal with Large Sour Plum (*Ximenia caffra*) Seed Meal on Erythrocyte Osmotic Fragility and the Packed Cell Volume of Growing Male Sprague Dawley Rats. *International Journal of Agriculture & Biology,* 14.

APPENDIX 3: SECOND MODIFICATION TO THE ETHICS CLEARANCE

Please note that <u>only typewritten applications</u> will be accepted.

UNIVERSITY OF THE WITWATERSRAND
ANIMAL ETHICS SCREENING COMMITTEE
MODIFICATIONS AND EXTENSIONS TO EXPERIMENTS

a.   Name: Ibrahim, Kasimu Ghandi

b.   Department: Physiology

c.   Experiment to be modified / extended   **AESC NO**

Original AESC number	2013	46	05
Other M&Es :			1

d.   Project Title: Effects of administration of *Hibiscus sabdariffa* aqueous calyx extracts on the gastrointestinal tract and neonatal programming of metabolic dysfunction.

		No.	**Species**
e.	Number and species of animals originally approved:	9 dams and 90 pups	*SD rats*
f.	Number of additional animals previously allocated on M&Es:	4 dams and 38 pups	SD rats
g.	Total number of animals allocated to the experiment to date:	13 dams and 128 Pups	SD rats
h.	Number of animals used to date:	12 dams and 128 pups	SD rats

Specific modification / extension requested:
1. 9 Additional pups
2. A Co-worker : Busisani Lembede

Motivation for modification / extension: The faculty postgraduate committee had previously reviewed my protocol and recommended that I investigate the effects of gender as previous studies have shown differences in susceptibility to the development of the metabolic syndrome. I had also subsequently asked for and got a modification to my ethics approval to accommodate the increase in the number of animals required to balance the gender.

Thus far, I have received a total of 12 dams and 128 pups. I allocated 44 pups to the first phase of the study (three treatment groups with a minimum of 7 males and 7 females).

Out of the 84 remaining pups allocated for the second phase, one pup did not thrive as well as his peers and so was removed from the study. Due to technical problems we were unable to utilise the plasma samples collected from three pups. Thus The eighty pups remaining (33males and 47 females) were allocated into six treatment groups. Each group should have a total of 14 pups (7 males and 7 females). To achieve this, a total of 42 males and 42 females are required. Thus, an
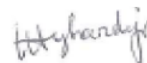
additional 9 male pups are needed to complete the groups. I had achieved the original numbers with 12 dams however my protocol was approved for 13 dams. I will thus just require extra pups; the dam would be as originally approved.

I would also want to list Busisani Lembede as a co-worker to assist with the running of the osmotic fragility tests.

All other procedures will remain as originally approved by the AESC.

Date: 30th April, 2014   Signature:

RECOMMENDATIONS: Approved.
i. Nine additional rat pups.
ii. Inclusion of Mr B Lembede as a co-worker.

Date: 2 May 2014   Signature:

Chairman, AESC

APPENDIX 4: PLANT IDENTIFICATION

**Department
of PHARMACOGNOSY & ETHNOPHARMACY**

Faculty of Pharmaceutical Sciences
USMANU DANFODIYO UNIVERSITY, SOKOTO

VICE CHANCELLOR: Prof. R. A. Shehu, OON, B.Sc (Unisok), Ph.D (Essex)
HEAD OF DEPARTMENT: H. E. Mshelia B.Sc, PGDE, M.Sc.

Email: pharmacognosyudus@yahoo.com
Phone: +2348069221840

Date: 31/10/2013

PLANT IDENTIFICATION

The plant *Hibiscus sabdariffa* (common name: Roselle) has been identified in our Department via Taxonomic means by: HALILU, Emmanuel Mshelia. The plant belongs to the family: Malvaceae and has been given Voucher number: PCG/UDUS/Malv/0001. The voucher specimen has been deposited in the Departmental Herbarium for reference purpose.

Halilu E. Mshelia

HEAD OF DEPT.
DEPT. OF PHAMACOGNOSY &
ETHNOMEDICINE
ACULTY OF PHAMACEUTICAL CIENCES
U. D. U. SOKOTO

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