IN VITRO AND *IN VIVO* CHEMICAL CHARACTERIZATION OF *KIGELIA AFRICANA, MIMUSOPS ZEYHERI, TERMINALIA SERICEA* AND *XIMENIA CAFFRA* NUTS AND NUT MEALS

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A thesis submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Doctor of Philosophy.

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DECLARATION BY CANDIDATE

I, **Eliton Chivandi**, hereby declare that this thesis is my own work and effort and that it has not been submitted to any other institution for an award. Where other sources of information have been used, they have been appropriately cited and acknowledged.

Signature:……

Date:……

ABSTRACT

Soyabean meal (SBM), the major protein source in feeds in sub-Saharan Africa, is in short supply. The shortage is a major constraint to intensified animal production to meet increased demand hence the dire need to search for alternatives. *Kigelia africana*, *Mumisops zeyheri*, *Terminalia sericea* and *Ximenia caffra* are indigenous fruit bearing trees (IFBTs) whose seeds' potential as alternative protein sources in feeds were evaluated. The evaluation consisted of an initial physico-chemical characterization of the seeds followed by determining *in vitro* the safety of seed oils on cell lines. Based on the physico-chemical and *in vitro* evaluation, the most suitable seed was selected, defatted and its meal used as a dietary substitute to SBM in the *in vivo* trials using adult and weanling male Sprague Dawley rats.

The *T. sericea* seed yield was not viable. Chemically *K. africana* and *X. caffra* seed demonstrated potential as protein sources in feeds. *M. zeyheri* seed demonstrated potential as an energy source. The IFBTs seeds oil yield surpassed that of some traditional oilseed crops. Oleic and linoleic acid were the major fatty acids contained in the oils. *In vitro*, *K. africana*, *M. zeyheri* and *X. caffra* seed oils suppressed Caco-2 and HEK-293 cell proliferation without causing cell death.

X. caffra seed, deemed the most suitable, was defatted and its seed meal used in the *in vivo* trials. In mature rats, dietary substitution of SBM with the defatted *X.*

caffra seed meal did not affect ($P > 0.05$) dry matter intake, apparent digestibility of nutrients and nitrogen absorption and retention. In weanling rats, the defatted *X. caffra* seed meal had no effect on termination (body mass at the end of the feeding trial) and empty carcass mass and linear growth of the rats. Metabolic substrate storage, fasting blood glucose concentration and the general health profile of the growing rats were not altered by dietary *X. caffra* seed meal. The defatted *X. caffra* seed meal increased the mass of the stomach and small intestine ($P = 0.0071$; $P = 0.0001$) of rats on the test diet where a 100% dietary crude protein (CP) from SBM was substituted by CP from the defatted *X. caffra* seed meal.

Defatted *X. caffra* seed meal could substitute SBM in rat and possibly monogastrics feeds without compromising digestibility, nitrogen balance, growth and general health.

DEDICATION

To the memory of my late father Mr. Svinurayi Chivandikwa

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Table of Contents

LIST OF ABBREVIATIONS

ADF:	Acid detergent fibre
ADG:	Average daily gain
ALP:	Alkaline phosphatase
ALT:	Alanine transaminase
ANFs:	Anti-nutritional factors
ANOVA:	Analysis of variance
BM:	Blood meal
CF:	Crude fibre
CLA:	Conjugated linoleic aid
CLM:	Cassava leaf meal
CP:	Crude protein
Cu:	Copper
CSC:	Cotton seed cake
DES:	Dietary energy supply
DM:	Dry matter
DMB:	Dry matter basis

EAAs:	Essential amino acids
ECL:	Ensiled cassava leaves
EE:	Ether extract
EFAs:	Essential fatty acids
EPA:	Eicosapentaenoic acid
FAO:	Food and Agricultural Organisation
$FeS0_4$:	Ferrous sulphate
FM:	Fish meal
GE:	Gross energy
GIT:	Gastrointestinal tract
IFBTs:	Indigenous fruit bearing trees
KI:	Potassium iodide
KTI:	Kunitz trypsin inhibitor
METABO:	Meat and bone meal
$MnS0_4$:	Manganese sulphate
n3-PUFA:	Omega-3 polyunsaturated fatty acid
N:	Nitrogen
NDF:	Neutral detergent fibre

NRC:	National Research Council
OA:	Oleic acid
OM:	Organic matter
PATs:	Plant associated toxins
SBA:	Soyabean agglutinin
SBM:	Soyabean meal
Se:	Selenium
SSA:	Sub-Saharan Africa
SSC:	Sunflower seed cake
TMUFA:	Total monounsaturated fatty acids
TPUFA:	Total polyunsaturated fatty acids
TSFA:	Total saturated fatty acids
XCSM:	defatted *Ximenia caffra* seed meal
Zn:	Zinc

LIST OF FIGURES

LIST OF TABLES

RESEARCH OUTPUTS

Journal publications

i. **Chivandi, E**., Davidson, B.C. and Erlwanger, K.H**.** 2012. The Red Sour Plum (*Ximenia caffra*) Seed: A Potential Non-conventional Energy and Protein Source for Livestock Feed. *International Journal of Agriculture and Biology*, 14: 540-544.

ii. **Chivandi, E**., Cave, E., Davidson, B.C., Erlwanger, K. H., Moyo, D. and Madziva, M.T. 2012. Suppression of Caco-2 and HEK-293 cell proliferation by *Kigelia africana*, *Mimusops zeyheri* and *Ximenia caffra* seed oils. *in vivo*, 26: 99-105.

iii. **Chivandi, E**. Davidson, B.C. and Erlwanger, K.H. 2011. *Kigelia africana* seed: proximate, mineral, vitamin E, fibre, amino acid and fatty acid composition. *International Journal of Food Science and Technology*, 46: 2153-2158.

iv. **Chivandi, E**. Pretorius, B., Davidson, B.C. and Erlwanger K. H. 2011. Proximate, Mineral, Amino Acid, Fatty Acid, Vitamin E, Phytate Phosphate and

Fibre Composition of *Mimusops zeyheri* (Red Milkwood) Seed. International *Journal of Food Science and Technology*, 46: 555-560.

Published abstracts

Chivandi, **E**., Cave, E., Davidson, B.C., Erlwanger, K.H., Moyo, D. and Madziva, M.T. 2011. Effect of *Kigelia africana*, *Mimusops zeyheri* and *Ximenia caffra* seed oils on Caco-2 and HEK-293 cells. *Scientific Research and Essays*, pp: 68.

Chivandi, E., Davidson, B.C. and Erlwanger, K.H. 2012. Effect of dietary substitution of soyabean meal with *Ximenia caffra* (Red Sour Plum) seed meal on apparent nutrient digestibility and nitrogen balance in Sprague Dawley rats. *Proceedings of the South African Society of Animal Science*, pp: 21.

Conference presentations

i. **Chivandi, E**., Davidson, B.C. and Erlwanger, K.H. 2012. Effect of dietary substitution of soyabean meal with *Ximenia caffra* (Red Sour Plum) seed meal on apparent nutrient digestibility and nitrogen balance in Sprague Dawley rats. **Oral/Theatre** presentation at the **45th Congress of the South African Society of Animal Science,** East London, South Africa, 9th-13th July 2012.

ii**. Chivandi, E**., Cave, E. Davidson, B.C. Erlwanger, K.H., Moyo, D. and Madziva M.T. 2011**.** Suppression of Caco-2 and HEK-293 Cell Proliferation by *Kigelia africana*, *Mimusops zeyheri* and *Ximenia caffra* Seed Oils. Poster presented at the **39th Congress of the Physiology Society of Southern Africa**, Cape Town, 28th-31st August, 2011.

iii. **Chivandi, E.** Davidson, B.C. and Erlwanger, K.H. 2010. Chemical Analysis of the Nutritive Value of the Transvaal Red Milkwood (*Mimusops zeyheri*) and Large Sourplum (*Ximenia caffra*) Seed as Potential Non-conventional Feed Resources. Poster presented at the **3rd Cross Faculty Postgraduate Symposium, University of the Witwatersrand**, 26-29th October 2010.

iv. **Chivandi, E.** Davidson, B.C. and Erlwanger, K.H. 2010**.** Nutritive Value of *Kigelia africana*, *Mimusops zeyheri* and *Ximenia caffra* Seed Determined by Chemical Analyses. Poster presented at the **38th Congress of the Physiology Society of Southern Africa**, East London, 27-29th September, 2010.

v. **Chivandi, E.,** Erlwanger, K.H. and Davidson, B.C. 2009**.** The Seeds of Indigenous Trees *Kigelia africana*, *Mimusops zeyheri*, *Terminalia sericea* and *Ximenia caffra*: Potential Sources of Lipids, Essential Fatty Acids, Vitamin E and Antioxidants. Poster presented at the **37th Congress of the Physiology Society of Southern Africa**, Stellenbosch, 7-9th September, 2009.

Papers submitted for publication

i. **Chivandi, E**. Davidson, B.C. and Erlwanger, K.H. 2012 *Terminalia sericea* seed: proximate, mineral, vitamin E, fibre, amino acid and fatty acid composition. *South African Journal of Botany*.

AUTHOR'S CONTRIBUTIONS

CHAPTER 1: INTRODUCTION AND JUSTIFICATION

I conceived the project idea, developed the idea and wrote a project proposal (with guidance from my supervisors) justifying the study.

CHAPTER 2: PHYSICAL AND CHEMICAL CHARACTERIZATION OF *KIGELIA AFRICANA*, MIMUSOPS *ZEYHERI*, *TERMINALIA SERICEA* AND *XIMENIA CAFFRA* SEEDS

I sourced the seeds, determined their physical traits, seed oil yield, and profiled the fatty acids for *M. zeyheri* (sourced from Matopos National Park, Zimbabwe) and *X. caffra* seed oils. I assayed the vitamin E and squalene content of all the tree seed oils. Other chemical assays were done at the Agricultural Research Council's Irene Research Institute Laboratories. I analysed data and wrote and published 3 journal papers.

CHAPTER 3: EFFECTS OF *KIGELIA AFRICANA*, *MIMUSOPS ZEYHERI* AND *XIMENIA CAFFRA* SEED OILS ON CELL VIABILITY

I designed the experiment and executed the experiment with the help of Ms Eleanor Cave and Dr. Michael T. Madziva. I collected the data, analysed data with the help of Mr. Davison Moyo; wrote and published 1 journal paper and 1 published abstract.

CHAPTER 4: DIGESTIBILITY AND NITROGEN BALANCE TRIAL

I designed the experiment, executed the experiment and the collected data with the assistance of Ms. Rachael Dangarembizi. Proximate analyses of the feed, faeces and urine samples were done at the Agricultural Research Council's Irene Research Institute Laboratories. I analysed the data and wrote the chapter.

CHAPTER 5: GROWTH PERFORMANCE, GASTROINTESTINAL MORPHOMETRY AND GENERAL HEALTH PROFILE TRIAL

I designed the experiment, executed the experiment and the collected data with the assistance of Ms Rachael Dangarembizi who assisted with assays for the general health profiles. I analysed the data and wrote the chapter.

CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

I summed up the major findings of the study based on the major objectives of the study and also highlighted areas of possible further research.

CHAPTER 7: REFERENCES

I compiled the list of references cited in the thesis.

CHAPTER 1: INTRODUCTION AND JUSTIFICATION

1.0 Introduction

Availability and access to nutritionally balanced food is one of the fundamental tenets of the United Nations charter that is enshrined in the constitutions of member states signatory to the charter [Food and Agriculture Organisation (FAO), 1996]. In sub-Saharan Africa (SSA) the staple human foods include cereals (largely maize and small grain cereals), roots and tubers (potatoes and cassava) which are sources of energy and farmed legumes and animal products (sources of protein) (Amuna et al., 2000; FAO, 2011). Amjad et al. (2006) contend that in excess of 170 million pre-school children and nursing mothers in the developing Afro-Asian countries suffer from protein-energy malnutrition. Although malnutrition is a complex condition that can involve multiple, overlapping deficiencies of protein, energy and micronutrients; protein malnutrition predisposes the population at risk to disease and is reported as the underlying cause of one out of every two deaths of children under the age of five in the developing world (Murray and Lopez, 1997; Bryce et al., 2005). The prevalence of protein malnutrition is high in the SSA in recently weaned children who are subjected to inadequate cereal-based diets that are protein and micronutrient deficient (Van de Poel et al., 2008). Proteins are sources of amino acids and they (proteins) constitute 16.36% of the average human body mass (Brožek et al., 1963). They (proteins) are necessary for the synthesis of and maintenance of the enzymes, hormones, neurotransmitters, the blood, skin, muscle and bone. Animal-derived products (protein) supply essential amino acids (EAAs) and essential fatty acids (EFAs). A deficiency of EAAs in children leads to stunting, subnormal

mental development, kwashiorkor and a predisposition to disease. Essential fatty acids (EFAs) constitute some of the omega-3 polyunsaturated fatty acids (n-3 PUFAs) that are a pre-requisite for normal foetal brain and visual development (Neuringer et al., 1998). Omeg-3 polyunsaturated fatty acids (n-3 PUFAs) play an important role in the reduction of plasma triglycerides, platelet aggregation, blood viscosity and blood pressure, and are known to have an inhibitory effect on the growth of prostate and breast cancer (Simopolous, 2000; Tample, 1996; Rose, 1997). Animal product-derived essential nutrients (EAAs and EFAs) are thus critical for growth and development, and are vital to normal early childhood growth and maintenance of health in both the young and elderly. In the developing world, including the SSA, EAAs and EFAs could be easily supplied from animal products if production met demand. However, it has been noted that animal products (eggs, meat, milk, fats and oils) are in short supply in human diets in the least developed countries, including in the SSA region (Amuna et al., 2000). Ideally, human diets, particularly in the developing world where most communities cannot afford micronutrient-dense supplements to fortify foods, need to have animal protein as a dietary component to meet the dietary protein and essential micro-nutrient requirements of the nutritionally vulnerable groups particularly pregnant women, nursing mothers, recently weaned children, adolescents, the elderly and the sick.

1.1 Protein sources for humans in sub-Saharan Africa

Forty-six percent of the dietary energy supply (DES) per capita per day in SSA comes from a combination of cereals, other grains and legumes with 15.6% coming from roots and tubers (Amuna et al., 2000). In the former Western Europe (part of the developed world), 33% of the DES in an average diet comes from animal products versus 7% of the DES from animal products in Africa (FAO, 2011). Cereals, roots and tubers (maize, small grain cereals, sweet potatoes and cassava) thus form the major proportion of the diets of communities in SSA supplemented with inadequate quantities of farmed legumes and protein from animal products. The dominance of inadequate low-protein carbohydrates in the diets of communities in SSA exposes the population, particularly the vulnerable groups to protein malnutrition. Compared to plant-derived protein, animal-derived protein is of high quality in terms of the balance between amino acid composition and proportion that leads to a higher protein efficiency ratio (PER) and digestibility (Schaafsma, 2000; Table 1.1). Protein efficiency ratio (PER) is determined by dividing the gain in body mass (g) by the quantity (g) of the protein consumed (McDonald et al., 2002), thus it is a measure of the efficiency of the utilization of absorbed protein.

Table 1.1: Protein efficiency ratio and digestibility of some selected protein sources

Protein	Protein Efficiency Ratio	Digestibility (%)
Egg	3.8	98
Cow's milk	3.1	95
Beef	2.9	98
Soy	2.1	95
Wheat	1.5	91

Source: Schaafsma, 2000

Natural pasture land in the SSA region has given way to cropping and urbanisation. The decrease in natural pasture land has led to a concomitant reduction in off-veld ruminant animal protein production (Thornton, 2010). The decrease in off-veld ruminant animal production has resulted in a failure to meet the increased demand of animal products. In SSA the increased demand for animal products is driven by urbanisation, increased incomes (Tadelle et al., 2003) a preference for a more nutritious diet by the increasing urban middle class (Delgado et al., 1999), and the heavy dependency burden as a result of the HIV/AIDS pandemic. The decreased ruminant animal protein production off-veld (Thornton, 2010) coupled with increased animal product (protein) demand in the SSA region (Tadelle et al., 2003), requires an intensification of animal protein production in order to meet the increased demand. The shortage of quality animal-derived protein in SSA has compromised the nutritional status of the vulnerable (recently weaned children and the aged) thus exposing them to increased risk of contracting diseases (Van de Poel et al., 2008).

1.2 Protein: a major feed ingredient in animal production

An intensification of animal product production hinges on the availability of quality feed resources to fuel the process. Under intensified animal production, feed costs account for about 70-80% of the cost of animal production (Brand 2000; Chipa et al., 2010 citing Henning; 1999Beken and Şahin, 2011). Feed costs account for 80-90% in intensive broiler production, 70-80% in pig production and

75-85% in intensive dairy farming, respectively, of the total variable costs (Brookes, 2001). Protein and energy are vital nutrients for livestock. Energy is important for normal functioning of the animal body (Jafarnejad and Sadegh, 2011). Protein, a constituent of all bodily tissues, influences animal productive and reproductive performance and is the most expensive nutrient in broiler and any intensified animal production enterprise (Kamran et al., 2004).

1.2.1 Protein sources in animal feeds in sub-Saharan Africa

Plant-derived and animal-derived products constitute the major sources of protein in livestock feeds in the SSA region. Undecorticated oil-seed cakes, largely sunflower (*Helianthus annuus*) and cotton (*Gossypium hirstum*) seed cakes constitute the major conventional plant-derived protein sources in intensive ruminant animal production (McDonald et al., 2002). Soyabean meal (SBM), a by-product of oil extraction from soyabean (*Glycine max*), decorticated sunflower and cotton seed cakes and fishmeal (FM) are largely the conventional plant-derived and animal-derived protein sources, respectively, used in intensive monogastric animal (broiler, pullet, piggery and aquaculture) production in the SSA region.

1.2.2 Nutritional characteristics of conventional protein sources

Sunflower and cotton seed cakes (SSC and CSC, respectively) are generated from the pressing of oil from the seeds (Jabbar et al., 2009; McDonald et al., 2002). Oil expulsion from the undecorticated sunflower and cotton seeds generally produces protein cakes that have a high content of different fibre fractions (McDonald et al., 2002). The high fibre content of such oil seed cakes (and meals) lowers digestibility and generally are not recommended as protein sources in simple-stomached (monogastric) animal feeds (McDonald et al., 2002). The fibre in protein cakes and meals produced from undecorticated oil seeds complexes with protein making it unavailable to monogastric animals. Such "high-fibre" oilseed protein cakes and meals are, in general, used in intensive ruminant animal production, such as dairy and pen-fattening of ruminants, that are physiologically and anatomically more adapted to digest fibre (cellulose and hemicelluloses) and thus can access some of the protein complexed with the fibre components.

The decortication of sunflower and cotton seed followed by oil extraction produces protein concentrates of low fibre content which can be utilised in diet formulation for monogastric animals. The degree of decortication determines both the crude protein (CP) and fibre content of the resultant seed cake (Jabbar et al., 2009), for example, the CP content of decorticated sunflower seed cake ranges from 24 to 40% (Jabbar et al., 2009). Compared to the 45% CP in decorticated

CSC (McDonald et al., 2002), undecorticated CSC has a lower (23.1%) CP content (Sharma et al., 1978), thus processing has an effect on protein quality particularly when the cake is intended to be used in monogastric animal feed formulation.

Although decorticated CSC and SSC are generally of good quality, their major disadvantage is the low concentration of lysine and methionine (McDonald et al. 2002). Lysine and methionine are usually the first and second limiting amino acids, respectively, in monogastric feeds (McDonald et al., 2002). In mammals, lysine and methionine are precursors of carnitine (Hoppel, 2003). Carnitine has an obligate role in the mitochondrial oxidation of long-chain fatty acids through the action of specialized acyltransferases (Hoppel, 2003). Additionally, methionine is an essential component for protein synthesis, a precursor for cysteine and a methyl donor (Graber and Baker, 1971), thus use of decorticated CSC and SSC in monogastric animal feeds, despite their low fibre content, requires methionine and lysine supplementation. Research has demonstrated the protein sparing effect of using synthetic lysine and methionine to balance low crude protein diets of monogastrics (Tuitoek et al., 1997).

Soyabean meal (SBM) is the major plant-derived protein source used in animal feeds for intensive animal production in the SSA region and the world over (Storebakken et al., 2000). It is one of the most nutritious of plant protein sources

in feeds (Lovell, 1988) with a high protein content and relatively well balanced amino acid profile and as such is widely used in feed formulation (Storebakken et al., 2000). Soyabean meal has a much higher concentration of the essential amino acids chiefly lysine and methionine than other plant-derived protein concentrates (McDonald et al., 2002). The high concentration of lysine and methionine in SBM is crucial in the formulation of least cost diets since less of the costly synthetic lysine and methionine is required to balance the diets.

Although blood meal (BM) and meat and bone meal (METABO) are animal-derived protein sources in feeds, fish meal (FM) is the major animal-derived protein source in livestock feeds. On average BM dry matter contains 90-95% CP (Buraczewski, 1990) and is characterized by protein rich in lysine and leucine. Despite BM's high concentration of lysine and leucine, routine processing of blood from slaughter houses into quality BM in SSA countries is bedevilled with technological challenges. Furthermore, BM inclusion in diets is limited by its unpalatability which negatively affects feed intake, thus is not routinely used. Additionally, the outbreak of *Bovine spongiform* encephalopathy (mad cow disease) has also limited (in some parts of the world) and / or resulted in a total ban (in Europe) of use of BM and METABO as protein sources in animal feeds (Brookes, 2001).

Fish meal is regarded as the protein ingredient with the highest protein (60-64%) concentration but the most expensive (Brookes, 2001). It (FM) is produced by pressing cooked fish in order to remove most of the water and oil; the residue is then dried (McDonald et al., 2002). Well produced FM is of high quality; contains the ideal composition and proportion of amino acids (including EAAs), with digestibility ranging from 93-95% and is a good source of calcium and phosphorus (McDonald et al., 2002). FM is generally used in the formulation of diets for younger animals, chiefly chicks (broilers and or pullets) and piglets.

1.2.3 Antinutritional factors in plant protein sources and effects in animals

1.2.3.1 Types of antinutritional factors

The use of legume and oil-seed derived cakes and meals as well leaf protein meals and concentrates as protein sources for livestock feeds is limited by the presence of a wide array of antinutritional factors (Francis et al., 2001). Antinutritional factors (ANFs) can be endogenous natural compounds found within the plant material usually produced for purposes of defence against herbivory and or insect pest attack or they can be secondary metabolites produced during processing of feeds and foods. Endogenous ANFs include, among others, protease inhibitors and phytohaemaglutinins found in most leguminous seeds, chelating agents (phytates and oxalates), gossypol, phorbol esters, saponins, tannins, oligosaccharides, lignin, cyanogenic glycosides and toxic amino acids (Francis et

al. 2001). Process-generated ANFs include products of the browning and Maillard reactions. Examples of toxic ANFs produced by browning and Maillard reactions include acrylamide and hydroxymethyl furfural (Capuano et al., 2009).

Soyabean, one of the three major protein sources in livestock feeds in the SSA contains protease inhibitors, Kunitz trypsin inhibitor (KTI) and Bowman-Birk inhibitor and phytohaemaglutinins (Mikić et al., 2009). It also contains saponins, tannins and the oligosaccharides, stachyose and raffinose (Francis et al., 2001). Some varieties of cotton seed contains the toxic gossypol while sunflower seed if used undecorticated contains tannins in the seed hull.

1.2.3.2 Effect of processing on antinutritional factors

The use of grain legumes (peanut, pea, beans and soyabeans) and oilseeds (rapeseed, sunflower and cotton) as protein and energy sources in feeds and foods is generally limited by the presence of thermo-labile and thermo-stable ANFs (Hajos and Osagie, 2004). The ANFs need to be eliminated, inactivated and or reduced to concentrations that do not elicit negative reactions before use of the grains and oilseeds for animal and human consumption (Muzquiz, 2004). Protease inhibitors and phytohaemoglutinins (lectins) are thermo-labile, thus can be largely inactivated via controlled heat treatment during processing of soybean seed into soyabean meal and or cake. Moist heat treatment is reported as a more effective

method of inactivating thermo-labile ANFs when compared to dry heating (Carlini and Udedibe, 1997). While heat treatment reduces the effects of protease inhibitors and phytohaemaglutinins in soyabean and other grain legumes, it does not completely eliminate them (Carvalho et al., 1998). Heat treatment causes a decline in protein quality by making lysine unavailable and reducing protein solubility and digestibility, hence the need for great care when using thermal treatment to inactivate thermo-labile ANFs. Thermo-stable ANFs, for example, saponins, gossypol and tannins present challenges in utilisation of plant-derived animal feed protein sources. However, dehulling and decortication of seeds has been shown to reduce polyphenols such as tannins while soaking, boiling and washing was reported to reduce phytates, stachyose, raffinose and saponins (Muzquiz, 2004).

1.2.3.3 Effects of antinutritional factors in animals

Antinutritional factors reduce the nutritional value of feed ingredients either by complexing with nutrients, forming insoluble complexes (precipitates) in the gastrointestinal tract (GIT) thus making them indigestible or unabsorbable (Waghorn, 2008). They may also inactivate digestive enzymes hence reducing their efficacy in catalysing hydrolytic reactions that are vital to digestion (Reyden and Selvendran, 1993; Ravindran et al., 1995; Waghorn, 2008). The negative effects of ingestion of ANFs in grain legumes (including soyabean) and oilseeds

on the physiology, productive and reproductive performance of livestock has been subject of many papers (Abou-Donia and Dieckert, 1974; Morgan, 1989; Pusztai et al. 1990; Lesake et al., 1995; Kensil, 1996; de Oliveira et al., 2001; Haridas et al., 2001; Knight and Walter, 2003; Badawy et al., 2007; Mikić et al., 2009).

1.3 Constraints to use of soybean meal and fish meal in animal feeds

Intensive animal protein production (meat, milk, eggs and fish) depends heavily on the availability of protein sources (oil seeds, especially soyabean) and energy sources (cereals) to formulate nutritionally balanced and adequate feeds (Thornton, 2010). In the SSA region, soyabean the major protein source in animal feeds, is also used to a large extent as a dietary ingredient in foods. Consequently this has created competition between humans and animals (Siddhuraju and Becker, 2001). The demand for soyabean to satisfy human and animal requirements in the SSA region is against a deficit in the region's combined soyabean production making the region a net importer if it were to meet both human and animal requirements for soyabean. Sub-Saharan Africa currently produces 1.4 million metric tonnes of soyabean but experiences a net SBM shortfall of 1 million metric tonnes annually (HighQuest Partners, 2011). As the per capita gross domestic product increases in SSA and the current high demand of animal protein continues, the shortfall of SBM in SSA will grow substantially (HighQuest Partners, 2011). The shortage of soyabean has resulted in an

escalation of the price of livestock feeds (Barros et al., 2002), thus negatively affecting animal protein production that translates into reduced intake of animal protein in the diets of communities in the region.

Droughts, recurrent mid-season dry spells (Kumar et al., 2010) and the high susceptibility of soyabean to insect pest attacks (Kawuki et al., 2003) have impacted negatively on efforts to intensify soyabean production in the SSA region. Mid-season dry spells and insect pest attacks require costly irrigation and pest management interventions, respectively, that most of the farmers in the region cannot afford. The use of fishmeal as a protein base in animal feeds is limited by problems of availability and its high cost (Forster et al., 2003) as well as the odour-tainting it imparts to animal products if used in finisher diets.

1.4 Justification

Competition and drought-induced soyabean shortages in the SSA region and the limitation in the use of FM mean that there is a great need for scientific search for non-conventional substitutes to soyabean and FM that are adapted to the climatic and edaphic environment of the region. The global increase in the cost of fossil fuels has resulted in an increased use of food crops as feeder material for biofuels. The use of food crops as feeder material for biofuels has exposed human

populations to famine and has exacerbated the shortage of plant-derived protein sources in feeds.

Indigenous fruit-bearing trees (IFBTs) are adapted to the eco-environment of the SSA region and generations of humans have harvested non-timber-tree products especially honey and fruit from them. The pulp of fruit from IFBTs has been and, continues to be, used as food. The fruit seeds which are potential sources of protein for animal feed, oils and essential oils are generally discarded after utilization of the fruit pulp. Currently, there is dearth of information on the chemical, *in vitro* and *in vivo* potential of the IFBTs' seeds as protein sources in animal feeds despite their (i.e. IFBTs) ready availability in the SSA eco-environment and the lack of competition between animals and humans for the IFBTs. To ensure wholesome use of the IFBTs seed meals as protein sources in feeds, it is important that the lipids in their seeds be quantified and profiled in order to determine their potential applications. Such a holistic approach would result in IFBTs contributing more to human livelihoods in a sustainable manner leading to community based conservation of the indigenous tree resources (Akinnifesi et al., 2006). Research efforts to broaden the animal feed ingredient base have focused on farmed grain crops and plantation products and their by-products. Some research on the contribution of browse species from trees indigenous to the region has been done (Katjiua and Ward, 2006). Research into the potential of seeds from IFBTs opens up new avenues of finding alternative sources of nutrients, especially protein, which might lead to a reduction of the current burden of human-livestock competition for protein sources in foods and

feeds. Such research has potential to generate raw (by-products in the form of oils, fatty acid and essential oils) materials for industry over and above generating innovative livestock feed ingredients.

Kigelia africana, *Mimusops zeyheri, Terminalia sericea* and *Ximenia caffra* are IFBTs trees widely distributed in SSA. The trees yield a crop of fruit whose fruit pulp (*M. zeyheri* and *X. caffra*) is used as a food. *K. africana* fruit is used in traditional beer brewing while the gum from *T. sericea* is consumed. While research has focused on the ethno-medicinal potential and to some extent the fruit pulp composition of *K. africana*, *M. zeyheri*, *T. sericea* and *X. caffra*, there is a dearth of information on the potential of their seeds as alternative protein sources in feeds.

1.5 Statement of objectives

1.5.1 Broad objective

Broadly, the study sought to evaluate the potential of *K. africana*, *M. zeyheri*, *T. sericea* and *X. caffra* seeds as alternative protein sources in feeds. The evaluation was planned to involve several stages: a determination of the physical traits of the seeds and quantification of the oil yield from shelled/dehulled seeds and profiling of the fatty acid content of the seed oils. This would then be followed by chemical

characterization of the the nutrient, fibre (neutral and acid detergent fibre) and phytate-phosphate composition of the shelled/dehulled seed meals. The seed oils would then be tested *in vitro* for biological activity using established cell lines. After the *in vitro* assessment of the seed oils, an *in vivo* evaluation would be executed to determine the effects of dietary substitution of SBM with the defatted IFBTs seed meal (most suitable seed on the basis of physical, chemical and *in vitro* evaluation results). The *in vivo* evaluation would be split into two components: determination of the effects of dietary substitution of SBM with the most suitable defatted IFBTs seed meal on apparent nutrient digestion and nitrogen balance in mature Sprague Dawley rats and a determination of the effects of substituting SBM with defatted IFBTs seed meal on growth performance, gastrointestinal (GIT) morphometry, liver metabolic substrate storage and function, and the general health profile in male Sprague Dawley rats weaned onto the IFBTs seed meal based diets.

1.5.2 Specific objectives

Specifically, the study sought to:

i. physically characterize the seeds by determining the seed mass, size (length and width), shelling/dehulling percentage and estimate the ease of shelling/dehulling.

ii. extract and quantify oil from the four IFBTs seeds and profile the fatty acids (saturated, monounsaturated and polyunsaturated), vitamin E and squalene content of the seed oils.

iii. determine the proximate [dry matter (DM), organic matter (OM), crude protein (CP), ether extract (EE) / lipid, ash and gross energy (GE)] and mineral (calcium, magnesium and phosphorus) content of the seeds.

iv. determine the neutral detergent fibre (NDF), acid detergent fibre (ADF) and the phytate-phosphate content of the seeds.

v. test *in vitro* the biological effects of IFBTs seed oils on established cell lines (Caco-2 and HEK-293), then select on the basis of the physical and chemical traits and *in vitro* evaluations, the most suitable seed; de-fat it and use its meal as a dietary substitute to SBM for the two *in vivo* trials.

vi. determine, in the first *in vivo* trial, the effects, in mature Sprague Dawley rats, of dietary substitution of SBM with the defatted IFBT seed meal on apparent nutrient (DM, OM, GE and CP) digestibility and nitrogen retention.

vii. determine, in the second *in vivo* trial in male Sprague Dawley rats weaned onto the IFBT seed based diets the effects of dietary substitution of SBM with the defatted IFBT seed meal on:

   a. the growth performance (body mass gain, average daily gain, terminal body mass, empty carcass mass and tibia and femur length).

b. gastrointestinal macroscopic morphometry (masses and lengths).

c. liver metabolic substrate storage (liver glycogen and total lipid content) and markers of liver function [serum alanine transaminase (ALT) and alkaline phosphatase (ALP) activity].

d. fasting circulating blood metabolic substrate (cholesterol, glucose and triglyceride) concentration.

e. the general health profile (serum urea, creatinine, phosphorus, calcium, total protein, albumin, globulin, total bilirubin, and amylase).

1.6 Hypotheses

Physical and chemical evaluation of the nutritive value of *K. africana*, *M. zeyheri*, *T. sericea* and *X. caffra* seeds was planned to focus on simple characterization of each seed without statistical comparisons between the seeds from the four IFBTs. However for the *in vitro* and *in vivo* studies comparisons were planned thus the key hypothesis for the *in vitro* and *in vivo* studies were:

i. H_0: The IFBTs seed oils have no effect on the proliferation and survival of Caco-2 and HEK-293 cells in culture.

ii. H_0: The graded substitution of SBM as the protein source in the diet with the most suitable defatted IFBT seed meal does not reduce apparent nutrient

digestibility and nitrogen retention in mature male Sprague Dawley rats.

iii. H_0: The graded substitution of SBM as the protein source in the diet with the most suitable defatted IFBT seed meal does not reduce the growth performance of weanling male Sprague Dawley rats.

iv. H_0: The graded substitution of SBM as the protein source in the diet with the most suitable defatted IFBT seed meal does not reduce liver metabolic substrate storage, does not cause liver toxicity neither does it alter fasting blood metabolic substrate concentration in weanling male Sprague Dawley rats.

v. H_0: The graded substitution of SBM as the protein source in the diet with the most suitable defatted IFBT seed meal does not affect the macroscopic morphometry of the GIT viscera and other visceral organs in weanling male Sprague Dawley rats.

vi. H_0: The graded susbstitution of SBM as the protein source in the diet with the most suitable defatted IBFT seed meal does not alter the serum concentration and activities of the markers of the general health profile in weanling male Sprague Dawley rats

CHAPTER 2: PHYSICAL AND CHEMICAL CHARACTERIZATION OF *KIGELIA AFRICANA, MIMUSOPS ZEYHERI, TERMINALIA SERICEA* AND *XIMENIA CAFFRA* SEEDS

2.0 Introduction

Indigenous fruit bearing trees supply fruit to rural societies of the SSA region (Saka and Msonthi, 1994). The fruits are sources of minerals, vitamins, protein and energy critical for maintenance of human health (Saka and Msonthi, 1994). Though undomesticated, the trees contribute to the improvement in household food security of rural communities in the region by yielding a crop of fruit, even in years characterized by poor rainfall (Mojeremane and Tshwenyane, 2004). Apart from yielding edible oils, plant-derived oils (including IFBTs seed oils) have been and continue to be used in the production of soaps, cosmetics, surfactants, lubricants and in the treatment of ailments among others dandruff, wounds and varicose veins (Lee, 1973; Goldberg and Williams, 1999; van Wyk, 2002; van Wyk and Gericke, 2003 and Mohammad and Mahmood, 2005). Additionally the IFBTs supply a habitat for the source of honey, browse for both game and domesticated ruminant animals. In some parts of SSA, for example in Zimbabwe, IFBTs such as *Mimusops zeyheri*, *Parinari cuatelifolia*, *Uapaca kirkiana* and *Strychnos cocculoide*s are left standing in crop fields where over and above supplying fruit also help in the sustenance of soil fertility through nutrient recycling (Hilhorst and Muchena, 2000).

2.1 The indigenous fruit bearing trees

The Sausage tree (*Kigelia africana*), the Red Milkwood (*Mimusops zeyheri*), the Silver leaf (*Terminalia sericea*), and the Large Sour Plum (*Ximenia caffra*) are some of the IFBTs that are widely distributed in SSA (Janick and Paull, 2008). Their fruit seeds' potential as feed and food resources has not been explored although currently the trees are put to multiple uses: timber and non-timber

2.1.1 *Kigelia africana*

K. africana, family Bignoniaceae, is a tropical tree native to and widely distributed in Africa where it grows in open woodlands and wet areas including river banks (Owolabi and Omogbai, 2007, Janick and Paull, 2008). The tree, with a smooth grey stem bark, has a rounded dense crown and grows to about 25 m in height (Janick and Paull, 2008). The compound leaves of *K. africana* with three to four pairs of leaflets are hairy, yellowish green above and pale green below and are held on petioles that are about 15 cm long (Janick and Paull, 2008). The tree flowers from June through to October in SSA. The large cup-shaped dark red asymmetric flowers have yellow veins on the outside, and hang approximately 90 cm from the canopy. The flowers are bisexual, emit a foul smell and are pollinated by bats (Janick and Paull, 2008). Its (*K. africana*) fruit, an indehiscent wooden berry, 30-100 cm long weighs between 5-10 kg (Joffe, 2003) and takes 4-9 months to mature (Janick and Paull, 2008). On average each *K. africana* tree

yields about 184.5 kg of seed (Jackson and Beckett, 2012). Mature *K. africana* seeds are greyish-brown in colour and ovoid (Grace et al., 2002).

K. africana is widely used in African ethnomedicine (Olalye and Rocha, 2007). Ogbeche et al. (2002) indicated that an alcohol and or aqueous mixture of *K. africana* leaves and fruit is used to alleviate fertility problems while Kokwaro (1976) cited by Azu et al. (2010) reported that a concoction of roasted *K. africana* seeds mixed with beer enlarges male sexual organs; a probable folklore legend in SSA. Root and bark extracts of the tree contain dihydrisocoumarin, kigelin, lapachol and naphthoquinone (Grace et al., 2002); phyto-chemicals that may explain the widespread use of the tree in ethnomedicine. The antibacterial fatty acids isolated from *K. africana* fruit and the cytotoxic gamma-sitosterol from the root bark support the tree's use in the management of bacterial infections (Asekun, 2007). While unripe *K. africana* fruit pulp is said to be poisonous (Azu et al., 2010), roasted ripe *K. africana* fruit is used to flavour beer and aid in the fermentation process (Coates-Palgrave, 1988). During periods of famine, in some parts of SSA, *K. africana* seeds are roasted and eaten (Janick and Paull, 2008; Azu et al., 2010). In Ghana young K. africana leaves are used to make palm-nut soup consumed as an accompaniment to staple foods such as yams and maize by lactating mothers (Glew et al., 2010).

2.1.2 Mimusops zeyheri

M. zeyheri, family Sapotaceae, also known as the Red Milkwood, is an indigenous fruit bearing tree native to Africa and is widely distributed in SSA though found in other parts of the globe. In SSA its distribution stretches from Tanzania and Angola through Botswana, Zimbabwe, Mozambique, the north-eastern regions of the republic of South Africa and Swaziland (Lemmens, 2005, Janick and Paull, 2008). Evergreen, *M. zeyheri,* grows to 6-10 m in height when mature and has a dense spreading crown. *M. zeyheri*'s rough grey to brown coloured stem bark exudes white latex (Janick and Paull, 2008). Its leaves (10-15 cm long; 3-6 cm wide) that form clusters at the end of shoots are pointed at both ends and are glossy but generally darker green on top and paler below. The sessile clusters of *M. zeyheri* flowers occur along branches between the shoot tip and trunk (Janick and Paull, 2008). The round to epileptic *M. zeyheri* fruit (weighing 50-250 g) is surrounded by a green leathery skin in the immature fruit which (skin) turns light yellowish when the fruit ripens. Each fruit on maturity has 1-5 shiny brown to black seeds and contains a sweet floury-tasting white fruit pulp (Janick and Paull, 2008; Motlhanka et al., 2008). While flowering occurs throughout the year, the major flowering season occurs from September to November with pollination thought to be facilitated by bees (Janick and Paull, 2008). In Zimbabwe, the tree is left standing in crop fields where in addition to supplying fruit for human consumption, it is reported to improve soil fertility (Wilson, 1989).

Ripe, fresh *M. zeyheri* fruit gathered from communal areas is sold in urban open markets. While the tree is well known for producing edible fruit, Amusan et al. (2002) pointed out that in Swaziland, a root infusion of *M. zeyheri* is used in the treatment of candidiasis. In Botswana, after extraction of the seed from the ripe fruit, the seedless fruit pulp is sun-dried and the dry residue is eaten in winter (Motlhanka et al., 2008). According to Janick and Paull (2008), *M. zeyheri* fruit pulp with an ascorbic acid content of 50-80 mg 100 g^{-1} pulp is a good source of vitamin C. Lemmens (2005) also highlighted the high vitamin C content (90 mg per 100 g) of *M. zeyheri* fruit pulp and observed that the fruit pulp is generally low in protein, fat and carbohydrate. The vitamin C rich *M. zeyheri* fruit pulp is used in jams, jellies and production of fermented juices (Lemmens, 2005).

2.1.3 *Terminalia sericea*

T. sericea also known as the Silver leaf tree or Silver Terminalia, family Combretaceae, is widely distributed in tropical Africa and in some warm temperate regions (Hutchings et al., 1996). *T. sericea* is found scattered in most of the Savannah woodlands of east, central and southern Africa where it occurs as a dominant or co-dominant species in mixed forests. The tree thrives in most soil types with proper drainage but is more abundant in areas with deep sandy soils and moderate rainfall (Coates-Palgrave, 1988). Palmer and Pitman (1972) point out that the tree is drought tolerant, moderately adapted to saline soils and tolerates some degree of frost. *T. sericea* occurs as a shrub and or bush that grows on average to a height of 6 to 9 m although some trees my reach a height of 23 m

(Coates-Palgrave, 1988). The stem and branch barks are reddish-brown in colour (Drummond, 1981). *T. sericea* leaves, covered in silvery hairs, are obovate-elliptic, crowded at the end of the branch, are bluish-green above and paler below (Drummond, 1981). The pale-yellow to creamy-white flowers are in axillary spikes. *T. sericea's* winged oval fruit is pinkish on maturity turning dark with advancement in age and contains one seed. In communities where it is found, the tree is of multipurpose value. It is used as a fuel source (fuel wood and charcoal), fencing posts, and carving of hand tools and in construction (Eckman and Deborah, 1993). The tree contributes to both domestic and wildlife production through provision of browse especially during the hot dry season in SSA (Katjiua and Ward, 2006). *T. sericea* is widely used in ethnomedicine: root bark extracts are used to treat diarrhoea, colic, pneumonia and bilharzia while its leaf extract is used in the management of stomach disorders (Coates-Palgrave, 1988). The use of *T. sericea* in ethnomedicine is premised on its reported biological activities that include antifungal and antibacterial activity (Fyhrquist et al., 2004) and ability to inhibit topoisomerase II (Wall et al., 1996). The enzyme topoisomerase II is critical for chromosome structure and segregation and plays a role in DNA replication, recombination and transcription in cells (Osheroff et al., 1991). Phytochemicals including resveratrol-3-*O*-β-rutinoside, a stilbene glycoside, and oleanane-based pentacyclic triterpenes and their glycosides have been isolated from *T. sericea* (Joseph et al., 2007). A *T. sericea* derived sericoside has been patented and is used in skin lightening preparations in Japan (Maeda and Fukuda, 1996). Resveratrol and its derivatives including the wine derived β-D-glucoside of

resveratrol are considered protective against coronary heart disease (Jeandet et al., 1991).

2.1.4 *Ximenia caffra*

The large Sour Plum, *Ximenia caffra* var. *caffra* (*X. caffra*), family Olacaceae, is a Southern African plant species whose distribution spans across Tanzania, Zambia, Zimbabwe, Botswana, Namibia, Mozambique and South Africa (Lee, 1973). *X. caffra* is small tree or shrub that grows to about 6 m, is sparsely branched with a shapeless crown. Its stem bark is brown to pale grey, smooth to slightly scaly and has thick axillary spines (Janick and Paull, 2008). *X. caffra* leaves (2.5-9 cm long; 1.2-5 cm wide) are simple, alternate, and elliptic to lanceolate (Orwa et al., 2009). The leaves are leathery, blue-green and hairy when young and turn shiny green when older. *X. caffra* flowers towards the end of the dry season (September to October) giving greenish, creamy-white blossoms that at times have a reddish tinge (Orwa et al., 2009). Fruiting occurs in December to January in southern Africa (Orwa et al., 2009) with immature fruit green in colour while the ripe fruit is orange or red (Janick and Paull, 2008). Each fruit (when ripe) contains a single yellowish-brown to reddish oval-shaped seed that is about 2.5 cm long and 1 cm wide (Janick and Paull, 2008; Orwa et al., 2009). The tree can withstand moderate frost and is drought resistant when mature and is largely common in the dry bushveld and wooded grasslands particularly on rocky soils (Orwa et al., 2009). The flesh of the fruit of *X. caffra* immediately around the stone is sour. The fruit pulp has a high protein value and is rich in ascorbic acid at 27mg100mg^{-1}

(Roodt, 1998). People add the dried flesh or the fresh juice to porridge to add taste and protein. The fruit also produces good quality jelly that is useful in making tarts (Roodt, 1998). The kernel is edible and is also used to make jam. Sixty-five percent of the kernel is oil (Venter and Venter, 1996): the oil, which is yellow, viscous and non-drying, is used as biofuel in lamps (Venter and Venter, 1996). Roodt (1998) indicated that ximenic acid, an unsaturated acid has been isolated from the kernel oil. The Khoi San tribe uses the oil for softening skin and also rub it on chapped hands and feet. van Wyk and Gericke (2003) indicated that *X. caffra* seeds contain tannins and that oil extracted from the seed is used in softening clothing leather such as for leather boots and skirts. *X. caffra* root extracts are used to treat abscesses, colic, malaria, cough and bilharzias (Janick and Paull, 2008; Orwa et al., 2009).

2.1.5 Gaps in IBFTs research

Research on *K. africana*, *M. zeyheri*, *T. sericea* and *X. caffra* has focused on the ethno-medicinal and pharmacological potential of the root, bark, leaf and fruit extracts (Coates-Palgrave, 1988; Olalye and Rocha, 2007; Janick and Paull, 2008; Orwa et al., 2009) and to some extent on the fruit pulp composition (Lemmens, 2005; Janick and Paull, 2008). Research on IFBTs seeds has been neglected despite their being potential sources of macro- and micro-nutrients, essential oils and protective phytochemicals. There is a dearth of information on the potential of *K. africana*, *M. zeyheri*, *T. sericea* and *X. caffra* seeds as feed and food ingredients. The shortage of feed ingredients in SSA region, especially protein,

exacerbated by the competition for feed/food ingredients between humans and livestock in SSA, justified my evaluation of the nutritive potential (alternative protein sources in feeds) of *K. africana*, *M. zeyheri*, *T. sericea* and *X. caffra* seeds.

2.1.6 Study objectives

The specific objectives of the study were to:

i. physically characterize the seeds from the four IFBTs by determining their seed mass, size (length and width), shelling/dehulling percentage and estimate the ease of shelling/dehulling.

ii. extract and quantify oil from the four IFBTs seeds and profile the fatty acids (saturated, monounsaturated and polyunsaturated), vitamin E and squalene content of the seed oils.

iii. determine the proximate [dry matter (DM), organic matter (OM), crude protein (CP), ether extract (EE) / lipid, ash and gross energy (GE)] and mineral (calcium, magnesium and phosphorus) content of the seeds.

iv. determine the neutral detergent fibre (NDF), acid detergent fibre (ADF) and the phytate-phosphate content of the seeds.

2.2 Materials and methods

2.2.1 Seed source and processing

The seeds (*K. africana*, *M. zeyheri*, *T. sericea* and *X. caffra*) were imported into the Republic of South Africa (permit number P0039683) from Zimbabwe for the analyses. Before harvesting the fruit for the experiments, branches and fruit samples of each of the four IFBTs were collected and submitted to the National Botanical Gardens of Zimbabwe for verification and confirmation of their identity. Ripe *K. africana* fruit falls to the ground (Janick and Paull, 2008) thus it was picked from the ground. Harvesting *M. zyheri* and *X. caffra* fruit was executed by initially shaking the trees wtih ripe fruit followed by picking fruit from the ground. Ripe fruit in was collected through farmer field groups organised by the local agricultural extension services officers.

2.2.1.1 Kigelia africana seeds

Ripe *K. africana* fruits were harvested from trees in Gutu District, Chitsa Communal area, Zimbabwe. The area which lies within latitude and longitude 19º41′S, 31º09′E is characterized by granitic soils, and has annual rainfall and temperature ranges of 650-800 mm and 20.5-30.0ºC, respectively (Government of Zimbabwe, 2000). Fifty fruit bearing *K. africana* trees, within an area of about

two square kilometres, were randomly identified out of which a subset of ten trees was selected as a source of ripe fruit. From each of the ten *K. africana* trees, ten ripe fruits were harvested. Fruits from ten trees (out of the fifty randomly selected trees) were used as a source of seeds for the experiment. The seeds were manually extracted and dried in the shade. The seeds were then all composited into a single sample, thoroughly mixed and stored in dark sealed sample bottles for 3 weeks, at 4ºC in a refrigerator until the time of assaying. Prior to assaying, the seeds were dehulled by hand via an initial cracking the seed hull using side-cutting pliers which was followed by extraction of the seed from hull. The dehulled seed was then crushed using a blender (Waring; Lasec Pty Ltd, Johannesburg, South Africa) to produce a composite meal from which the various assays were done.

2.2.1.2 Mimusops zeyheri seeds

The fruits from which *M. zeyheri* seeds were harvested were from two different locations in the republic of Zimbabwe. The first batch of fresh ripe *M. zeyheri* fruits were harvested from trees in Matopos National Park, Zimbabwe [latitude: 20º25′S, longitude: 28º29′E, altitude: 1320 m, rainfall: 609 mm, mean maximum temperature: 25.9ºC, mean minimum temperature: 10.8ºC and soil type: shallow sandy lithosol (Mushove et al., 1995)]. The second batch of fresh ripe *M. zeyheri* fruit was harvested from trees in Gokwe district, (18º13'S and 28º56'E**),** in North West Zimbabwe, in the Machakata Community Forest area of the Mapfungautsi Plateau. The area is characterized by granitic deep gusu sandy soils, an average

annual rainfall of 819 mm and an annual average temperature of 26ºC (Gwimbi, 2009). Two kilograms of ripe fruit was harvested from each of ten random .ly selected trees out of a sample of fifty identified *M. zeyheri* trees in each area. The seeds were manually extracted, dried in the shade and stored in dark sample bottles at 4ºC in the refrigerator until the time of assaying. Prior to assaying, the seeds were hand shelled and then crushed using a blender (Waring; Lasec Pty Ltd, Johannesburg, South Africa) to produce a composite meal from which the various assays were done.

2.2.1.3 Terminalia sericea seeds

Fresh ripe *T. sericea* fruit were collected from farms surrounding Fletcher High School, in Gweru District, Zimbabwe (latitude 19º25′S; longitude 29º5′E). Gweru District is characterised by an average annual rainfall of 643 mm and an average annual temperature of 28ºC (Moyo, 2006). The soils are basalt and granitic. Two kilograms of ripe fruit was harvested from each of ten randomly selected trees out of a sample of fifty identified *T. sericea* trees. The seeds were manually extracted from the tough fruit coat, dried in the shade and stored in dark sample bottles at 4ºC in the refrigerator until the time of assaying. Before the chemical assays, the seed were crushed using a blender (Waring; Lasec Pty Ltd, Johannesburg, South Africa) to produce a composite meal from which the various assays were done.

2.2.1.4 Ximenia caffra seeds

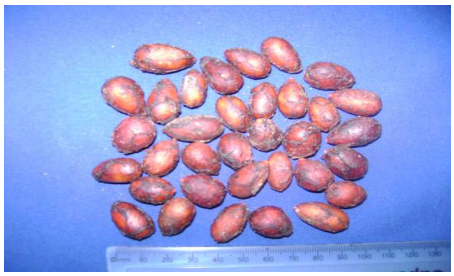
Fresh ripe *X. caffra* fruit were collected from Zhombe District, Zimbabwe (latitude 14º45/S; longitude 26º50/E) in the Agricultural and Rural Development Authority's Fare Acres estate and the surrounding communal areas of Silobela. Zhombe district is characterized by low annual rainfall (mean 550 mm per annum) and a mean annual temperature of 26ºC. The *X. caffra* fruit for chemical analyses were harvested from twenty trees randomly selected from a sample of one hundred identified *X. caffra* trees. One hundred ripe fruit were picked from each of the twenty trees. Out of the 100 fruit picked from each tree, twenty were randomly selected. The fruit pulp was removed from the selected fruit and the fruit stones (containing the seeds) were dried in the shade and stored separately in dark sample bottles at 4ºC in the refrigerator until the time of assaying. Prior to assaying, the seeds were manually extracted from the fruit stones/shells, and were crushed using a blender (Waring; Lasec Pty Ltd, Johannesburg, South Africa) to produce a composite meal from which the various assays were done.

Figure 2.1 shows photographs of the seeds and or fruit of the *K. africana, M. zeyheri, T. sericea* and *X. caffra* used in the study.

panel A

panel B panel C

panel D



panel E panel F

Figure 2.1: Photographs of the IFBTs fruit/seeds used in the study. Panel A: undehulled *K. africana* seeds; panels B and C: unshelled and shelled *M. zeyheri* seeds, respectively; panel D: *T. sericea* fruit; panel E: *X. caffra* fruit stone; panel F: shelled *X. caffra* seed.

2.3 Physical characterization of the seeds

The shape and colour of *K. africana*, *M. zeyheri*, *T. Sericea* and *X. caffra* seeds was determined by visual inspection. In batches of 50 seeds and in triplicate, seeds of each species were weighed prior to shelling and or dehulling. After shelling/dehulling the mass of the 50 seeds minus the shell and or hull was determined. Mean seed mass (before and after shelling/dehulling) was computed for each seed batch. Shelling and or dehulling percent was computed as shown in the formula below:

$$\text{Shelling/dehulling \%} = \frac{\text{Mass of 50 shelled/dehulled seeds}}{\text{Mass of 50 seeds before shelling/dehulling}} \times 100$$

Seed size (length and width) was measured with a Veneer calliper (Servier Nederland b.v., Leiden, Netherlands). The degree of ease of seed shelling (extrication from the fruit stone/shell by hand) and dehulling (removal of hull by hand) was determined subjectively.

2.3.1 Chemicals and reagents for assays

All the chemicals and reagents used were of analytical grade. The chemicals and reagents were obtained from Sigma-Aldrich Chemie (Steinheim, Germany), unless otherwise stated.

2.3.2 Proximate determinations

The proximate, mineral, amino acid, fibre and phytate-phosphate determinations were performed at the Agricultural Research Council's Irene Analytical Services Laboratories, South Africa. The proximate components, that is, dry matter (DM), ash, crude protein (CP), and ether extract (EE) were determined as outlined by the Official Methods of Analysis of Analytical Chemists (AOAC) (2005: method numbers 934.01, 942.05, 954.01, and 920.39 respectively). Organic matter was estimated as the difference between dry matter and ash. The gross energy (GE) value of the seeds was determined using an MC-1000 Modular Calorimeter (Energy Instrumentation, Centurion, South Africa) equipped with a PC and MC1000 software.

2.3.3 Calcium, magnesium and phosphorus determination

Prior to the determination of the mineral concentration in the seed samples, 0.5 g the milled samples was digested in concentrated nitric acid and perchloric acid at 200^0C to generate the digest solution (Zasoski and Burau, 1977). From the digest solution an aliquot of the digest solution was used for the inductively coupled plasma optical emission spectrometric (ICP-OES) determination of calcium, magnesium and phosphorus on a Varian Liberty 200 spectrometer (Varian, Perth, Australia) as described by Huang and Schulte (1985).

2.3.4 Amino acid assay

The concentration of each of the assayed amino acids was determined as described by Einarsson et al. (1983). Briefly, the assay involved acid hydrolysis with 6M HCl at 110°C for 24 hours and pre-column fluorescence derivatization of amino acids with 9-flourenylmethyl chloroformate. The amino acids were extracted with pentane, and separated by gradient elution on a chromatograph. The chromatograph consisted of a SpectraSystem P4000 Quaternary HPLC (Rigas Labs S.A., Thessaloniki, Greece) equipped with a SpectraSystem FL3000 fluorescence detector and Rheodyne 7125 valve with 20 µl injection loop. The eluent was varied with a concave curve from sodium citrate buffer (pH 2.95)-acetonitrile (70:30) to sodium citrate buffer (pH 4.5)-methanol-acetonitrile (14:6:70) and a flow-rate of 1.4 ml/min. An OmniSper 5 C18 150x4.6 analytical column and guard-column were used for separation of the amino acids. Identification of the amino acids was done at an excitation wavelength of 264nm and an emission wavelength of 340nm. A PC equipped with TSP software was used for quantification. Quantification was performed by using an external calibration procedure.

2.3.5 Fibre determinations

The neutral detergent fibre (NDF) and acid detergent fibre (ADF) components of the seeds were determined, inclusive of ash, as described by Van Soest et al.

(1991). In summary, NDF determination involved refluxing a 0.5g sample for 1 hour in 100mL of neutral detergent solutions of sodium lauryl sulphate and ethylenediamine-tetraacetic acid to which heat-stable alpha-amylase (20 350 IU/ml) (dietary fibre kit, Sigma-Aldrich) was added. After refluxing for 1 hour, the mixture was filtered; the residue was dried and then weighed. ADF was determined by refluxing for 1 hour a 0.5g sample in acid detergent solution (20g cetyl-trimethyl ammonium bromide dissolved in 1L N H_2SO_4). After refluxing, the mixture was filtered and the residue was dried and then weighed.

2.3.6 Phytate-phosphate determination

Phytate-phosphate content of the seeds was determined colorimetrically as described by Wheeler and Ferrel (1971) using a Perkin Elmer Lambda25 UV/Vis Spectrometer (PerkinElmer, California, USA) equipped with a desk top computer and Lambda25 software. In summary, samples were treated with 3% trichloroacetic acid followed by addition of ferric chloride (2mg ferric iron per ml in 3% trichloroacetic acid) and the precipitate dissolved in 3.2N nitric acid. After addition of 1.5M potassium thiocynate the absorbance was read at 480nm.

2.3.7 Fatty acid profile determination

Fat was extracted from the respective seeds by the soxhlet method as described by AOAC (2005; method number 920.39). Methyl esters for capillary gas chromatography were prepared according to the method of Christopherson and Glass (1969). Briefly, the fat extracts were trans-methylated with 2M methanol-sodium hydroxide. The resulting fatty acid methyl esters were extracted in heptane, filtered and dried under nitrogen. The fatty acids were separated by a temperature gradient over 45 min on a GC with nitrogen as carrier gas on a DB-23 capillary column (90cm x 250 µm x 0.25 µm) (Supelco, Sigma-Aldrich). The gas chromatograph consisted of a HP6890 GC (Hewlett Packard, Bristol, United Kingdom) with flame ionization detector (FID). Both the detector and injector temperatures were set at 300°C. A PC equipped with Chemstation software was used for quantification. Nonadecanoic acid (C19:0) was used as an internal standard.

2.3.8 Vitamin E and squalene determination

The lipid extracts used in the assays were prepared using standard lipid extraction procedures (Bligh and Dyer, 1959). After evaporation to dryness, the lipids were re-dissolved in an equal volume of the respective running solvent; methanol:water (95:5) for vitamin E and, hexane:propan-2-ol:water (98:2:0.02) for squalene prior

to injection into the HPLC system. Assays for vitamin E were done as described by De Leenheer et al. (1985) and Gratzfeld-Huesgen et al. (1992) whereby, following dissolution of the lipid extracts into the running phase solvent, the sample was injected into the HPLC system (LKB Bromma 2150 HPLC; LKB, Bromma, Sweden). The mobile phase ran at 2 ml min^{-1}. Vitamin E was separated using methanol:water (95:5) and a C18, 15 cm x 4.6 mm ID, 5µm particle size column; and detection at 290 nm by a Lambda-Max Model 481 LC spectrophotometer (Millipore Water Corporation, Ontario, Canada) with vitamin quantification using an HP 3390A integrator (Hewlett Packard, Bristol, United Kingdom). Squalene was assayed as described by Sulpice and Ferezou (1984). The sample was injected into an HPLC system (LKB Bromma 2150 HPLC). The mobile phase ran at 5 ml min^{-1} with squalene separation using hexane:propan-2-ol:water (98:2:0.02) and a silica gel C18, 25 cm x 4.6 mm ID, 5µm particle size column (Phenomenex, Torrance, USA); and detection at 215 nm by a Lambda-Max Model 481 LC spectrophotometer (Millipore Water Corporation, Ontario, Canada) with squalene quantification using an HP 3390A integrator (Hewlett Packard, Bristol, United Kingdom). Authentic vitamin E and squalene standards were used to identify and to quantitate the vitamin E and squalene, respectively.

2.3.9 Statistical analysis

For each of the chemical component assayed for in the seeds, mean values (assays done in triplicate) and standard deviations were computed. Results are reported as means and standard deviations.

2.4 Results

The physical characteristics of the four IFBTs seeds are shown in Table 2.1.

Table 2.1: Physical characteristics of the IFBTs seeds

Parameter	***K. africana***	***M. zeyheri***	***T. sericea***	***X. caffra***
Seed mass (mg)	125.07±2.97	351.18±13.14	nd	1634.96±157.67
Shelled seed mass (mg)	61.18±5.33	186.05±10.37	nd	1019.37±92.84
Seed length (cm)	0.97±0.05	1.38±0.08	nd	1.48±0.18
Seed width (cm)	0.59±0.04	0.49±0.03	nd	0.97±0.18
Shelling/dehulling (%)	48.92±4.09	52.96±1.71	nd	62.42 ±2.14
[a]Shelling/dehulling ease	difficult	easy	very difficult	very easy

nd: not determined, seeds broken to fragments. [a]Shelling/dehulling ease: refers to arbitrarily determined degree of easiness by which seed was manually extricated from the shell/fruit stone/hull.

K. africana seeds were greyish-brown, coloured kidney/ovoid-shaped with whitish kernels. *M zeyheri* had shiny brown oval shaped seeds with creamish-white kernels. *T. sericea* had winged brown coloured fruit with tiny yellowish seeds inside while *X. caffra* seed was oval shaped, brown coloured which when shelled gave cream-yellowish coloured kernels.

The toughness of the *T. sericea* fruit stone made it difficult to extricate intact seeds, thus it became impossible to determine the seed size (length and width) and mass from the broken seed pieces after using a side cutting pliers to extricate the seed.

Table 2.2 summarises results on the proximate, mineral, fibre, and vitamin E content of the seeds.

Table 2.2: **Proximate, mineral, fibre and vitamin E and squalene content of the IFBTs seeds**

	K. africana	*M. zeyheri*	*T. sericea*	*X. caffra*
Proximate component (g kg^{-1} DM)				
Dry matter (DM)	964.27±3.46	911.00±1.41	953.03±0.92	955.13±0.78
Organic matter (OM)	915.23±7.98	883.39±2.11	884.08±3.22	934.69±1.97
Crude protein (CP)	357.35±3.39	93.45± 0.54	462.32±5.49	182.55±0.52
Lipid/oil yield	492.20±1.68	212.50± 5.19	325.61±14.00	484.47±0.08
Ash	49.05±4.55	27.61± 0.70	68.95± 2.30	20.44±1.19
Mineral composition (mg $100g^{-1}$ DM)				
Calcium	56.05±1.53	587.40±10.11	795.20±17.82	17.85±0.74

Table 2.2 continued

Magnesium	531.09±4.41	102.30± 2.33	560.70±6.68	207.90±5.94
Phosphorus	1123.20±23.53	110.37± 0.00	1121.75±10.39	45.45±5.94
Vitamin and squalene content (µg g^{-1} DM)				
Squalene	nd	nd	nd	nd
Vitamin E	0.94±0.25	1.97±0.75	48.70±6.08	0.53±0.12
Fibre content (g kg^{-1})				
Neutral detergent fibre (NDF)	132.84±2.65	332.46± 6.61	23.30±2.16	213.31±5.45
Acid detergent fibre (ADF)	87.71±2.13	153.41±1.56	90.27± 4.68	51.17±1.70

nd = not detected, Data presented as mean ± standard deviation, n = 3 composite samples of seeds from the fruit of 10 trees for *K. africana*, *M. zeyheri* and *T. sericea*, respectively, and 20 trees for *X. caffra*.

The seeds' DM, OM and CP ranged from 91.10-96.43%, 83.33-93.47% and 9.35-46.23%, respectively (Table 2.2). Shelled full-fat *K. africana*, *M. zeyheri*, *T. sericea* and *X. caffra* seed had gross energy values of 29.6±0.06, 24.34±0.56, 21.96±0.01 and 32.1±0.04 MJ kg^{-1}, respectively and phytate-phosphate content of 0.09, 0.03, 0.10 and 0.04%, respectively (Table 2.2). Neutral detergent fibre and acid detergent fibre ranged from 13.3-33.24% and 5.12-15.34%, respectively (Table 2.2).

Table 2.3 shows the amino acid composition of the tree seeds.

Table 2.3: **Amino acid composition (g 100g^{-1} DM) of the IFBTs seeds**

Amino acid	K. africana	M. zeyheri	T. sericea	X. caffra
Alanine	0.97±0.06	0.79±0.05	3.68±1.70	1.17±0.04
Arginine	5.43±0.37	0.80±0.16	7.56±0.21	1.85±0.16
Aspartic acid	2.57±0.16	0.75±0.01	3.49±0.26	1.21±0.11
Glutamic acid	6.14±0.31	1.29±0.02	8.07±0.13	2.34±0.18
Glycine	1.72±0.10	0.38±0.02	2.14±0.01	0.58±0.05
Histidine	1.15±0.25	0.37±0.06	1.25±0.09	0.47±0.07
Hydroxyproline	0.11±0.06	0.04±0.01	0.32±0.08	0.24±0.01
Isoleucine	1.46±0.10	0.38±0.01	1.59±0.23	0.62±0.02
Leucine	1.95±0.05	0.58±0.01	2.43±0.19	1.03±0.05
Lysine	1.49±0.25	0.64±0.04	1.60±0.21	1.03±0.09
Methionine	0.49±0.08	0.21±0.01	0.65±0.11	0.16±0.02
Phenylalanine	2.09±0.17	0.34±0.01	1.19±0.03	0.55±0.04
Proline	0.94±0.04	0.57±0.06	1.88±0.13	0.79±0.00
Serine	1.41±0.13	0.36±0.04	1.62±0.01	0.64±0.04
Threonine	1.42±0.00	0.44±0.00	1.76±0.26	0.73±0.08
Tyrosine	1.69±0.39	0.71±0.13	2.34±0.02	0.75±0.13
Valine	1.72±0.08	0.49±0.00	1.79±0.31	0.71±0.04
Total	**32.75**	**9.05**	**44.08**	**14.87**

Data presented as mean ± standard deviation, n = 3 composite samples of seeds from the fruit of 10 trees for *K. africana, M. zeyheri* and *T. sericea*, respectively, and 20 trees for *X. caffra*.

Of the assayed 17 amino acids, glutamic acid was the highest concentrated amino acid in the seeds (Table 2.3) and constituted about 1.72%, 1.38%, 1.75% and 1.28% of the crude protein content of the full fat *K. africana*, *M. zeyheri*, *T. sericea* and *X. caffra* seed, respectively (Table 2.3).

The fatty acid profile of the tree seed oils is shown in Table 2.4 below. While squalene was not detected in any of the seeds, the vitamin E concentration of the tree seeds ranged from 0.50 to 48.70 µg g^{-1}. All the tree seed oils contained oleic acid (OA) and the essential fatty acids (EFAs) linoleic acid (LA) and α-linolenic acid (Table 2.4).

Table 2.4: **Fatty acid profile (% of total lipid yield) of the tree seed oils**

Parameter	***K. africana***	***[1]M. zeyheri***	***[2]M. zeyheri***	***T. sericea***	***X. caffra***
Fatty acid					
Saturated					
C12:0 (lauric acid)	0.03±0.00	nd	0.15±0.12	0.04	nd
C14:0 (myristic acid)	0.05±0.02	nd	0.13±0.02	0.12	0.02±0.01
C15:0 (pentadecanoic acid)	nd	nd	0.04±0.01	nd	nd
C16:0 (palmitic acid)	7.54±0.25	15.25±0.97	14.06±0.01	9.22	1.47±0.20
C17:0 (margaric acid)	0.11±0.01	nd	0.12±0.01	0.15	nd
C18:0 (stearic acid)	4.56±0.15	nd	6.92±0.01	6.18	0.45±0.10
C20:0 (arachidic acid)	0.89±0.02	nd	0.73±0.00	0.57	nd
C21:0 (heneicosanoic acid)	0.22±0.28	nd	0.09±0.10	0.04	nd
C22:0 (behenic acid)	0.43±0.02	nd	0.32±0.01	0.15	0.56±0.24
C23:0 (tricosanoic acid)	0.03±0.02	nd	0.03±0.01	nd	nd

Table 2.4 continued

C24:0 (lignoceric acid)	0.23±0.02	nd	0.18±0.00	0.04	17.84±1.46
TSFA	**14.10±0.23**	**15.25±0.97**	**22.73±0.08**	**16.51**	**20.19±1.07**
Monounsaturated					
C14:1n7 (myristoleic acid)	nd	nd	0.01±0.00	0.04	0.03±0.01
C16:1n7 (palmitoleic acid)	0.10±0.07	nd	0.08±0.01	0.11	nd
C17:1n8 (8-heptadecenoic acid)	0.03±0.00	nd	0.02±0.00	0.04	nd
C18:1n9 (oleic acid)	17.58±0.59	84.59±0.90	54.41±0.76	14.05	62.84±2.05
C20:1 (11-eicosenoic acid)	0.27±0.03	nd	0.02±0.00	0.14	nd
C22:1n9 (erucic acid)	nd	nd	0.01±0.00	nd	nd
C24:1n9 (nervonic acid)	nd	nd	0.30±0.01	nd	8.64±2.76
TMUFA	**17.99±0.69**	**84.59±0.90**	**54.85±1.12**	**14.38**	**71.48±0.99**
Polyunsaturated					
C18:2n6 (linoleic acid)	12.88±0.61	nd	21.86±1.37	68.63	nd
C18:3n3 (α-linolenic acid)	54.29±1.08	nd	0.44±0.04	0.41	7.80±0.84
C18:3n6 (γ-linolenic acid)	0.28±0.02	nd	0.02±0.00	nd	nd

Table 2.4 continued

C20:2n6 (11,14-eicosadienoic acid)	0.33±0.39	nd	nd	0.06	nd
C20:3n3 (11,14,17-icosatrienoic acid)	0.08±0.00	nd	nd	nd	nd
TPUFA	**67.83±0.91**	**----**	**22.47±1.12**	**69.10**	**7.80±0.84**
Cis fats	30.46±1.19	nd	76.27±0.61	82.68	nd
Omega-3 fats	54.38±1.08	nd	0.54±0.17	0.41	7.80±0.84
Omega-6 fats	13.20±0.57	nd	21.88±1.37	68.63	nd
Omega-9 fats	17.60±0.61	nd	54.43±0.76	14.05	62.84±2.05
TPUFA:TSFA	4.81:1	----	0.99:1	4.19:1	0.39:1
n3PUFA:n6PUFA	4.13:1	----	0.02:1	0.01:1	----

[1]Fatty acid profile determined with seed batch from Matopos National Park, Zimbabwe; [2]Fatty acid profile determined with seed batch from Gokwe District, Zimbabwe; TSFA: Total saturated fatty acids; TMUFA: Total monounsaturated fatty acids; TPUFA: Total polyunsaturated fatty acids; n3PUFA: Omega-3 polyunsaturated fatty acids; n6PUFA: omega-6 polyunsaturated fatty acids; nd = not detected. Data presented as mean ± standard deviation; n = 3 fatty acid assays done in triplicate except for *T. sericea* where each value is a mean of assays done in duplicate. Samples of composite seed meals from the fruit of 10 trees for *K. africana*, *M. zeyheri* and *T. sericea*, respectively, and 20 trees for *X. caffra*.

2.5 Discussion

The discussion for the chapter focused on the proximate, mineral and fibre composition of the seeds viz potential as feed and food resources. Additionally it (discussion) also focused on the seed fatty acid content and potential as sources of industrial raw materials. Lastly, the anti-oxidant (vitamin E and squalene) concentration in the seed oils was discussed in comparison to other plant sources of anti-oxidants.

2.5.1 Proximate, mineral and fibre composition

K. africana, M. zeyheri, T. sericea and X. caffra seeds' lipid content (range: 21.3-49.2%) was comparable to that of *Glycine max* (Soyabean: 15-25%), *Gossipium hirstum* (cotton seed: 35-40%) and *Helianthus annuus* (sunflower seed: 35-40%), three of the major traditional oil seed crops (Cheftel and Cheftel, 1977; Manga et al., 2000). Oil derived from conventional oilseeds, for example, soyabean is used for culinary purposes and in the manufacture of thousands of industrial products (Fabiyi, 2006). In comparison to conventional oil seed crops, oil yield from the four tree seeds is high enough to warrant their possible commercial exploitation as available plant oil sources for the manufacture of industrial products. The industrial use of the oils from the trees would cover the cost of processing of seeds into feed ingredients.

M. zeyheri seed's CP content of 9.3% was low in comparison to the CP of legume seeds (major protein sources in feeds), but was comparable to the CP content of ordinary maize varieties, that, according to the Food and Agriculture Organization (FAO) (1992) range from 8–11%. The low CP content of *M. zeyheri* seed was in agreement with reported low CP content of *M. zeyheri* fruit pulp (Lemmens, 2005). Due to its low protein content, as is the case with ordinary maize grain, full-fat *M. zeyheri* seed cannot be used a protein source in the formulation of animal feeds. Even if *M. zeyheri* seed were to be defatted, its CP content is not likely to increase substantially considering that its seed had the least oil content. The shelled/dehulled full-fat seeds of *K. africana*, *T. sericea* and *X. caffra* (CP: 35.7, 46.2 and 18.2%, respectively) could potentially be used as protein sources in animal feeds. Sunflower seed (*Helianthus annuus*) and soyabean (*Glycine max*) are major protein concentrates used in the formulation of animal feeds. Solvent-extraction produced SBM accounts for 80% of the total protein supplements used in non-ruminant feeds (Herkelman and Cromwell, 1990). *K. africana* seed's CP content (35.7%) is higher than the 18.8% CP content reported for undefatted sunflower seed (Wahlstrom, 1985 as cited by FAO 1993) and the 26.4% CP in sunflower kernels (Sistach and Diaz, 1974 as cited by FAO 1993) as well as the 30.3% CP of raw cowpea (*Vigna unguiculata*) reported by FAO (1993). Although the CP content of full fat *K. africana* seed (35.7%) is lower than the 45% CP content of solvent-extraction produced SBM (FAO, 1993), the potential of *K. africana* as a protein concentrate in feed formulations is high considering that the seed has a high fat content. Defatting *K. africana* seed is likely to increase its CP

content to a value higher than the CP content of solvent extracted SBM. Full fat *T. sericea* seed's CP (46.2%) was comparable to the 45% CP solvent extracted SBM (FAO, 1993) thus making the seed a potential substitute and or compliment to SBM. Defatting *T. sericea* seed could result in a seed meal that has CP content much higher than that of SBM. Although the 18.3% CP in full fat *X. caffra* seed was lower than the CP content of SBM, it was comparable to the 18.8% CP content reported for undefatted sunflower seed (Wahlstrom, 1985 as cited by FAO 1993). Sunflower seed is used as one of the major protein sources for animal feeds in SSA. The potential of *X. caffra* seed as a protein source in animal feeds could be high considering that the seed had a high fat/oil yield (48.4%). Defatting *X. caffra* seed would most likely increase its CP content to a value much comparable to the CP content of solvent extracted SBM. Additionally, of the four tree seeds, *X. caffra* seed was the largest in terms of mass and size thus most likely its exploitation as a potential alternative protein source in feeds could be more economic.

Fagbenro, (1999) reported that maize grain has a gross energy value of 17 MJ kg^{-1} DM. Maize is one of the major sources of energy in feeds and foods, (Fagbenro, 1999). The gross energy values of the full-fat tree seeds (range: 24.34±0.56-32.1±0.04 MJ kg^{-1} DM) was 30-47% higher than that of maize grain. The high energy content of tree seeds opens up potential to their possible utilization as dietary energy sources, particularly in energy-dense livestock fattening diets. However, utilization of high lipid/oil content seeds such as the, *K. africana*, *T. sericea* and *X. caffra* seeds as dietary energy sources would require some degree

of defatting prior to seed meal utilization in order to guard against possible rancidity of the resultant feeds.

Soyabean, a major protein source in animal and humans feeds and foods, respectively, has a mineral content of content of 7.48% (Hadjipanayiotou and Economides, 2001). In comparison, the mineral (ash) content of *K. africana*, *M. zeyheri* and *X. caffra* seed, at about 5, 3 and 2%, respectively, was lower while that of *T. sericea* seed (6.89%) was comparable. However when comparing individual mineral elements, the tree seeds' calcium content except that for *X. caffra* seed was much higher than the 48.3±12.9 mg $100g^{-1}$ reported by the FAO (1992) for maize. Maize grain has a phosphorus content of 299.6±57.8 mg $100g^{-1}$ DM (FAO, 1992). While the phosphorus content of *M. zeyheri* seed at 107.9±9.40 mg $100g^{-1}$ was lower than the phosphorus content of maize, its content in the other three tree seeds (range: 345.45±5.94 to 1123.20±23.53 mg $100g^{-1}$) was higher compared to that of maize. While *K. africana*, *T. sericea* and *X. caffra* seed's magnesium content was higher (531.09±4.41, 560.70±6.68 and 207.90±5.94 mg $100g^{-1}$, respectively) compared to that of maize at 107.9±9 mg $100g^{-1}$ (FAO, 1992), *M. zeyheri* seed's magnesium content, 102.30±2.33 mg $100g^{-1}$ was comparable to that of maize. Thus, if used as a substitute to maize grain in feeds, *M. zeyheri* seed could provide adequate calcium. However, dietary supplementation with phosphorus would be required to make up for the low phosphorus content in the seed. *K. africana*, *T. sericea* and *X. caffra* seed if used to substitute maize in feeds could result in savings on the phosphorus component of the mineral supplement due their higher phosphorus content compared to that of maize grain. Similarly, use *K. africana*, *T. sericea* and *X. caffra* as energy

sources in place of maize could result in savings on the magnesium component of the mineral supplement due to the higher concentration of the mineral in the seeds.

Soyabean meal has a reported neutral detergent fibre (NDF) and acid detergent fibre (ADF) content of 15.74% and 10.70%, respectively (Hadjipanayiotou et al., 1985). The respective NDF and ADF content of maize grain is 10.80% and 2.8% (FAO, 1992). Compared to that of SBM, the NDF content of *K. africana* seed (13.38%) was slightly lower while the NDF content of *T. sericea* and *X. caffra* seed (22.33 and 21.30%, respectively) was moderately higher compared to that of SBM. The ADF content of *K. africana, T. sericea* and *X. caffra* seed (8.77, 9.03 and 5.12%, respectively) was lower compared to the ADF concentration in SBM. The fibre content of *M. zeyheri* seed (NDF and ADF: 33.2% and 15.3%, respectively) was higher compared to its concentration in maize and SBM. High fibre content usually limits inclusion levels of protein and energy sources in monogastric animal feeds (due to physiological limitation to digest highly fibrous feeds). The medium fibre content in *M. zeyheri* seed could be useful in providing the necessary bulk for the facilitation of normal gastrointestinal motility, while the relatively lower fibre content (NDF and ADF) of *K. africana, T. sericea* and *X. caffra* seed could mean that their use in monogastric animal feeds could be without challenges of the fibre content.

2.5.2 Amino acid content

When compared to the amino acid profile of soyabean (Kapsiotis, 1968; Cerny et al., 1971) the concentration of both non-essential and essential amino acids in *K. africana, M. zeyheri, T. sericea* and *X. caffra* seed was lower. In comparison to the amino acid profile of SBM, the individual amino acid concentration (essential and non-essential) in *K. africana, T. sericea and X. caffra* seed was in the range of 50-80%, 70-90% and 30-60%, respectively, of that in solvent extracted SBM (FAO, 1993). The concentration of the essential amino acids (EAAs) arginine, histidine, isoleucine, leucine, lysine and valine in solvent extracted SBM were reported to be 3.45%, 1.41%, 1.98%, 3.29%, 2.90%, and 2.15%, respectively (FAO, 1993). The respective concentrations of the corresponding EAAs in *K. africana, T. sericea* and *X. caffra* were generally lower compared to their concentration in SBM except arginine that was higher in *K. africana* and *T. sericea* seed. While full fat *T. sericea* seed could be a better source of the stated EAAs compared to SBM, based on the fact that diets are formulated from several ingredients, full fat *K. africana* seed could supply a significant amount of the EAAs in animal and human diets. The high oil/lipid content of full fat *X. caffra* seed (48.4%) could be masking the potential of *X. caffra* seed to supply EAAs. Defatting *X. caffra* seed to about 1% residual lipid could produce a meal whose CP content and hence both non-essential and EAAs concentration could be substantially higher and probably comparable to that of SBM. The EAAs content of *M. zeyheri* seed was also lower compared to that of the FAO/WHO reference protein (FAO, 1992). From a nutritional stand point, utilization of *M. zeyheri* seed

as a feed or food ingredient would require its supplementation with a protein concentrate to mitigate against protein malnutrition since it is inherently poor in both essential and non-essential amino acids. Solvent extracted *K. africana* and *X. caffra* seed meals could be used as protein sources in animal feeds with minimal supplementation with synthetic lysine and methionine.

K. africana and *T. sericea* seeds had a high concentration of arginine (5.43 ± 0.37 and 7.56±0.21 g $100g^{-1}$) compared to the 3.45% arginine in soyabean meal (CNPSA, 1991 as cited by FAO, 1993). *K. africana* and *T. sericea* seed, due their high concentration of arginine, when used as a food ingredient or supplement, could potentially increase the body's physiological pool of L-arginine, a physiological precursor of nitric oxide (NO), thus offering some degree of cardiovascular protection. An improvement in endothelium-dependent vasodilation subsequent to exogenous administration of the physiological precursor of NO (L-arginine) has been noted (Creager et al., 1992). L-arginine administration restored endothelium-dependent relaxation in hypercholesterolemic humans (Creager et al., 1992) and decreased aortic lesion formation in cholesterol-fed rabbits (Jeremy et al., 1996) all indicative of cardiovascular protection. However, the potential of *K. africana* and *T. sericea* seed to offer cardiovascular protection when used as food supplements requires further investigation.

2.5.3 Fatty-acid profile

The fatty acid profiles of the *K. africana*, *M. zeyheri*, *T. sericea* and *X. caffra* seed oils were interesting from a nutritional, health and industrial view point (Table 2.4). The OA content of the two batches of *M. zeyheri* seed oil was 54% and 85% for the Gokwe and Matopos National Park sourced *M. zeheri* seed, respectively. The OA content of the Matopos National Park sourced *M. zeyheri* seed was comparable to the 70-80% OA reported in *Sclerocarya birrea* (Marula tree) kernel oil (Burger et al., 1987) an IFBT that flourishes in the same eco-environment as *M. zeyheri* in southern Africa. Similarly, virgin olive oil contains 70-80% OA (Terès et al., 2008), thus making the *M. zeyheri* (Matopos National Park provenances) seed oil a comparable source of OA. *K. africana* and *T. sericea* seed oils had lower OA content compared to *M. zeyheri* seed oil and to that reported in *Sclerocarya birrea* kernel oil (Burger et al., 1987) and virgin olive oil (Terès et al., 2008). The *X. caffra* seed oil OA content (63%) was intermediate between that reported for Gokwe sourced *M. zeyheri* seed and *Sclerocary*a *birrea* kernel oils, respectively. The differences in the OA content of the Matopos National Park and Gokwe District sourced *M. zeyheri* seed could be probably due to differences in the microclimates (environmental effects) surrounding the plants (Ladipo et al., 1996). Plant micronutrient content is influenced by differences in the moisture content and nutrient status of the soils in which the plants grow (Ladipo et al., 1996).

Long term intake of olive oil with its high OA content has been reported to attenuate blood pressure and the risk of developing hypertension (Alonso and Martinez-Gonzàlez, 2004; Ruiz-Gutièrrez et al., 1996). At a molecular level OA and olive oil have been shown to regulate G protein-associated signalling both *in-vivo* (humans) and in cell culture (Yang et al., 2005; Alemany et al., 2004). The potential use of *K. africana*, *M. zeyheri*, *T. sericea* and *X. caffra* seed oil as alternative dietary sources of OA in the attenuation of hypertension and the development of cardiovascular disease requires further investigation. Additionally, oils with high oleic acid content have high oxidative stability which (high oxidative stability) is a vital characteristic of lubricants (Cahoon, 2003). The potential of *K. africana*, *M. zeyheri*, *T. sericea* and *X. caffra* seed oils as raw materials for industrial lubricants (due to their high OA content) needs further investigation. The possibility of the tree seed oils generating some useful industrial raw materials would justify the processing of the seeds into dietary ingredients (protein and energy concentrates) since the envisaged by-products would help cover the processing costs.

The EFAs (linoleic acid, α-linolenic acid and γ-linolenic acid) constituted about 67.83±0.91%, 22.47±1.12% and 69.10% of the *K. africana*, *M. zeyheri* (from Gokwe District) and *T. sericea* seed oil, respectively with α-linolenic acid as the most abundant EFA in the seed oils. No EFAs were detected in the *M. zeyheri* seed oil extracted from seeds sourced from Matopos National Park. The presence and absence of EFAs in the seed oils of the Gokwe and Matopos *M. zeyheri*

provenances, respectively, could be due to environmental differences that impact on seed nutrient (fatty acid) content (Ladipo et al., 1996).

Omega-3 polyunsaturated fatty acids (n-3 PUFA) play an important role in the reduction of plasma triglycerides, platelet aggregation, blood viscosity and pressure, and are known to have an inhibitory effect on the growth of prostate and breast cancer (Tample, 1996; Rose, 1997; Simopolous, 2000). Furthermore, n-3 PUFA are a pre-requisite for normal foetal brain and visual development (Neuringer *et al.*, 1998). The potential use of *K. africana*, *M. zeyheri* and *T. sericea* seed as dietary sources of the n-3 PUFA, for the aforementioned health benefits needs to be further investigated.

2.5.4 Vitamin E and squalene

Like vegetables, wild fruits and their seed are valuable sources of vitamins that are essential for the maintenance of good health (Saka et al., 1994). Vitamin E was detected (but not squalene) in all the seeds. All photosynthetic organisms synthesize vitamin E (Grusak, 1999; Sattler et al., 2004) thus the presence of the vitamin in *K. africana, M. zeyheri, T. sericea and X. caffra* seed was not surprising. Ladan et al. (2010) point out that *Helianthus annuus* (Sunflower) and *Sesamun indicum* (Sesame) seed, both commercial seed oil sources have a vitamin E concentration of 41.1 mg g^{-1} and 1.4 mg g^{-1}, in their respective seed oils;

concentrations that are much higher than the 0.50 to 48.70 µg g^{-1} vitamin E concentration reported for *K. africana, M. zeyheri, T. sericea and X. caffra* seed oils. Vitamin E, *in-vivo*, is reported to function as a recyclable chain reaction terminator of polyunsaturated fatty acid free radicals that are generated from endogenous oxidation of lipids (Kamal-Eldin and Appelqvist, 1996). The mopping up free radicals by vitamin E gives protection to membranes and cells against oxidative damage from the reactive oxygen species. *K. africana*, *M. zeyheri, T. sericea and X. caffra* seed due to the presence of vitamin E, albeit at a low concentration, could potentially, if used as dietary ingredients, increase the systemic antioxidant pool thus protecting the body against potential oxidative damage. Squalene, an isoprenoid antioxidant, found in large concentration in shark liver oils (Farvin et al., 2004), and also found in some plants (e.g. Amaranth seeds and olives) (Vázquez et al., 2007), is reported to protect cells and cell membranes against harmful effects of free radicals. However, none of the tree seed oils contained squalene.

2.6 Conclusion

This study established that *K. africana, T. sericea and X. caffra* seed are energy dense and have significant amounts of protein, phosphorus and lipids. Additionally, it was found that *K. africana, M. zeyheri* (Matopos National Park and Gokwe provenances) and *T. sericea* seed oils are rich in OA and the EFAs linoleic, α-linolenic and γ-linolenic acids. The Matopos National Park *M. zeyheri*

provenance was found not to contain EFAs in its seed oil. *K. africana, T. sericea and X. caffra* seed have potential as dietary protein and energy sources in feeds. The seed oils could be exploited as sources of OA, for potential use as health supplements and as raw materials for lubricants, and as sources of EFAs (n3-PUFA). *M. zeyheri* seed was found to have a low crude protein content and cannot be used as a potential protein concentrate in feeds but it could be exploited as an energy source in feeds and for its high oil yield and high OA content. Exploitation of *K. africana* seed as a protein source in feeds would however require the development of technical competence to dehull the seed. The tough *T. sericea* fruit stone made it difficult to determine its seed size as use of a side cutting pliers to extricate the seed led to the breakage of its seed into small pieces. Although *T. sericea* seed had the highest CP content, potential exploitation as a protein concentrate in feeds would be militated against by its very small seed size that makes its yield unviable economically.

Having determined the chemical composition of *K. africana* and *X. caffra* (alternative protein sources) and *M. zeyheri* seed (alternative energy source), it was imperative to undertake *in vitro* studies using established cell lines before *in vivo* studies. *In vitro* studies are a critical phase after chemical assessements in the evaluation of potential alternative feed and or food ingredients (protein and energy concentrates) since residual oils in seed cakes of some trees/crops have been found to contain toxic elements (Lai et al., 1990, Martínez-Herrera et al., 2006, Goel et al., 2007, Sirisha et al., 2008, Savanam and Bhaskara Rao, 2011). In animal and human nutrition studies the *in vitro* studies are designed and

undertaken to establish the safety of the test materials before their evaluation *in vivo*. The next chapter is a description of the *in vitro* study undertaken to evaluate the effect(s) of *K. africana*, *M. zeyheri* and *X. caffra* seed oils on Caco-2 and HEK-293 cells in culture.

CHAPTER 3: EFFECTS OF *KIGELIA AFRICANA, MIMUSOPS ZEYHERI* AND *XIMENIA CAFFRA* SEED OILS ON CELL VIABILITY

3.0 Introduction

Cell culture involves the dispersal of cells in an artificial environment that contains requisite nutrients and provides a surface to support the growth of the cells under ideal conditions (Biotecharticles, 2012). Ballard (1994) reported mammalian cell culture as a powerful tool in the investigation of biochemical mechanisms and regulatory processes. Cells in culture have been used to study, among other things, receptor-binding interactions, post-receptor reactions, and responses following transfection of foreign materials and transport of nutrients across membranes (Ballard, 1994). The need to determine the bioavailability, efficacy and possible toxicity of new drugs have been the major drivers in the pharmaceutical industry's interest in cell-based bioassays (Cheli and Baldi, 2011). In the feed/food industry cell-based bioassays are critical to research regarding the safety and efficacy of feed/food additives and new functional feeds and foods (Cheli and Baldi, 2011) and it is acknowledged that cultured cells have provided ground breaking information in nutrition (Ballard, 1994).

Traditional methods of determining nutritive value of alternative feed and food ingredients require animal and or human feeding trials (Glahn et al., 1998). These *in vivo* methods of feed evaluation are labour intensive, time consuming and are prone to ethical objections in some instances (Glahn et al., 1998). In contrast, cell-based bioassays (animal cell culture) are quick, less expensive, and highly repeatable. Additionally, cell culture studies help provide a sound framework

upon which *in vivo* studies can be undertaken (Glahn et al., 1998). In Europe *in vitro* cellular models as alternatives to animal studies have become a legislative issue (Cheli and Baldi, 2011).

3.1 Limitations and approaches in cell-based bioassays

Cells in culture contain the same genetic machinery and metabolic pathways characteristic of cells in *in vivo*. They are, however, dissociated to some extent from cell-cell interactions and totally from control exerted by tissues and organs one to another (Ballard, 1994). The dissociation, especially from organ and tissue control, results in cell-based (cell culture) bioassays' failure to completely mimic the *in vivo* conditions; a recognized limitation in the methodology. However cell culture models, for example, the *in vitro* digestion/Caco-2 model for divalent metal ion absorption, have been successfully utilized in nutritional studies (Glahn et al., 1996). Similarly the alternative tetrazoilum salt MTT-assay for cytotoxical determination of mycotoxins has been successfully used in toxicological evaluations (Mosmann, 1983).

In vitro cell culture methods can be employed in a two-tiered approach (Cheli and Baldi, 2011). The first is the simplistic approach whereby simple effects of a metabolite or compound of interest on cell viability and proliferation are assessed. Examples of the simple approach include the colorimetric cell culture assay using the tetrazolium salt MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium

bromide (Mosmann, 1983) and the trypan blue dye exclusion test (Paul, 1975) which are used to measure cell proliferation and survival. The second, a more complex approach, entails the elucidation of mechanisms of action for the compound of interest (Cheli and Baldi, 2011). Determination of cell proliferation and survival (simple cell-based approach) was used in the experiment described in this chapter.

3.2 IFBTs seeds and *in vitro* research

The major research focus on IFBTs has been highlighted in Chapter 2. *In vitro* research on IFBTs has largely focused on anti-bacterial, anti-fungal and anti-plasmodial activity (potential) of the tree root, stem bark and leaf extracts (Moshi and Mbwambo, 2005; Owolabi et al., 2007; Nyiligira et al., 2008). Seeds from IFBTs are largely discarded after utilisation of their fruit pulp despite their being potential sources of oils, essential oils, and EFAs with a multiplicity of possible applications. Very little if any research has been conducted targeting the biological effects of seed oils from IFBTs from SSA on established cell lines *in vitro*.

3.3 Justification: *in vitro* testing of IFBTs seed oils

One of the major objectives of my study was to test, *in vitro*, the biological effects of seed oils, from the IFBTs under study, on established cell lines. The outcomes of the *in vitro* study together with findings from the physico-chemical characterization would be used to select the most suitable IBFT seed and de-fat it and use its meal as a dietary substitute to SBM for the *in vivo* trials. Seed oils at times contain lipo-soluble toxic compounds such as the tumour-promoting phorbol esters found in *Jatropha curcas* seed oil (Goel et al., 2007), the toxic gossypol found in crude cotton (*Gossypium hirsutum*) seed oil (Savanam and Bhaskara Rao, 2011) and the azadirachtin (compound with pesticidal activity) and lead laden encephalopathy-causing Neem seed oil (Lai et al., 1990). Despite its high crude protein content, use of phorbol ester-tainted *Jatropha curcas* seed cake has resulted in toxicity in livestock and poultry (Martínez-Herrera et al., 2006, Sirisha et al., 2008). Thus some oil seed cakes; both conventional and non-conventional; have to be detoxified if their nutrient potential is to be harnessed. The possibility of having lipo-soluble toxins in residual oils in potential (non-conventional) seed cakes that could cause toxicity demands that *in vitro* assessment, using cells in culture, of the oils from the seeds be executed before undertaking *in vivo* trials.

Biological characterization of oils using cells in culture has largely focused on animal-derived oils and purified fatty acids with little if any investigations being

carried out with seed oils from IFBTs. Given their natural content seeds of IFBTs could be used to generate non-conventional feed ingredients in the form of oil seed cakes and or meals. However due to their high oil content the oil would have to be extracted and there is usually residual oil in the seed cake after oil extraction which could cause deleterious effects on animals if the seed cakes are used as feed ingredients. A preliminary screening study of *K. africana*, *M. zeyheri* and *X. caffra* seed oils *in vitro* using human colon adenocarcinoma (Caco-2), a common immortalised gastrointestinal cell line, and Human Embryonic Kidney (HEK-293), an epithelial immortalised cell line was undertaken in order to establish the safety of testing *in vivo* the potential of oil cakes from the three IFBTs. Caco-2 cells are known to form tight junctions (Rousset, 1986, Rousset et al., 1985) and express structural properties similar to those of ileal enterocytes (Vachon and Beaulieu, 1992) hence they are useful models as they mimic the gastrointestinal tract. The HEK-293 cell line has been used extensively (Durocher et al., 2002, Schlaeger and Christensen, 1999) as a laboratory work horse in cell culture studies.

3.4 Study objective

The specific objective of the study was to test *in vitro* the biological effects of *K. africana*, *M. zeyheri* and *X. caffra* seed oils on the viability and proliferation of Caco-2 and HEK-293) in culture and then select on the basis of the physical and chemical traits and *in vitro* evaluations, the most suitable seed; de-fat it and use its meal as a dietary substitute to SBM for the two *in vivo* trials. *T. sericea* seed oil was not considered for the *in vitro* studies for reasons discussed in Chapter 2.

3.5 Materials and methods

3.5.1 Seed source and oil extraction

The sources of the IFBTs seeds (*K. africana*, *M. zeyheri* and *X. caffra*) have been described in Chapter 2. The IFBTs seed oils used in the *in vitro* study were extracted from the respective seeds as described in Chapter 2.

3.5.2 Fatty acid profile determination

The fatty acid profiles of the seed oils used in the *in vitro* study were determined as described in Chapter 2.

3.5.3 Cell culture

The Caco-2 and HEK-293 cell lines used in the study as well Dulbecco's Modified Eagle's Medium (DMEM) and the foetal bovine serum (FBS) used to maintain the two cell lines were obtained from Highveld Biologicals (Pty) Ltd., Johannesburg, South Africa. Each cell line was grown in DMEM containing 10% FBS (v/v) in 75 cm^2 flasks and incubated in an Autoflow IR-Water-Jacketed CO_2 Incubator [Lasec SA (Pty) Ltd, Johannesburg, South Africa] under 10% CO_2 at 37^0C. To maintain the characteristics of each cell line, cells were passaged (inside an ESCO Class II Biosafety Cabinet; ESCO, Johannesburg, South Africa) at less than 80% confluent and put in fresh DMEM containing 10% FBS.

3.5.4 Oil treatment and viability determination

Prior to dosing of the cells with the seed oils, any contaminants in the oils were removed by microfiltration using Millipore HA 0.45 µm filters (Millipore Corporation, Massachusetts, Bedford, USA). The micro-filtered seed oils were solubilised in 99.5% ethanol (Merck Chemicals, Johannesburg, South Africa). A pilot study (at the maximum solubilisation dose) indicated that the ethanol itself at that concentration had no effect on cell viability (data not shown). The seed oil/ethanol solutions were prepared to give final oil concentrations per culture plate well of 0, 20, 40, 80, 100 and 120 mg l^{-1} of culture medium. Ethanol (99.5% purity) was used in the control wells at a concentration of 120 mg.l^{-1}. The cells

were detached (lifted) from the flasks through the addition of phosphate buffered saline containing 1mM EDTA. EDTA is a calcium chelator that is used to detach cells (Öberg et al., 2011).

Cells in medium were seeded into 24 well tissue culture plates at 1×10^5 cells per well followed by addition of 1ml of medium (DMEM containing 10% FBS), and then incubated in an Autoflow IR-Water-Jacketed CO_2 Incubator [Lasec SA (Pty) Ltd, Johannesburg, South Africa] under 10% CO_2 at 37ºC for 24 hours to recover prior to treatment (dosing) with the oils. Following the 24-hour recovery period, cells in each well were then individually dosed, inside an ESCO Class II Biosafety Cabinet (ESCO, Johannesburg, South Africa), with the appropriate tree seed oil (treatments) at the requisite concentrations. Each treatment dose of the respective seed oils was replicated three times for each cell line. After application of the oils, the cells were incubated for 48 hours (Giangregorio, 1992; Girao, 1988). The Trypan Blue exclusion method (Paul, 1975) was then used to assess cell viability.

3.5.5 Statistical analysis

A two-way analysis of variance using the GraphPad Prism 5 for Windows statistical package (GraphPad Software Inc., San Diego, USA) was performed. The Bonferroni's *post hoc* test was used to compare the effects of each tree seed

oil on the two cell lines at the same oil concentration level and also compared to the control. The model used in the analysis of variance was:

$Y_{ijk} = \mu + T_i + C_j + W_k + e_{ijk}$; where;

Y_{ijk} = response variable of interest (cell proliferation, cell death),

μ = overall mean to all observations,

T_i = fixed effect of the i^{th} oil treatment (i = 1, 2...6),

W_k = fixed effect of the k^{th} cell type (k = 1,2) and

e_{ijk} = random residual error.

3.6 Results

The number of non-viable (dead) cells between treatments and in comparison to the control was low, ranging from 0 to 2 dead cells per count per treatment across treatments. More importantly there were no statistically significant differences in the number of dead cells across treatments compared to the control, an indication that the oils did not cause cell death.

Figure 3.1 shows the effect of *K. africana* seed oil on Caco-2 and HEK-293 cell proliferation compared to the control.

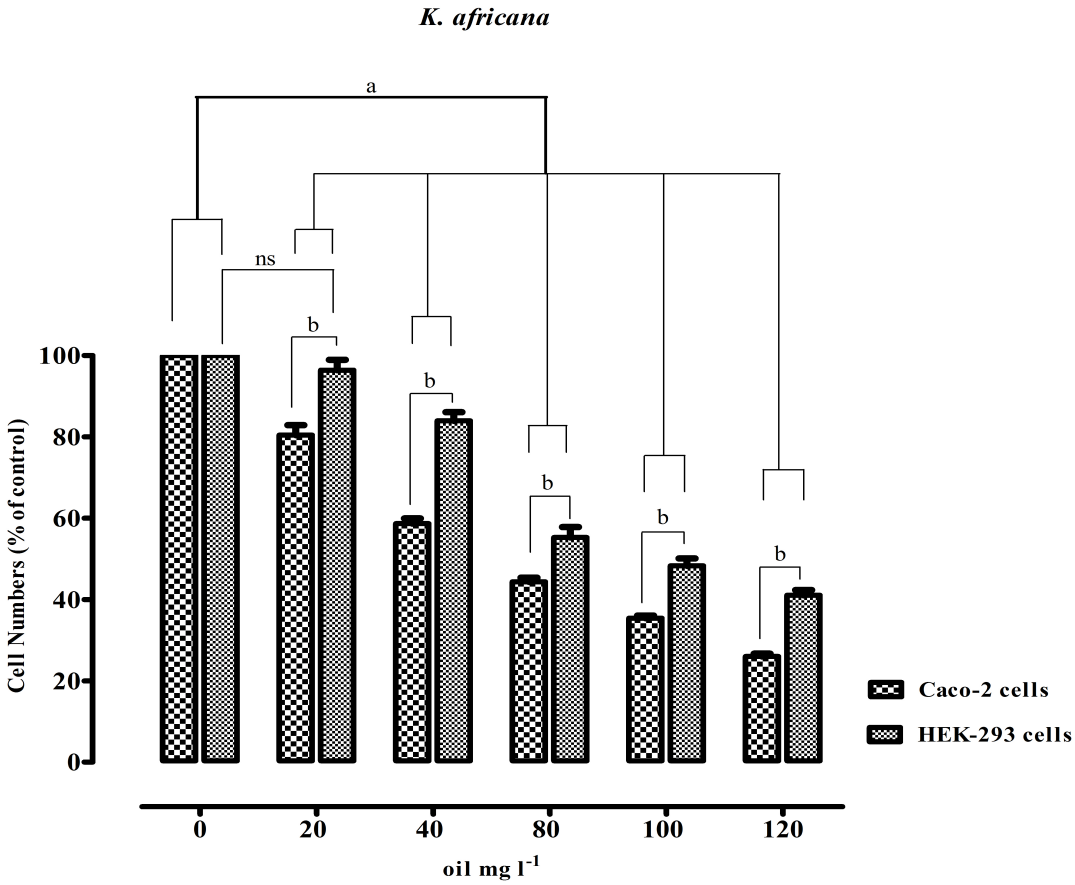


Figure 3.1: Comparison of the effects of *K. africana* oil on Caco-2 and HEK-293 cells.

1 x 10^5 cells were plated per well and treated with the indicated oil concentrations. The cells were counted using the Trypan Blue Dye Exclusion Method as described under materials and methods. Cell numbers (percent of control) for the Caco-2 and HEK-293 cells are shown for each oil concentration. The assays were done in triplicate. [ns]Non-significant differences at $P>0.05$. [a]*K. africana* oil significantly ($P<0.05$) suppressed both Caco-2 and HEK-293 cell growth at all oil concentrations compared to control. [b]*K. africana* oil significantly ($P<0.05$) suppressed Caco-2 cell growth compared to HEK-293 cell growth at all oil concentrations.

K. africana seed oil significantly ($P<0.05$) suppressed Caco-2 and HEK-293 cell growth at all oil concentrations compared to control (Figure 3.1). While there were no significant differences ($P>0.05$) in HEK-293 cell growth between the control and cells treated with *K. africana* seed oil at 20 mg l^{-1}, *K. africana* seed oil significantly ($P<0.05$) suppressed HEK-293 cell growth at higher doses and it suppressed Caco-2 cell growth more compared to HEK-293 cell growth at all levels of the *K. africana* oil concentration (Figure 3.1).

Figure 3.2 shows the effect of *M. zeyheri* seed oil on Caco-2 and HEK-293 cell proliferation compared to the control.

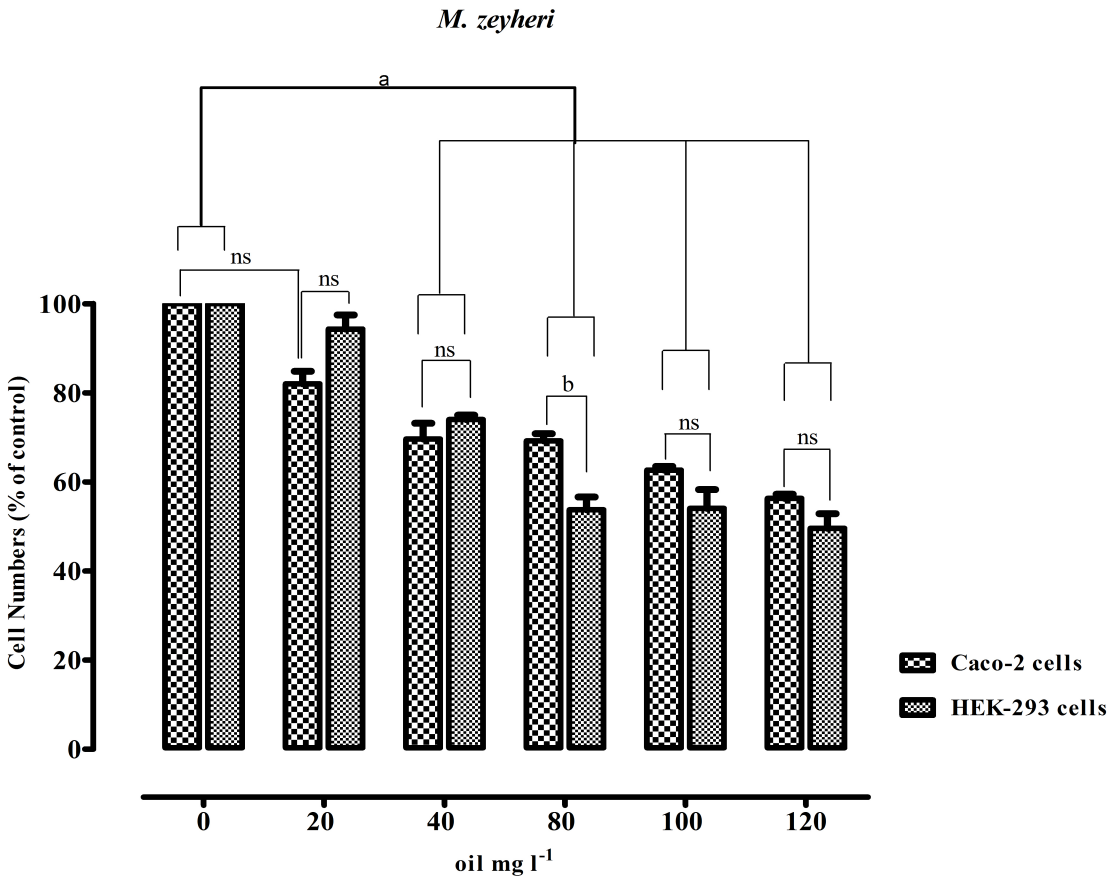


Figure 3.2: Comparison of the effects of *M. zeyheri* seed oil on Caco-2 and HEK-293 cells.

1 x 10^5 cells were plated per well and treated with the indicated oil concentrations. The cells were counted using the Trypan Blue Dye Exclusion Method as described under materials and methods. Cell numbers (percent of control) for the Caco-2 and HEK-293 cells are shown for each oil concentration. The assays were done in triplicate. ^{ns}Non-significant differences with $P>0.05$. ^{a}*M. zeyheri* oil significantly ($P<0.05$) suppressed both Caco-2 and HEK-293 cell growth at oil concentrations $\geq$ 40 mg l^{-1}. ^{b}*M. Zeyheri* oil significantly ($P<0.05$) suppressed HEK-293 cell growth compared to Caco-2 cell growth at 80 mg l^{-1} oil concentration.

M. zeyheri seed oil significantly suppressed both Caco-2 and HEK-293 cell growth at oil concentrations greater or equal to 40 mg l^{-1} compared to control (Figure 3.2). The degree of suppression of the growth of the two cell lines by *M. zeyheri* seed oil was similar at all concentrations except at a concentration of 80 mg l^{-1} where it significantly suppressed HEK-293 cell growth compared to Caco-2 cell growth (Figure 3.2).

Figure 3.3 shows the effect of *X. caffra* seed oil on Caco-2 and HEK-293 cell proliferation compared to the control.

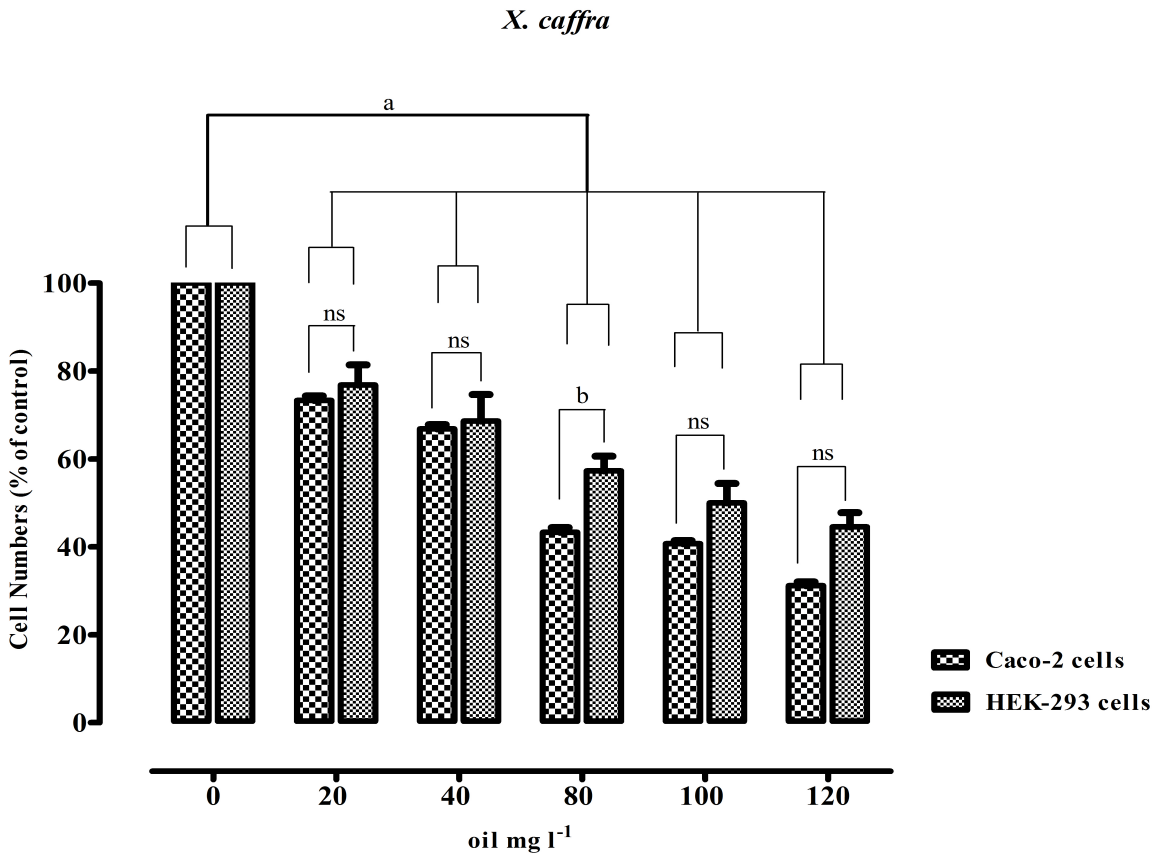


Figure 3.3: Comparison of the effects of *X. caffra* oil on Caco-2 and HEK-293 cells

1 x 10^5 cells were plated per well and treated with the indicated oil concentrations. The cells were counted using the Trypan Blue Dye Exclusion Method as described under materials and methods. Cell numbers (percent of control) for the Caco-2 and HEK-293 cells are shown for each oil concentration. The assays were done in triplicate. [ns]Non-significant differences at $P>0.05$. [a]*X. caffra* oil significantly ($P<0.05$) suppressed both Caco-2 and HEK-293 cell growth at all oil concentrations compared to control. [b]*X. caffra* oil at 80 mg l^{-1} concentration significantly ($P<0.05$) suppressed Caco-2 cell growth compared to HEK-293 cell growth.

X. caffra seed oil significantly ($P<0.05$) suppressed Caco-2 and HEK-293 cell growth at all the *X. caffra* seed oil concentrations used compared to control (Figure 3.3). There were no significant differences between Caco-2 and HEK-293 cell growth at all the *X. caffra* seed oil concentrations, other than at 80 mg l^{-1} where the *X. caffra* seed oil, unlike *K. africana* and *M. zeyheri*, significantly ($P<0.05$) suppressed Caco-2 cell growth compared to HEK-293 cell growth (Figure 3.3).

The growth of both Caco-2 and HEK-293 cells showed an overall trend of decreasing with an increasing seed oil concentration (Figures 3.1-3.3).

3.7 Discussion

The three IFBTs seed oils consistently suppressed the growth of the two cell lines (Caco-2 and HEK-293) in a seemingly dose-dependent manner. In a similar study using shark liver oils, Davidson et al. (2007) reported a trend towards growth inhibition of Caco-2 cells treated with liver oils from the great white and spinner shark species and attributed the trend to the ratio of n3 to n6 (n3:n6) polyunsaturated fatty acids of the shark liver oils. The ratio of n3:n6 polyunsaturated fatty acids (4.13) for *K. africana* seed oil falls midway between the n3:n6 polyunsaturated fatty acid ratio for the great white (9.74) and spinner (2.62), shark liver oils, (Davidson et al., 2007) while the 0.02 n3:n6 ratio of *M.*

zeyheri seed oil is lower compared to that observed in the shark liver oils. The observed Caco-2 cell growth suppression trend cannot be adequately explained by the n3:n6 ratio of the tree seed oils, since *M. zeyheri* seed oil with an n3:n6 polyunsaturated fatty acid ratio outside the observed ratios in shark liver oils also resulted in a suppression of Caco-2 cell growth.

In animal models, fish oil, a rich source of eicosapentaenoic acid (EPA), an n3-polyunsaturated fatty acid, has been reported to reduce the induction of colorectal cancer by mechanisms that are thought to involve suppression of mitosis and increased apoptosis (Clarke et al., 1999). In culture, EPA at 10 and 15µg ml^{-1} reduced the growth rate of adherent HT29 cells, a human colonic adenocarcinoma cell line (Clark et al., 1999). *K. africana*, *M. zeyheri* and *X. caffra* seed oils, did not contain EPA but contained α-linolenic acid, an n3-polyunsaturated fatty acid that could have similar effects on the cells as EPA. In cells, α-linolenic acid is a substrate for longer chain more unsaturated n-3 fatty acids including EPA and docosahexaenoic acid (Burdge, 2006) thus the α-linolenic acid from the tree seed oils could have been metabolised to EPA (in the cells) that then could have led to suppressed Caco-2 and HEK-293 cell growth. Dommels et al. (2002) reported growth inhibition of Caco-2 cells on incubation with linoleic acid and α-linolenic acid. All three tree seed oils contained α-linolenic acid whilst only *K. africana* and *M. zeyheri* also contained linoleic acid. Although Dommels et al. (2002) made use of purified linoleic and α-linolenic acid in their study that demonstrated growth inhibitory effects on Caco-2 cells, the presence, in large concentrations, of linoleic and α-linolenic acid in the seed oils could account for the suppression of both

Caco-2 and HEK-293 cell growth in a dose-dependent manner. The Caco-2 cells over and above forming tight junctions (Rousset, 1986, Rousset et al., 1985) and expressing structural properties similar to those of ileal enterocytes (Vachon and Beaulieu, 1992) also express various levels of transporters on their cell membranes (Mandagere et al., 2002). In this study, the depressed Caco-2 cell growth with the increase in tree seed oil concentration (*K. africana*, *M. zeyheri* and *X. caffra* respectively) could have resulted from a possible alteration of the cells' membrane traits by the oils. Alteration of the cell membrane characteristics could lead to interference and or partial blockage of channels that are responsible for nutrient uptake and excretion of metabolic wastes by the cells (Kirk and Strange, 1998) thus leading to subnormal levels of nutrient uptake and abnormal waste accumulation; conditions that might lead to growth suppression.

The trypan blue dye exclusion method, though a rapid and easy method of measuring cell growth and viability has got shortcomings. The method indirectly measures viability from cell membrane integrity, hence it is possible to have a cell whose viability has been compromised in its ability to grow and or function yet its membrane integrity can be transiently maintained. There is also the possibility that cell membrane integrity could be seen as compromised (by letting the dye into the cell), but the method fails to cater for cells whose membranes (seemingly compromised) repair themselves and become viable. It has to be pointed out, however, that the focus of this study was to do a preliminary determination of the effects of the three tree seed oils on cell proliferation as a basis for further in depth studies. In addition, as previously mentioned, there was the possibility that the oils

altered membrane structure and so the trypan blue dye exclusion method would be useful in detecting these changes of membrane fluidity and permeability.

3.8 Conclusion

The suppression of cell proliferation by the tree seed oils strongly indicated anti-proliferative effects of the *K. africana, M. zeyheri* and *X. caffra* seed oils on the two cell lines *in vitro*. Further work focusing on the measurement of DNA synthesis and metabolic profiles of treated cells could assist in the delineation of mechanisms responsible for the observed growth suppression.

Differences in cell dynamics occur *in vivo* compared to *in vitro* (Ballard, 1994). Due to the potential differences in cell behaviour *in vitro* compared to *in vi*vo, it is not plausible to extrapolate the observed anti-proliferative effects of the seed oils observed *in vitro* to anti-proliferative effects of the oils on gastrointestinal mucosal cells *in vivo* should the tree seed cakes, with residual oils, be used as feed ingredients. The fact that the seed oils did not cause cell death means that the oils were not toxic and therefore use of the seed cakes, with residual oils, is not likely to cause deleterious effects in animals.

X. caffra seed oil's lack of toxicity to cells *in vitro* justified further evaluation of the potential of defatted *X. caffra* seed meal as a protein source in feeds *in vivo*.

The seed (*X. caffra)* was found to be easy to shell (Chapter 2). Additionally, chemical analyses had revealed (Chapter 2) the potential of *X. caffra* seed as an alternative protein source in feeds and foods. Despite the chemical potential (Chapter 2) and the lack of toxicity of its seed oil to cells *in vitro* (Chapter 3), the current lack of appropriate technology to dehull *K. africana* seed militated against its evaluation *in vivo*. Chapter four is a description of the first set of *in vivo* experiments where the effects of substituting SBM with defatted *X. caffra* seed meal on apparent nutrient digestibility and nitrogen balance were investigated.

CHAPTER 4: *XIMENIA CAFFRA* SEED MEAL AS A DIETARY PROTEIN SOURCE: EFFECTS ON NUTRIENT DIGESTIBILITY AND NITROGEN BALANCE IN SPRAGUE DAWLEY RATS

4.0 Introduction

The scarcity and seasonality of feed availability, high feed costs and competition for feed ingredients between man and livestock are key constraints to intensified animal production in SSA. Optimal livestock production levels hinge on providing high quality nutrition. The major target when feeding animals is to be able to supply the requisite amount of nutrients to meet the productive and reproductive requirements of different classes of animals. Animal-dependent factors such as age, (pre-weaning, brooding, post-weaning, post-brooding, fattening/finishing) and physiological status (e.g. pregnant, pregnant and lactating, dry and open, laying and moulting) influence nutrient requirements and proportions of nutrients for optimal production levels at the different animal ages and physiological states.

The chemical composition of feed ingredients has a major impact on animal performance. Whilst the knowledge of the chemical composition of feed ingredients is of value, by itself is not very useful since it does not always translate into increased bioavailability of nutrients and a concomitant improvement in animal performance. Feed ingredients when ingested by animals interact in a manner that either increases nutrient availability (positive associative effective) or in a way that reduces nutrient availability (negative associative effects), for example, tannins and phytic acid cause negative associative effects in animal feeds: they complex with dietary protein and digestive enzymes in the GIT

negatively impacting on protein digestion and its availability (Waghorn, 2008; Reyden and Selvendran, 1993). Kumar et al. (2002) reported that combining mustard seed cake and SBM (50:50) as protein sources resulted in positive associative effects on growth in lambs than when either of the protein source was used individually.

4.1 Approaches to feed evaluation

Feed evaluation is generally undertaken both to assess and compare the value between feeds and or feed ingredients and their effect on production levels (Ribeiro and Moreira, 1998). Methods employed in the assessment of feed value are designed in such a manner that they mimic the effect of digestion on a feedstuff (Ribeiro and Moreira, 1998). Chemical, enzymatic and biological approaches constitute the major methods used in feed evaluation. Despite their simulation of the digestive process, each of the major feed evaluation methods comes with some margin of error.

4.1.1 Chemical methods of feed evaluation

Chemical methods, for example, the proximate analysis (McDonald et al., 2002), fibre and modified fibre assays (Van Soest, 1967; Van Soest et al., 1991), nuclear magnetic resonance and near infrared reflectance spectroscopy (Givens and

Deaville, 1999), generally quantify the different nutrients in a feed. Compared to enzymatic and biological methods, chemical methods of feed evaluation, though they give useful data for predicting feed value, are far removed from what happens *in vivo* thus they are less reliable (Ribeiro and Moreira, 1998).

4.1.2 Enzymatic methods of feed evaluation

Enzymatic methods of feed evaluation make use of synthetic enzymatic preparations (Donefer et al., 1963), enzyme-bearing GIT secretions, for example pancreatic extracts and rumen liquor containing microbial digestive enzymes (McDonald et al., 2002). Although developed largely to assess forage feed values, enzymatic methods have also been applied in the determination of the feed value of mixed feeds and concentrates (Ribeiro and Moreira, 1998). Enzymatic methods of feed evaluation have some advantages: they are not time consuming and are relatively less costly compared to reference *in vivo* methods (Ribeiro and Moreira, 1998), have a high degree of accuracy due to their repeatability and reproducibility (Aufrère and Guérin, 1996) and have a higher feed value predictive capability when compared to chemical methods.

4.1.3 Biological methods of feed evaluation

Functionally, biological methods of feed evaluation were designed to represent and mimic a part of or a series of parts of the GIT and the digestive and absorptive processes in animals (Ribeiro and Moreira, 1998). Although all different biological approaches generate useful information, their applicability is situation specific. The key biological methods include the *in vitro* two stage technique (Tilley and Terry, 1963), the *in situ* technique (Meherez and Ørskov, 1977, Ørskov and McDonald, 1979), the *in vitro* gas production (Menke and Steingass, 1988) and *in vivo* digestibility trials. With the exception of the *in vivo* digestibility trials, the other biological methods though giving a good prediction of the feed value, are limited by their failure to completely mimic the dynamics of the GIT that include digesta movement, pH changes, secretion and absorption.

Determination of feed quality using *in vitro* methods is of essence to animal nutritionists: it has advantages, among others, of allowing more control of the experimental conditions compared to *in vivo* determination. It is less expensive (no need to keep and feed large numbers of animals), less labour intensive and takes relatively less time to get results (Makkar, 2005). Additionally, *in vitro* techniques allow animal nutritionists to execute preliminary screening tests for potential feed materials such as breeder's and foundation seeds that are not yet produced commercially. The syringe-based *in vitro* gas method (Menke et al., 1979) is a more suited tool to evaluate feedstuffs in the less resourced developing

countries because it is less capital intensive (Makkar, 2004). It is recommended that data generated from in *vitro gas* methods be validated against *in vivo* nutrient utilization and animal performance (Makkar, 2005).

In vivo digestibility trials give a better prediction of the feed value as a result of the integration of digestion and absorption; two critical processes that occur in the GIT. The *in vivo* digestibility trials also allow for the measurement of nitrogen (N) balance. The latter is important in the assessment of nutritional response and is a useful tool in the evaluation of the adequacy of protein intake and determination of protein requirements. *In vivo* digestibility trials are however saddled with some criticisms (McDonald et al., 2002). Scientific technological advancements have made it possible, albeit at an increased cost, to eliminate most of the criticisms associated with basic digestibility trials by making it possible to generate true as opposed to apparent digestibility coefficients of feeds.

4.2 Study justification: test chemical and *in vitro* potential *in vivo*

X. caffra seed's potential as a protein source in feeds has been partially demonstrated by chemical analyses, a relatively viable seed yield, ease of processing (Chapter 2) and lack of toxicity of its oil on cells *in vitro* (Chapter 3). However, as discussed earlier, chemical analyses and *in vitro* studies do not accurately predict the nutritional value of feeds. Additionally, in view of the constraints imposed on intensification of livestock production in SSA as a result of shortages and high cost of feed ingredients, particularly protein and the

possibility of using *X. caffra* seed as a non-conventional protein source in feeds, it thus became imperative to determine, *in vivo*, the feed (protein) value of solvent extracted *X. caffra* seed meal as a substitute to SBM on apparent nutrient digestibility and nitrogen balance.

4.3 Study objectives

The specific objectives of the study were to:

i. determine *in vivo*, the effects, in mature male Sprague Dawley rats, of dietary substitution of SBM with defatted *X. caffra* seed meal (following the selection of *X. caffra* seed as the most appropriate protein source) on apparent nutrient (DM, OM, CP and GE) digestibility.

ii. determine *in vivo*, the effects, in mature male Sprague Dawley rats, of dietary substitution of SBM with defatted *X. caffra* seed meal (following the selection of *X. caffra* seed as the most appropriate protein source) on nitrogen balance.

4.4 Materials and methods

4.4.1 *X. caffra* seed: source, preliminary processing and importation

Sourcing, preliminary processing and importation of *X. caffra* seed used to in the *in vivo* trials was described in Chapter 2.

4.4.2 Defatting of *X. caffra* kernels

The dry shelled *X. caffra* kernels were defatted at the Centre for Scientific and Industrial Research's Biosciences Modderfontein Experiment Station, Kempton Park, Johannesburg, Republic of South Africa. Briefly, the *X. caffra* kernels (34 kg) were pressed in 500 g aliquot portions at a pressure 25 000 kPa for 12.5 minutes using a hydraulic press. The pressed solid paste was divided into three feed batches. Each feed batch (pressed *X. caffra* kernel paste) and hexane (96%), at a ratio of 4:1 (hexane: solid paste) was then charged into a 60L reactor and stirred for 1 hour at room temperature. This was followed by centrifugation of the mixture for 30 minutes to separate the solid and liquid components. Recovered "solids" were subjected to an additional 5 cycles of extraction/centrifugation. On completing the 6^{th} extraction, the defatted *X. caffra* seed meal (XCSM) (solids) was air dried under shade. The dry XCSM was then put in hessian bags and stored in dark cupboard prior to chemical analyses and subsequent use for diet

formulation. The recovered oil/hexane mixtures were pooled and the hexane was removed using a Buchi rotary evaporator (BUCHI Labortechnik AG, Flawil, Switzerland) at 65ºC.

4.4.3 Proximate, mineral and fibre analyses of defatted *X. caffra* seed meal

Prior to use in the formulation of the experimental diets, proximate components DM (method number 934.04), CP (method number 954.01, EE (method number 920.39), ash (method number 942.05) and CF, were determine as outline by AOAC (2005). The minerals calcium, magnesium and phosphorus, were assayed as described by Zasoski and Burau (1977). The fibre fraction (NDF and ADF), inclusive of ash, of the defatted XCSM was determined as decribed byVan Soest et al. (1991). Gross energy (GE) value of the defatted XCSM was determined using an MC-1000 Modular Calorimeter equipped (Energy Instrumentation, Centurion, South Africa) with a PC and MC1000 software. The chemical assays on the defatted XCSM were performed at the Agricultural Research Council's Irene Analytical Services Laboratories, Irene, South Africa.

4.4.4 Feed ingredients, diet formulation and diets

Yellow maize, wheat bran, gluten feed and feed-grade limestone were supplied by Optic-Feeds Private Limited, Lichtenburg, South Africa; the vitamin-mineral premix was supplied by ADVIT Animal Nutrition, Johannesburg, South Africa, tallow was supplied by Energy Oil, Johannesburg, South Africa and brewers' yeast was sourced from Anchor Yeast, Gweru, Zimbabwe. The Least cost feed/ration formulation software, WinFeed, was used in the formaulation of the diets. The control and experimental diets were formulated in such a manner that they met the National Research Council (NRC) requirements for rats (NRC, 1995). The control diet (Diet 1) was SBM based with the test diets formulated in such a manner that XCSM substituted SBM on a crude protein basis to generate iso-calorific and iso-nitrogenous diets. Ingredient and chemical composition of the diets is shown in Tables 4.1.

Table 4.1: Ingredient and chemical composition of the control and test diets

	Diet 1	**Diet 2**	**Diet 3**	**Diet 4**	**Diet 5**
Ingredients (g kg^{-1})					
Maize meal	614.0	607.5	600.5	593.5	587.0
Wheat bran	10.0	10.0	10.0	10.0	10.0
Gluten feed	71.5	71.5	71.5	71.5	71.5
Tallow	20.5	20.5	20.5	20.5	20.5
SBM	164.0	122.5	82.0	41.0	0.00
XCSM	0.00	48	95.5	143.5	191.0
	(0)	(25)	(50)	(75)	(100)
[1]Yeast	102.5	102.5	102.5	102.5	102.5
[2]Limestone	13.5	13.5	13.5	13.5	13.5
Salt	2.0	2.0	2.0	2.0	2.0
[3]Premix	2.0	2.0	2.0	2.0	2.0
Total	**1000**	**1000**	**1000**	**1000**	**1000**

Table 4.1 continued

Chemical composition					
DM (%)	89.87	89.90	89.99	90.05	90.24
OM (% DM)	85.61	85.52	85.58	85.91	86.33
*CP (% DM)	18.55	18.74	18.25	18.60	18.18
EE (% DM)	4.47	4.93	4.61	4.99	4.71
Ash (% DM)	4.26	4.38	4.41	4.14	3.91
Ca (% DM)	0.59	0.58	0.55	0.52	0.57
P (% DM)	0.43	0.43	0.39	0.44	0.44
Energy (MJ kg^{-1}DM)					
**GE	15.78	15.62	15.67	15.61	16.13

Figures in parentheses indicate the percentage substitution of SBM CP by XCSM CP; [1]Brewers' yeast; *Diets iso-nitrogenous; **Diets iso-energetic. DM: dry matter; OM: organic matter, CP: crude protein; EE: ether extract; Ca: calcium; P: phosphorus; GE: gross energy; SBM: soyabean meal; XCSM: defatted *X. caffra* seed meal; [2]Feed grade limestone; [3]Vitamin-mineral premix: each kg contained vit A 30 000 000 IU, vit D3 166 667 IU, vit E 20 500 IU; vit K3 1.67 g, vit B_1 5.67 g, vit B_2 3.33 g, Niacin 15 g, Calcium pantothenate 6.67 g, vit B_{12} 0.01 g, vit B6 2.5 g, Choline 333.33 g, Folic acid 0.33 g, Biotin 0.04 g, Rovimix Stay C 10.83 g, $MnSO_4$ 10 g, Zn 10 g, Cu 2.33 g, KI 0.17 g, $FeSO_4$ 41.67 g, Se 0.05 g.

4.5 Animal ethical clearance and study site

The study, conducted in the Central Animal Services Unit (CAS) Animal Unit at the Faculty of Health Sciences, University of the Witwatersrand, was approved by the Animal Ethics Screening Committee (AESC) of the University of the Witwatersrand, Johannesburg, South Africa (AESC number: 2008/46/01; see appendix 1).

4.5.1 Animals and experimental design

Nineteen (19) mature male Sprague Dawley rats with a mean body mass of 287.32±12.14 g were used. The rats were randomly assigned to the five dietary treatments [Diet 1 to Diet 5 (D1to D5]). The control diet (D1) was replicated on three occasions while the test diets (D2 through to D5) were each replicated four times.

4.5.2 Animal housing and feeding

Each rat was individually penned in a polyethylene metabolism crate. The metabolism crates (Tecniplast S.p.A., Buguggiate, Italy) were designed in a manner that allows for separation of urine and faeces to facilitate clean sample

(faeces and urine) collection. The rats had *ad libitum* access to drinking water and were fed at a rate of 87.01 g kg^{-1} body weight per day. Lighting was set on a 12-hour light-dark cycle with lights on from 0700 hours. The ambient temperature was set at 22±2ºC.

4.5.3 Animal adaptation

The experimental period lasted for 17 days. The rats were adapted to the experimental conditions (metabolism crate environment and the dietary treatments) for 12-days before commencement of total sample (faeces and urine) collection. During the adaptation period, faecal and urine samples from each rat were removed (but not quantified) and disposed of on a daily basis. On the last day of the adaptation period, each metabolism crate was cleaned and the rats were returned to their respective crates. Fresh feed was weighed and offered to each rat on the basis of its dietary treatment group.

Figure 4.1 below shows the metabolism crates designed to facilitate clean urine and faecal sample collection during the digestibility and nitrogen balance trial.

Figure 4.1: **Photograph of metabolism crates set up during the digestibility and nitrogen balance trial.**

4.5.4 Faeces and urine sample collection and storage

After the 12-day adaptation period, measurements of feed offered, refusals, faeces and urine sample collection were done daily and continued for 5 days. Each rat was weighed using an electronic balance [Snowrex Electronic Scales, (PTY), Ltd., Johannesburg, South Africa) at the commencement of the 5-day total collection period. Samples of the feed and refusals for each rat were taken and bulked daily to generate composite samples from which chemical assays were to be done. Feed intake and refusals per rat on each of the dietary treatments were recorded daily for the 5-day collection period. Daily faecal samples from each rat were collected, weighed fresh and stored in a freezer (-20ºC) as described by Osuji et al. (1993). Each rat's bulked faeces were kept frozen pending proximate analysis. Urine from each rat was similarly collected, the volume and mass determined and stored in plastic sample bottles to which 0.5ml of concentrated H_2SO_4 was added to prevent loss of nitrogen (Osuji et al., 1993). The urine samples were also stored in a freezer (-20ºC) pending proximate analysis. After collection of the last batches of faecal and urine samples (day 5), the rats were returned to the Central Animal Services stock.

4.5.5 Proximate analysis of feed, faeces and urine

A representative sample of each dietary treatment was ground using a Wiley Laboratory Mill (GMI, Minnesota, USA) to pass through a 1 mm screen prior to

the analysis. The bulk faecal sample of each rat was freeze dried [Specht Scientific Freeze Drier, United Scientific (Pty) Ltd, Johannesburg, South Africa] and the faecal DM output determined prior to grinding through a 1 mm screen. The ground dietary samples and faeces were each stored in air tight sample dark bottles in a fridge at 4ºC pending analysis.

The proximate analyses for the dietary treatments (diet 1 to diet 5), faecal and urine samples were performed at the Agricultural Research Council's Irene Analytical Services Laboratories, Irene, South Africa. The proximate components for the diets, that is, dry matter (DM), ash, crude protein (CP), and ether extract (EE) were determined as outlined by the Official Methods of Analysis of Analytical Chemists (AOAC) (2005: method numbers 934.01, 942.05, 954.01, and 920.39 respectively). Organic matter was estimated as the difference between dry matter and ash. Prior to determination of nitrogen (N) content of the urine, each frozen urine sample was thawed then thoroughly mixed. A 5 g urine sample was then taken and used in the determination of urinary N as described by AOAC (2005: method number 954.01). The GE value of each dietary sample, faecal sample and urine sample was determined using an MC-1000 Modular Calorimeter equipped with a PC and MC1000 software (Energy Instrumentation, Centurion, South Africa).

4.5.6 Computations: apparent digestibility coefficients and nitrogen balance

Computations of nutrient intake (DM, OM CP, N, and GE) and nutrient output via faeces and urine, apparent digestibility coefficients for DM, OM, CP and GE and N balance (absorption and retention) were done as described by Osuli et al. (1993) and McDonald et al. (2002), respectively.

4.5.7 Statistical analysis

An analysis of variance (ANOVA) using the GraphPad Prism 5 version for windows statistical package (GraphPad Software Inc., San Diego, USA) was performed on the effects of graded dietary substitution of SBM with XCSM on feed (DM) and nutrient intake, nutrient coefficients of digestion and N balance (absorption and retention) for the rats. The comparisons among the diets' effects on nutrient intake, faecal and urinary nutrient output, apparent nutrient digestibility and N balance were done using the Bonferroni's *post hoc* test. Differences among means with $P < 0.05$ were accepted as representing statistical differences. Results are reported as means ± standard deviation. The model used in the analysis of variance was: $Y_{ij} = \mu + T_i + A_j + e_{ij}$; where:

Y_{ij} = dependent variable of interest (nutrient intake, nutrient output, nutrient coefficient of digestibility, nitrogen balance),

μ = overall mean common to all observations,

T_i = fixed effect of dietary treatment (i = 1,2,..5),

A_j = fixed effect of individual rat (j = 1,2,3,...19) and

e_{ij} = random residual error.

4.6 Results

Table 4.2 shows the effects of defatting (solvent extraction) *X. caffra* kernels on their nutrient composition.

Table 4.2: Proximate, mineral and fibre content of full fat *X. caffra* seed and solvent extracted *X. caffra* seed meal

Nutrient content	**Full fat *X. caffra* seed**	**Defatted *X. caffra* seed meal**
DM (g kg^{-1})	955.13	911.70
Proximate composition (g kg^{-1} DM)		
OM	934.69	858.17
CP	182.55	432.71
EE	484.47	40.36
Ash	20.44	53.53
Mineral composition (mg 100g^{-1} DM)		
Calcium	17.85	50.67
Magnesium	207.90	659.21
Phosphorus	345.45	959.75

Table 4.2 continued

Fibre composition (g kg^{-1} DM)		
CF	nd	90.16
NDF	213.31	235.27
ADF	51.17	102.56
Energy content (MJ kg^{-1} DM)		
GE	32.10	18.83

DM: dry matter; OM: organic matter; CP: crude protein; EE: ether extract; CF: crude fibre; NDF: neutral detergent fibre; ADF: acid detergent fibre; GE: gross energy. nd: not determined.

Defatting *X. caffra* seed resulted in a significant increase in the CP, ash, calcium, magnesium, phosphorus, NDF and ADF content, respectively (Table 4.2). However, there was a 41.34% decreased in GE content in the defatted *X. caffra* kernel meal when compared to the GE content in the full-fat *X. caffra* kernels (Table 4.2).

Nutrient intake (DM, OM, CP, N and GE) and faecal nutrient output (DM, OM, CP, N and GE), urinary N excretion, apparent digestibility of DM, OM, CP, and GE and N balance (absorption and retention) in rats on the SBM-based control diet and on XCSM-based test diets are shown in Table 4.3 below.

Table 4.3: Effect of a graded dietary substitution of soyabean meal (SBM) with defatted *X. caffra* seed meal (XCSM) on nutrient intake, faecal and urinary nutrient output, apparent digestibility of nutrients and nitrogen balance in male Sprague Dawley rats

Parameter	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Significance level
5-day nutrient intake						
DM intake (g)	82.15±15.89	96.37±28.96	101.28±13.69	106.12±3.50	109.82±2.76	n.s.
OM intake (g)	78.61±15.20	92.22±27.71	96.86±13.09	101.80±3.36	105.54±2.65	n.s.
CP intake (g)	15.38±2.97	18.20±5.47	18.43±2.49	19.86±0.66	19.92±0.50	n.s.
Energy intake (MJ)	1.31±0.25	1.52±0.46	1.60±0.22	1.66±0.05	1.77±0.04	n.s.
5-day faecal nutrient output						
DM output (g)	12.33±3.53^{a}	16.64±4.09^{ab}	18.07±3.03^{ab}	19.13±1.28^{ab}	22.71±3.78^{b}	*

Table 4.3 continued

OM output (g)	11.14±3.09[a]	14.97±3.69[ab]	16.18±2.82[ab]	7.13±1.14[ab]	20.72±3.76[b]	*
CP output (g)	3.74±0.96[a]	5.04±1.26[ab]	5.56±0.90[ab]	5.71±0.40[ab]	6.88±1.30[b]	*
GE output (MJ)	0.25±0.07[a]	0.33±0.08[ab]	0.36±0.06[ab]	0.38±0.02[ab]	0.45±0.08[b]	*
Digestibility						
Apparent DM digestibility (%)	85.14±1.74	81.68±3.64	82.08±2.35	81.97±2.35	79.57±3.50	n.s.
Apparent OM digestibility (%)	85.95±1.60	83.29±2.71	83.22±2.29	83.17±0.95	80.31±3.95	n.s.
Apparent CP digestibility (%)	75.80±3.09	71.38±5.53	69.65±4.09	71.28±1.60	65.41±6.86	n.s.
Apparent GE digestibility (%)	81.16±2.12	77.60±3.73	77.53±3.02	77.24±1.25	74.27±5.03	n.s.
Nitrogen utilization						
N intake (g day^{-1})	0.49±0.10	0.59±0.17	0.59±0.08	0.64±0.02	0.64±0.02	ns
Nitrogen excretion						
Faecal N (g day^{-1})	0.12±0.03[a]	0.16±0.04[ab]	0.18±0.03[ab]	0.18±0.01[ab]	0.22±0.04[b]	*

Table 4.3 continued

Urinary N (g day^{-1})	0.31±0.08	0.21±0.06	0.27±0.05	0.22±0.02	0.22±0.05	n.s.
Nitrogen balance						
Absorption (g day^{-1})	0.37±0.07	0.43±0.14	0.41±0.07	0.45±0.02	0.42±0.05	n.s.
Retention (g day^{-1})	0.06±0.04	0.21±0.11	0.14±0.08	0.23±0.03	0.20±0.09	n.s

n.s. = not significant, $P > 0.05$. *P ≤ 0.05. [ab]Within row means with different superscripts are significantly different at $P < 0.05$. The faecal DM, OM, CP, N and GE output of rats on Diet 5 were significantly higher ($P = 0.0140$, $P = 0.0149$, $P = 0.0178$, $P = 0.0159$ and $P = 0.0149$, respectively) compared to those of rats on Diet 1. Diet 1 = 100% SBM as protein source; Diet 2 = 75% SBM + 25% XCSM as protein source; Diet 3 = 50% SBM + 50% XCSM as protein source, Diet 4 = 25% SBM + 75% XCSM as protein source; Diet 5 = 100% XCSM as protein source. Data presented as Mean±SD; n = 4, except for Diet 1 where n = 3.

Dietary DM, OM, CP, N and GE intake of rats on the control and test diets was statistically similar (Table 4.3). While urinary N output was similar ($P > 0.05$) for the rats across the dietary treatments (D1 through to D5), rats on Diet 5 (100% substitution of CP from SBM by CP from XCSM) had consistently higher ($P < 0.05$) faecal DM, OM, CP, N and GE output compared to their counterparts on control diet (Diet 1; Table 4.3). There were no statistically significant differences in the apparent digestibility of DM, OM, CP and GE in rats on the control and test diets (Table 4.3). Additionally, there were no differences ($P > 0.05$) in N balance (absorption and retention) in rats across the dietary treatments (Table 4.3).

4.7 Discussion

4.7.1 Effect of defatting *X. caffra* kernels on nutrient composition

Oilseed crops (sunflower, cotton, soyabean) constitute the major protein sources in animal feeds. Upon dehulling (sunflower and cotton seed) and defatting, the resultant cakes and or meals are used as protein concentrates in both monogastric and ruminant animal feeds. Undefatted sunflower seed and sunflower seed kernels have a CP concentration of 18.8% and 26.4% %, respectively (FAO, 1993). When defatted, their respective CP concentrations increase to about 36 and 40%. Solvent extracted SBM has a reported CP content of 45% (FAO, 1993) that is greater than the 34-38% CP in full fat soyabean meal. Defatting *X. caffra* kernels, as is the case with defatting of conventional oilseeds, increased the CP in the resultant

meal from 18.3% in the full fat *X. caffra* kernels to 43.3%, a concentration comparable to the 45% CP in SBM. While defatting of *X. caffra* kernels removed the diluting effect of the kernel oil on CP, ash and minerals, the decrease in the GE of the *X. caffra* seed meal (defatted) compared to the full fat *X. caffra* kernels, was ascribed to the massive reduction in the energy dense oil from the meal. It is critical to note that the defatting over and above improving the status of *X. caffra* kernel meal as a potential non-conventional protein source, also served to remove most of the oil which would otherwise lead to rancidity in feeds if full fat *X. caffra* kernels were to be used as a feed ingredient.

4.7.2 Effect of dietary *X. caffra* seed meal on feed and nutrient intake

Rotger et al. (2005) reported similarity of the intakes of DM, OM, CP, and NDF in heifers fed diets constituted from different ingredients but that, chemically, were iso-calorific and iso-nitrogenous. In the current study despite the differences in dietary ingredient composition, the SBM-based control diet and the *X. caffra* seed based test diets were iso-calorific and iso-nitrogenous. The similarity ($P > 0.05$) in nutrient (DM, OM, CP, N and GE) intake observed in the rats across dietary treatments, as was the case with the observations of Rotger et al. (2005), could have been due to the similarity in dietary energy content of the diets since animals eat to satisfy their energy requirements (Cheeke and Dierenfield, 2010). Results of the current study suggested that SBM could be substituted with defatted

X. caffra seed meal without negative impacts on energy availability to the rats, hence the observed similarity in nutrient intake.

4.7.3 Defatted *X. caffra* seed meal versus other alternative plant protein sources: effects on digestibility and nitrogen utilization

A number of studies have been conducted assessing the effect of substituting SBM with non-conventional protein sources in feeds on nutrient digestibility and N balance. A 100% substitution of SBM with chickpea (*Cicer arietinum*) meal, as a protein source in rat diets resulted in significantly lower N digestibility compared to those on the SBM-based diet (Rubio et al., 1998). In contrast, in the current study, replacement of SBM with defatted *X. caffra* seed meal did not statistically significantly decrease apparent nutrient (including CP) digestibility. While use of chickpea meal as an alternative protein source to SBM resulted in decreased N retention in rats (Rubio et al., 1998), the graded replacement of SBM with defatted *X. caffra* seed meal did not significantly influence N absorption and retention; an indication that defatted *X. caffra* seed meal might not have negative effects on N balance in rats and possibly in other monogastric animals.

In another study to evaluate the nutritional quality of heat treated baobab (*Adansonia digitata*), iron wood (*Prosopis africana*), Senegal lilac (*Lonchoarpus sericeus*), ear pod tree (*Enterolobium cyclocarpium*), sesbania (*Sesbania pachycarpa*) and camwood (*Pterocarpus osun*) seeds, rats were used in a growth

and balance study to determine digestibility, N and energy balance (Proll et al., 1998). Each seed meal was used as the sole source of protein in place of casein (Proll et al., 1998). The *P. africana* and *L. sericeus* based diets caused reduced feed intake. However, *S. Pachycarpa* and *A. digitata* seed based diets had apparent digestibilities of 74.8 and 66.3%, respectively compared to the 91.2% apparent digestibility of the casein-based diet (Proll et al., 1998). In the current study apparent OM digestibility (85.95±1.60, 83.29±2.71, 83.22±2.29, 83.17±0.95 and 80.31±3.95%) for substituting SBM CP with defatted *X. caffra* seed meal (XCSM) at 0, 25, 50, 75 and 100% on a CP basis, respectively) were much higher than the 74.8 and 66.3%, apparent OM digestibility when *S. Pachycarpa* and *A. digitata* seed, respectively, were used to replace casein. Apparent OM digestibility in the test diets with XCSM replacement of SBM (80.31-83.29%) approached the 91.2% OM digestibility in a casein-based diet reported by Proll et al. (1998), a possible indicator of the high potential of defatted *X. caffra* kernel meal as an alternative protein source in monogastric animal feeds. Casein is an animal-derived protein source. Animal-derived protein sources are of high quality in terms of the balance between amino acid composition and proportion that leads to a higher PER and digestibility (Schaafsma, 2000). However, the OM digestibility range of 80.31-83.29% in diets containing defatted *X. caffra* kernel meal pointed to a possible high quality plant-derived protein source in feeds.

Phuc et al. (2000) reported that replacement of SBM on a CP basis with cassava leaf meal (CLM) and ensiled cassava leaves (ECL) in growing pig diets resulted

in a linear decrease in the apparent digestibility coefficients of OM, CP and CF and a decrease in N utilization and retention with increasing concentrations of either the CLM and ECL in the diet. OM, CP and CF digestibility coefficients were 49% , 51% and 44% , respectively, with CLM-based diet and 52%, 51% and 59%, respectively, with ECL-based diet in pigs (Phuc et al., 2000); coefficients which are much lower when compared to when XCSM was used albeit in rat diets. Similarly, replacement of SBM with a mixture of Taro (*Colocasia esculenta* L) leaf silage and water spinach in Mong Cai pigs resulted in a linear decrease in apparent total tract digestibility of DM, OM, CP and CF with increasing concentration of the mixture of the Taro leaf silage and water spinach in the diet (Malavanh et al., 2008). Use of Rubber seed (*Hevea brasiliensis*) meal to replace SBM at 10%, 20% and 30% in growing pig diets, on a CP basis, resulted in lower apparent digestibilities of DM, GE and N at 20% level of replacement (Babatunde et al., 1990). The observations by Babatunde et al. (1990), Phuc et al. (2000), and Malavanh et al. (2008) are at variance with the results observed with graded substitution of SBM with XCSM on a CP basis, in the current study, where an increase in concentration in XCSM in the test diets had statistically (P>0.05) no negative effect on nutrient digestibility and N absorption and retention.

A number of plant-derived non-conventional protein sources are used as protein concentrates in feeds despite their generally lower nutritive value viz effects on nutrient digestibility and N utilization. In the current study it was observed that defatted *X. caffra* meal, when used to substitute SBM on a CP basis, resulted in nutrient digestibilities approaching those observed by others when casein, an

animal-derived protein, was used a protein source, thus pointing to the high potential of defatted *X. caffra* seed as alternative plant-derived protein source in feeds.

Determination of true as opposed to apparent nutrient digestibilities, computation of nutrient digestibilities in the different GIT segments (e.g. ileal digestibility), determination of specific amino acid digestibilities and computation of other indicators of the nutritive value of XSCM such as biological value and net PER ratio could have improved results and given a clearer picture of the nutritive value of XCSM as a potential protein source in monogastric animal feeds. However, it has to be noted that the primary objective of the study was to execute a preliminary *in vivo* determination, using apparent digestibility and N balance trials, to evaluate the feed (protein) value of defatted *X. caffra* kernel meal as a protein source in animal (monogastric) feeds. In view of the stated objective and the need to generate preliminary data upon which to mount further and more in-depth studies, the approach taken was deemed sufficient to lay the foundation for further studies.

4.8 Conclusion

Differences exist in the physiologic anatomy of the GIT of rats and other monogastric animals such as pigs and rabbits. The differences impact on their ability to digest and utilize non-conventional plant-derived protein sources used to substitute SBM as a protein source in feeds. However, rats are a good model of monogastric animals in nutritional studies (NRC, 1995). The bulk of the cited research with monogastric animals has pointed to a decrease in the feed value with an increase in the concentration of non-conventional plant-derived protein substitutes of SBM. The similarity in nutrient intake (DM, OM, CP, N and GE), apparent nutrient digestion (DM, OM, CP and GE) and N absorption and retention across the dietary treatments (0-100% substitution of SBM CP by XCSM CP) pointed to the nutritional superiority of defatted *X. caffra* kernel meal when compared to most other non-conventional plant-derived dietary protein sources explored by others. Based on the results of the current study, it seemed possible that defatted *X. caffra* kernel meal could be used to completely replace SBM and or be used as a compliment to SBM in rat feeds, particularly mature rats, hence most likely in monogastric animal feeds, with no adverse effects on feed intake, apparent nutrient digestibility and N balance.

Weanling animals are sensitive to dietary manipulations, hence the need, to explore further the demonstrated potential of defatted *X. caffra* kernel meal as a substitute to SBM, in the digestibility and nitrogen balance trial, on the growth

performance, GIT macroscopic morphometry and general health profile of rats weaned onto XCSM-based diets. These experiments are described in the following chapter.

CHAPTER 5: *XIMENIA CAFFRA* SEED MEAL AS A DIETARY PROTEIN SOURCE: EFFECTS ON THE GROWTH PERFORMANCE, GASTROINTESTINAL MORPHOMETRY AND GENERAL HEALTH PROFILE OF SPRAGUE DAWLEY RATS

5.0 Introduction

Key indicators of dietary adequacy are growth and reproductive performance (NRC, 1995). The recommendation to researchers is to monitor experimental animal performance in relation to expected patterns of body mass gain and reproduction (NRC, 1995). Growth, an increase in size over time, is an inherent process in the development of living organisms including vertebrates (Melin et al., 2005). Typically, growth is measured as an increase in mass. It encompasses cell multiplication (hyperplasia) and/ or cell enlargement (hypertrophy) and an incorporation of definite components, such as the deposition of phosphate minerals, from the environment (Owens et al. 1993). In meat production, muscle accretion is of primary interest, but by definition, growth also includes fat deposition (Owens et al. 1993). Yan et al. (2012) contends that muscle hyperplasia occurs primarily prenatally while Bergen and Merkel (1991) point to a marginal (slight) increase in muscle fibre numbers post-natally. However Brameld et al. (2000) reported that hypertrophy is solely responsible for skeletal development post-natally. Post-natal muscle growth is largely ascribed to hypertrophy and satellite cell replication and incorporation (Goldspink, 1991). Embryonic tissue growth is by hyperplasia but as mammals mature, specialized cells, for example, nerves and skeletal muscles, lose their ability to replicate and thus grow by hypertrophy. However, blood cell precursors, hair follicles, gastrointestinal epithelia and digestive tract organs and the ectoderm retain the ability to divide throughout life (Bunting and Hawley, 2003). The difference between the synthesis and degradation of body tissue constitute net growth

(Owens et al., 1993). Tissue synthesis and degradation in living animals is a continuous process with total protein synthesis being 6-10 times net protein retention (Bergen and Merkel, 1991). Compared to skeletal muscle cell turnover, the primary GIT organs and accessory GIT organs, for example, the liver and pancreas cells, have a faster protein and cell turnover as exemplified by 10-30% daily fractional protein synthesis in the ruminant GIT (McBride and Kelly, 1990) while the half-life for myofibrillar protein is about 18-50 days (Swick and Song, 1974).

5.1 Factors affecting growth rate and body composition

Mature body size, nutrition and hormonal effects are among the major factors that affect body composition and growth rate (Owens et al., 1993). Although it is accepted that maximum body size is genetically determined, nutritional and hormonal factors, either individually and or in synergy can alter body size. There is debate on the extent and effect of nutritional restriction on mature animal size. However, it is known that severe nutrient restriction, particularly protein restriction, causes a reduction in the mature size of pigs and cattle (Pond et al., 1990, Widdowson and Lister 1991). Restriction of energy intake during the late pre-pubertal or early post-pubertal stage in steers markedly reduced the fat content of the finished steers (Lewis et al., 1990). Hormones and specific growth factors alter body composition and growth rate. Both endogenous [insulin, somatotropin, and Insulin-like-growth factors I and II (IGF-I and IGF-II)] and exogenous

hormones have been observed to promote transcription, translation and amino acid uptake, thus promoting lean growth (Owens et al., 1993). Although there is a decrease in the number of polysomes in muscle with a deficiency in insulin, a deficiency in growth hormone is associated with a decrease in both the number and activity of ribosomes which leads to a cessation of DNA production by muscle tissue (Owens et al., 1993). In pigs, lambs and cattle, administration of growth hormone (somatotropin) improved growth rate and feed:gain ratio while reducing fat content in pig and lamb carcasses (Early et al., 1990). The effects of growth hormone are dependent on locally produced somatomedins (IGF-1 and IGF-II); however the IGF response to growth hormone has been reported to be dampened when nutrient status and or insulin concentrations are low. Beta-adrenergic agonists, (compounds that are structurally related to adrenaline and noradrenaline) when they activate β-receptors in adipose tissue, stimulate lipolysis (Muir, 1988) yet in muscle tissue they decrease protein degradation (Morgan et al., 1989). Administration of β-adrenergic agonists has a net effect of reducing fat deposition while promoting protein (lean) accretion.

In growing animals, restriction of nutrient intake results in subnormal growth rates. When the restriction is lifted, the rate of body mass gain of such previously nutrient restricted growing animals is greater compared to their counterparts that were never restricted: a phenomenon called compensatory/catch-up growth (Owens et al., 1993). The growth rebound due to compensatory growth is due to a rapid hypertrophy of muscle tissue (Owens et al., 1993) and it (compensatory growth) has a major effect on body composition during growth and marginal if

any effect at maturity. Drouillard et al. (1991) point out that compensatory growth is greater following energy restriction than when it follows protein restriction. Literature is not however agreed on the phenomenon of compensatory growth. Tolley et al. (1988) contend that differences in digesta alone accounts for the phenomenon. In support, Williams et al. (1992) point out that the mass of the digestive tract contents can change within minutes or days yet the mass of the empty digestive tract and liver will take a few weeks to change. Despite the misgivings raised by Tolley et al. (1988) and Williams et al. (1992), the magnitude of compensatory growth depends a multiplicity of factors, among which include, age when restriction began, restriction severity, duration and nature of undernutrition, the realimentation diet and time and the breed type (Hogg, 1991).

5.1.1 Approaches to measurement of growth

It is observed and recognized that certain morphological features can be used as accurate estimates of overall growth (Garde and Escala, 1996, Gibson et al., 2000, Riek et al., 2001, Lu, 2003 and Melin et al., 2005). Without going into the complexities of the multivariate predictive mathematical models used to estimate and characterize the growth of individual species, organs and or tissues (Anderson, 1963, Bookstein, 1997), there are several simpler approaches to the measurement of animal growth. Although each different approach may have inherent weaknesses, the critical issue is that the approaches help give some idea

of the magnitude of growth and thus under experimental conditions give a basis for comparisons. Approaches to measure and characterize growth centre largely on body mass where total mass, lean mass, body mass gain, percent gain, average daily gain, and empty/dressed carcass mass can be made use of. Owens et al. (1993) found that full, shrunk and empty body mass could change independently of muscle mass. Changes in the GIT fill, body composition and hydration status make full body mass alone an unreliable indicator of growth (Cameron, 2002). Despite acceptance that shrunk body mass partly accounts for the GIT fill, the extent of shrink is diet-dependent (Tolley et al., 1988, Waldo et al., 1990 and Williams et al., 1992). Although empty body mass is a preferable and more reliable measure compared to full or shrunk body mass, an increase in empty body mass does not necessarily represent an increase in empty body protein (Laurenz et al., 1992). Owens et al. (1993) points out that some authorities refer to growth as an increase in protein mass or lean body mass. However, as observed by Carstens et al. (1991), GIT and liver protein stores may and can increase independently of carcass lean mass during catch-up (compensatory) growth, thus an increase in protein mass and an increase in lean body mass are not synonymous.

Measurement of the lengths of the tibia and femur, anti-gravity bones, has been used as a relatively reliable proxy to overall body growth. Butler et al., (1956) reported highly significant correlations between body weight (mass) and tibia and femur length in cattle. Cheeke and Hill (1974) contend that other growth evaluation methods are less reliable compared to using the length of long bones since they are the only ones that respond to growth hormone in a dose-dependent

manner. Use of long bone lengths to estimate growth employs techniques such mikro-knemometry (Hermanussen et al. 1995) where bone length is measured *in vivo*, radiology and or euthanasia of experimental animals followed by dissecting out the bones of interest.

5.1.2 The GIT: importance, growth and development

The degree of functional efficiency of the GIT influences animal growth. Through its digestive and absorptive role, the GIT plays a critical role in the supply of nutrients to the body (Guiloteau et al., 2010). Additionally, the GIT protects animals from potential injurious insults that originate from undesirable substances, harmful microbiota ingested with food, and GIT resident microbiota (Guiloteau et al., 2010). The gut-associated lymphoid tissue plays an important role in the protective function of the GIT. The physiologic anatomy of the GIT is characterized by the presence of many entero-endocrine cells to the extent that it is regarded as the largest endocrine organ in the body. The GIT endocrinocytes release many regulating factors that are involved in the regulation of the GIT and other associated organs downstream to the GIT and thus have direct and indirect effects on animal growth and development.

Compared to other organs, the GIT is formed relatively early and its embryonic, pre-natal and post-natal development is a complex process involving both an

increase in tissue mass, hypertrophy and hyperplasia of cells (growth) and, changes in structure and function of cells (maturation) (Trahair and Sangild, 1997). At birth the mammalian GIT is developed to a level where it can support a shift from parenteral to enteral nutrition in the neonate (Trahair and Sangild, 1997). The level of GIT development at birth differs in precocial and altricial mammals, particularly the stages of intestinal maturation (Guiloteau et al., 2010). In altricial mammals such as the rat and the mouse, where birth occurs after a short gestation period and the neonate is largely dependent on the dam for locomotion, nutrition, and thermoregulation, at birth, adult diets are poorly tolerated until late in postnatal life. The adult-type GIT also develops rapidly after weaning (Sangild, 2006, Guiloteau et al., 2010). Long-gestation precocial species such as humans and other primates have a precocious mode of GIT development where at birth the neonate GIT is sufficiently mature to digest significant amounts of non-milk derived carbohydrates and proteins (Guiloteau et al., 2010). The pig, sheep, cattle and horse are reported to have an intermediate type of GIT maturation where major intestinal development occurs both in *utero* and post-partum (Pacha, 2000, Sangild, 2006).

Many factors, both external and internal, influence the growth and development of the GIT. Dietary and endocrine factors play crucial roles in the regulation of GIT growth and development (Sangild, 2006). In *utero*, luminal factors from the ingested amniotic fluid, GIT accessory organs and wall secretions all contribute to the growth and development of the GIT (Trahair and Sangild, 1997). The ingested (in *utero*) amniotic fluid has been shown to modulate GIT tissue growth,

macromolecule and immunoglobulin transport, enterocyte differentiation, cell turnover and activity of the brush border hydrolases (Trahair and Sangild, 1997). The modulatory role of the ingested amniotic fluid is hypothesized to be mediated via IGF-1, gastrin releasing peptides, gastrin, epidermal growth factor and insulin (Trahair and Sangild, 1997), all of which are important regulatory peptides. Dietary influences on GIT growth and development are more pronounced at birth and weaning where rapid GIT maturation is experienced. At birth, in mammals, there is a change from the dependence on parenteral feeding to enteral nutrition chiefly milk. Similarly at weaning, there is a shift from largely milk to more solid feeds of different composition compared to milk. The growth-stage dependent dietary changes have been noted to cause an acceleration in the growth and maturation of the GIT (Trahair and Sangild, 1997, Sangild, 2006, Guiloteau et al., 2010) both of which are largely due to marked changes in the endocrinology, microbiology and immunity of the GIT at these time points (Sangild, 2006).

5.2 Clinical biochemistry reference values in animal production

Research institutions and toxicology laboratories collect large volumes of reference data in order to facilitate and support drug and chemical toxicology testing (Alemán, et al., 1998). For each animal species accurate interpretation of haematological and serum biochemical results is dependent on specific reference intervals for the particular species. Mohri et al. (2006) contend that the ability to interpret laboratory data stems from the knowledge of the normal physiologic

mechanisms upon which each test is based and an awareness of disease effects on the test results. Correct interpretation of laboratory data is a vital tool for diagnosis and treatment of disease (Thrall, 2004). Although the specific reference value of each analyte is influenced by many factors, among others, age, breed and physiological status, major changes in serum analyte values occur before puberty (Mohri et al., 2006).

5.3 Justification of study

The chemical potential, and relative economic viability of *X. caffra* seed (Chapter 2), lack of toxicity of *X. caffra* seed oil (Chapter 3) and the results obtained when defatted *X. caffra* seed meal was used as a substitute to SBM in the digestibility and N balance trial (Chapter 4) formed a firm base upon which to test further the potential of XCSM as an alternative protein source in growing animals. Growing animals are more sensitive to dietary manipulation. According to the NRC (1995) one of the key indicators of dietary adequacy is growth: thus evaluating XCSM as a substitute to SBM in rats weaned onto XCSM-based diets would give valuable information on the effects of XCSM on growth performance, GIT morphometry and the general health profile of the animals.

5.4 Study objectives

The specific objectives of the study were to determine, in weanling male Sprague Dawley rats, the effects of a graded dietary substitution of SBM with defatted *X. caffra* seed meal as a protein source on:

i. the growth performance as measured by body mass gain, average daily gain, terminal body mass, empty carcass mass and tibia and femur length.

ii. gastrointestinal macroscopic morphometry

iii. liver metabolic substrate storage (liver glycogen and total lipid content) and markers of liver function [serum alanine transaminase (ALT) and alkaline phosphatase (ALP)].

iv. fasting blood cholesterol, triglyceride and glucose concentrations.

vi. general health profile mrkers (serum urea, creatinine, phosphorus, calcium total protein, albumin, globulin, total bilirubin, and amylase).

5.5 Materials and methods

5.5.1 Ethical clearance for animal study and study site

The study, conducted in the Central Animal Services Unit (CAS) Animal Unit at the Faculty of Health Sciences, University of the Witwatersrand, was approved by

the Animal Ethics Screening Committee (AESC) of the University of the Witwatersrand, Johannesburg, South Africa (AESC number: 2008/46/01; appendix 1).

5.5.2 Diet formulation and diets

The diets used in the digestibility and nitrogen balance trials (Chapter 4) were used for the growth performance, GIT morphometry and general health profile trial. The ingredient and chemical composition of the diets are shown in Table 4.1.

5.5.3 Animals and experimental design

Forty (40) 21-day old weanling male Sprague Dawley rats of mean body mass 58.99±9.97 g at induction were used. The rats were randomly assigned to the five dietary treatments [Diet 1 (control) and Diet 2 to Diet 5 (test diets)]. The control and test dietary treatments were each replicated eight times (n = 8 for each diet).

5.5.4 Animal housing and feeding

The rats were individually penned in Perspex cages (Tecniplast S.p.A., Buguggiate, Italy). Wood shavings were used as bedding. The cages and bedding were

changed twice weekly. The rats had *ad libitum* access to drinking water and feed, and were fed on the respective diets for 38 days. Lighting was set on a 12-hour light-dark cycle with lights on from 0700 hours. The ambient temperature was set at 22±2ºC.

5.5.5 Body mass measurements

On commencement of the experiment, the body mass of each rat in all the dietary treatment groups was measured and thereafter twice weekly using an electronic balance [Snowrex Electronic Scales, (PTY), Ltd., Johannesburg, South Africa].

5.6 Terminal procedures and measurements

5.6.1 End body mass and fasting blood metabolite determination

After the 38 days long feeding trial, the rats were fasted for 12 hours overnight and then weighed [Snowrex Electronic Scales, (PTY), Ltd., Johannesburg, South Africa] in the morning to determine the fasting termination body mass. Immediately after weighing, two drops of blood were drawn from the tail, using a sterile 21-G needle, via a pin prick of the tail vein after disinfecting the tail with an alcohol infused gauze swab (Loxham et al., 2007). The blood was used to measure fasting blood glucose using a calibrated glucometer (ACCU-CHEK®

Active, Roche, Mannheim, Germany) and fasting triglycerides using a calibrated TG meter (Accutrend Plus Cobas, Roche, Mannheim, Germany). The determination of fasting blood glucose and fasting blood triglycerides using the respective meters was done as per the manufacturer's guidelines.

5.6.2 Euthanasia and blood collection

Following the determination of fasting blood glucose and triglycerides, the rats were then euthanased by anaesthetic overdose using Eutha-naze (Centaur Labs, Johannesburg, Republic of South Africa) at 200 mg kg^{-1} body mass administered intra-peritoneally. Immediately blood was collected into 5 ml plain blood collection tubes via cardiac puncture using 18-G needles and 5-ml syringes. Prior to putting the fresh blood into the plain blood collection tubes, heparinised microhaematocrit capillary tubes (IDEXX Laboratories Inc., USA) were used to draw blood from the syringe tip for use in the determination of the packed cell volume.

5.6.3 Dissection, macro-measurements, sample collection and storage

Immediately after blood collection, each rat was carefully dissected and the viscera removed for macroscopic morphometric (GIT organ component masses and lengths) determination. Prior to determination of the mass of the GIT

the residual digesta in them was gently squeezed out. Visceral organ masses were determined using a Presica 310M electronic balance (Presica Instruments AG, Switzerland). The length of the small and large intestines was measured by gently stretching them out on the dissection board.

The livers were stored in a freezer at -20ºC pending further assays. Each empty carcass was then weighed using a Presica 310M electronic balance (Presica Instruments AG, Switzerland).

5.6.4 Serum preparation and storage

In order to separate the serum from the blood cells, on clotting, the blood collected into plain blood collection tubes was centrifuged using a Thermo Sorvall® RT 6000B centrifuge (Du Pont Instruments, Connecticut, USA) at room temperature (22ºC) for 15 minutes at a force of 35,217 x *g*. A pipette (Thermo Scientific, Johannesburg, South Africa) was used to decant the serum from each tube into 1.5 ml microtubes/eppendorfs (Greiner Bio-One GmbH, Frickenhausen, Germany). The sera samples were immediately frozen and stored in a freezer at -20ºC pending determination of markers of the general health of the rats from the sera.

5.6.5 Haematocrit and general health profile determinations

To determine the packed cell volume of blood from rats on different dietary treatments, blood collected into the heparinised haematocrit microcapillary tubes at cardiac puncture was immediately spun at a force of 13,700 x *g* for 2 minutes in an IDEXX Statspin® VT centrifuge (IDEXX Laboratories Inc., USA). Packed cell volume (haematocrit) values were then read off a haematocrit reader.

The general health profile of the rats, as depicted by their serum concentrations of urea, creatinine, phosphorus, calcium, total protein, albumin, globulin, alanine transaminase (ALT), alkaline phosphatase (ALP), total bilirubin, cholesterol and amylase was determined using a colorimetric-based clinical chemistry analyzer (IDEXX VetTest® Clinical Chemistry Analyser, IDEXX Laboratories Inc., USA) as per the manufacturer's instructions. Briefly, each serum sample was thawed and allowed to warm to room temperature, gently inverted to mix the contents and the analyzer automatically drew up 150 µL of the serum. Ten microlitres (10 µL) of serum were then loaded onto each of the pre-loaded disks after which each sample was then analysed and print outs provided.

5.6.6 Determination of stored liver metabolic substrates

Liver glycogen content was determined by indirect acid hydrolysis as described by Passonneau and Lauderdale (1974). The glucose concentration in the hydrolysates was determined using a calibrated glucometer (ACCU-CHEK® Active, Roche, Mannheim, Germany). Liver lipid storage was determined by solvent extraction followed by gravimetric quantification as described by Bligh and Dyer (1959).

5.6.7 Determination of linear growth

The femur and tibia length were used to determine linear growth of the rats on the control and test diets. Briefly, the right femoral head was cut away from the acetabulum at the hip joint followed by removal of all non-calcified soft tissues from the femur and the tibia. Bone length was measured using a ruler. Femur length was determined by measuring the distance between the proximal-most point of the femur head and the distal-most point of the femur (from the distal femoral articular surface to the greater trochanter) while tibia length was determined by measuring the distance between the tibia head closest to femur and the medial malleolus. The bones were then dried in an oven (Salvis®, SalvisLab, Schweiz, Switzerland) at 40ºC until constant mass (7 days) and then weighed on a Presica 310M electronic balance (Presica Instruments AG, Switzerland) to

determine their dry mass. The femur and tibia densities were then estimated using the formula:

$$\text{Bone density (mg mm}^{-1}\text{)} = \frac{\text{dry bone mass (mg)}}{\text{bone length (mm)}}$$

5.7 Statistical analysis

An analysis of variance (ANOVA) using the GraphPad Prism 5 for windows statistical package (GraphPad Software Inc., San Diego, USA) was performed on the effects of graded dietary substitution of SBM with XCSM on growth performance (weekly body masses, termination and body mass gain, percent body mass gain, average daily gain, and empty carcass mass, tibia and femur lengths, mass and density), visceral organ masses and lengths, liver metabolite storage, fasting blood metabolite concentration and serum markers of the general health. Mean comparison on dietary effects of graded substitution of SBM with XCSM was done using the Bonferroni's *post hoc* test. Results are reported as means ± standard deviation. The model used in the analysis of variance was

$Y_{ij} = \mu + T_i + A_j + e_{ij}$; where:

Y_{ij} = is dependent variable of interest (growth performance, visceral organ mass/length, liver glycogen/lipid, AST, ALP, PCV, fasting blood metabolite concentration, serum concentration/activities of marker of general health profile),

μ = overall mean common to all observations,

T_i = fixed effect of dietary treatment (i = 1,2..5),

A_j = fixed effect of indidual rat (j = 1,2,3....40) and

e_{ij} = random residual error.

5.8 Results

5.8.1 Growth: body mass

The weekly growth profile of the rats on the control diet (Diet 1) and a test diets (Diet 2 to 5) is shown in figure 5.1.

At weeks 2 and 3 (days 14 and 21, respectively) of the growth trial, the body mass of rats on test diet 5 (100% substitution of SBM CP with XCSM CP) significantly (P = 0.0054; P = 0.0026, respectively) lagged compared to the body mass of rats on the diets 1 and 2, respectively (Figure 5.1). At day 28 (week 4), the mean body mass of rats on test diet 5 had caught up with the mean body mass of rats on diet 2 but their (rats on diet 5) mean body mass was still significantly lower (P = 0.0268) compared to the mean body mass of rats on diet 1. However, by day 35 the rats on test diet 5 showed compensatory growth such that their mean body mass was statistically similar (P>0.05) to the mean body mass of rats on the control diet (0% substitution of SBM CP) and at termination, on day 38 (Figure 5.1).

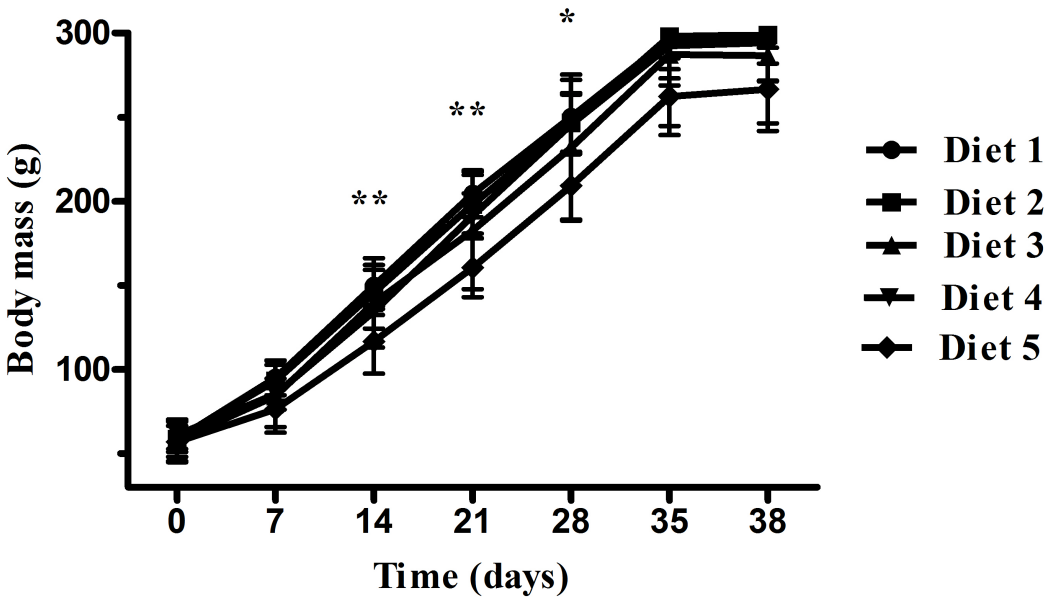


Figure 5.1: Effect of a graded dietary substitution of soyabean meal (SBM) with defatted *X. caffra* seed meal (XCSM) on mean weekly body masses (g) of male Sprague Dawley rats. On days 0 to 7 rats on Diets 1 through to Diet 5 had similar ($P > 0.05$) mean body masses. **On days 14 to 21 rats on Diet 5 had statistically significantly lower mean body masses ($P = 0.0054$ and $P = 0.0026$, respectively) compared to rats on Diet 1. *On day 28 rats on Diet 5 had a statistically significantly lower mean body mass ($P = 0.0268$) compared to rats on Diet 1. On days 35 to 38 rats on Diets 1 through to Diet 5 had similar ($P > 0.05$) body masses.

The mean induction body mass, mass gains (percent and average daily gain), termination body mass and empty carcass mass are shown in Table 5.1.

Table 5.1: Effect of a graded dietary substitution of soyabean meal (SBM) with defatted *X. caffra* seed meal (XCSM) on body mass (termination and body mass gain), average daily gain and empty carcass mass of male Sprague Dawley rats.

Parameter	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Significance level
Induction body mass (g)	58.75±7.73	59.88±10.07	58.00±12.35	61.12±8.24	57.20±12.61	n.s.
Termination body mass (g)	295.75±23.443	299.00 ±17.21	286.50±40.11	294.25±22.49	266.50±24.77	n.s
Body mass gain (g)	237.00±18.18$^{a\,b}$	239.13±11.72^{a}	228.05±28.07^{ab}	233.13±18.61^{ab}	209.30±15.79^{b}	*
Percent mass gain (%)	407.18±42.07	410.61±76.83	400.92±39.80	386.37±54.18	377.23±67.34	n.s.
Average daily gain (g day^{-1})	6.24±0.48^{ab}	6.29±0.31^{a}	6.01±0.74^{ab}	6.14±0.49^{ab}	5.51±0.42^{b}	*
Empty carcass (g)	226.65±20.21	225.85±14.28	217.77±31.46	223.75±16.36	199.49±19.68	n.s.

n.s. = not significant, $P > 0.05$). *$P \leq 0.05$. ^{ab}Within row means with a different superscript are significantly different at $P<0.05$. Rats on Diet 5 had a significantly lower body mass gain ($P = 0.0282$) and averagely daily gain ($P = 0.0282$) compared to rats on Diet 2. Diet 1 = 100% SBM as protein source; Diet 2 = 75% SBM +25% XCSM as protein source; Diet 3 = 50% SBM + 50% XCSM as protein source; Diet 4 = 25% SBM + 75% XCSM as protein source; Diet 5 = 100% XCSM as protein source. Data presented as Mean±SD; n = 8.

The mean termination body mass, mean body mass gain (%) and mean empty carcass mass of rats was not affected by the substitution of SBM with XCSM (Table 5.1).While the mean absolute body mass gain and mean average daily (ADG) of rats on the control diet (Diet 1) and those on test diets 3,4 and 5, were not affected by the concentration of XCSM in the diet (Table 5.1), rats on test diet 2 had significantly higher (P = 0.0282) mean absolute body mass gain and mean ADG (P = 0.0282) compared to rats on test diet 5 (Table 5.1).

5.8.2 Linear growth

Table 5.2 shows the effect of dietary substitution of SBM with defatted *X. caffra* seed meal as protein source on linear growth as measured by tibia and femur length.

Table 5.2: Effect of a graded dietary substitution of soyabean meal (SBM) with defatted *X. caffra* seed meal (XCSM) on long bone length, mass, and density of male Sprague Dawley rats.

Parameter	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Significance level
Tibia length (mm)	37.06±0.56	37.50±0.89	36.94±1.32	37.13±0.69	36.31±0.88	n.s.
Tibia mass (mg)	385.13±15.99^{a}	383.00±21.27^{a}	358.25±40.59^{ab}	348.63±20.35^{ab}	337.50±31.14^{b}	**
Tibia density (mg mm^{-1})	10.39±0.44^{a}	10.21±0.43^{ac}	9.68±0.81^{ad}	9.39±0.46^{bcd}	9.28±0.70^{bd}	**
Femur length (mm)	32.00±0.80	32.75±0.76	32.19±1.07	32.38±0.74	1.63±0.88	n.s.
Femur mass (g)	468.00±22.72^{a}	476.13±31.72^{a}	448.25±57.58^{ab}	449.13±21.12^{ab}	398.88±61.52^{b}	**
Femur density (mg mm^{-1})	14.62±0.49^{a}	14.54±0.89^{a}	13.89±1.41^{ab}	13.87±0.44^{ab}	13.00±1.18^{b}	*

n.s. = not significant, $P > 0.05$). *$P \leq 0.05$. ** $P \leq 0.01$. ^{abcd}Within row means with different superscripts are statistically significantly different at P<0.05. Rats on diet 5 had significantly lower tibia and femur masses compared to rats on Diets 1 and 2, respectively. Diet 1 = 100% SBM as protein source; Diet 2 = 75% SBM + 25% XCM as protein source; Diet 3 = 50% SBM + 50% XCSM as protein source, Diet 4 = 25% SBM + 75% XCSM as protein source; Diet 5 = 100% XCSM as protein source. Data presented as Mean±SD; n = 8.

Dietary substitution of SBM with XCSM did not affect tibia and femur length (Table 5.2). However, substitution of SBM with XCSM resulted in significantly ($P < 0.05$) lighter tibiae and femora for rats on test diet 5 compared to those on diets 1 and 2 (Table 5.2). Graded substitution of SBM caused a significant trend in the reduction of both the tibiae and femora density with increase in XCSM concentration in the diet (Table 5.2).

5.8.3 Visceral organs

The effect of graded dietary substitution of SBM with defatted *X. caffra* seed meal on the absolute and relative masses and lengths (where appropriate) of GIT organs of male Sprague Dawley rats weaned onto the control and test diets is shown in Table 5.3.

Table 5.3: Effect of a graded dietary substitution of soyabean meal (SBM) with defatted *X. caffra* seed meal (XCSM) on GIT visceral organ absolute and relative masses and lengths in male Sprague Dawley rats.

Visceral organ	**Diet 1**	**Diet 2**	**Diet 3**	**Diet 4**	**Diet 5**	**Significance level**
Stomach (g)	1.63±0.17	1.78±0.18	1.66±0.23	1.66±0.12	1.68±0.15	n.s.
(% body mass)	0.55 ± 0.05^{a}	0.59 ± 0.04^{ab}	0.58 ± 0.05^{ab}	0.57 ± 0.03^{a}	0.63 ± 0.04^{b}	**
(g mm^{-1} tibia)	0.0438±0.0046	0.0473±0.0039	0.0446±0.0053	0.0448±0.0033	0.0462±0.0040	n.s.
Small intestine (g)	7.71±0.94	8.42±0.83	8.56±1.66	9.18±0.70	9.32±1.09	n.s
(% body mass)	2.61 ± 0.23^{a}	2.81 ± 0.19^{ac}	2.98 ± 0.35^{ad}	3.13 ± 0.27^{bcd}	3.50 ± 0.32^{b}	***
(g mm^{-1} tibia)	0.2081 ± 0.0256^{a}	0.2245 ± 0.0203^{ab}	0.2312 ± 0.0397^{ab}	0.2474 ± 0.0199^{ab}	0.2567 ± 0.0296^{b}	*
Small intestine (mm)	1326.88±113.48	1308.75±67.02	1328.13±124.55	1370.00±80.40	1340.75±186.42	n.s.
Large intestine (g)	1.79±0.18	1.78±0.24	1.81±0.30	1.95±0.21	2.00±0.24	n.s.

Table 5.3 continued

(% body mass)	0.60±0.04[a]	0.59±0.07[a]	0.63±0.09[a]	0.66±0.05[ab]	0.75±0.08[b]	***
(g mm^{-1} tibia)	0.0480±0.0051	0.0473±0.0055	0.0490±0.0080	0.0525±0.0053	0.0550±0.0070	n.s.
Large intestine (mm)	213.75±15.06	213.75±15.98	208.13±19.99	231.25±15.53	220.00±10.69	n.s.
Caecum (g)	1.56±0.21	1.62±0.15	1.48±0.46	1.62±0.25	1.52±0.24	n.s.
(% body mass)	0.53±0.06	0.54±0.05	0.51±0.11	0.55±0.08	0.50±0.21	n.s.
(g mm^{-1} tibia)	0.0420±0.0056	0.0433±0.0046	0.0399±0.011	0.0435±0.0063	0.0367±0.0161	n.s.

n.s. = not significant, $P > 0.05$. *$P \leq 0.05$. **$P \leq 0.01$. ***$P \leq 0.001$. [abcd]Within column means with a different superscript are significantly different at P<0.05. Rats on diet 5 had statistically significantly larger ($P = 0.0071$) relative (% of body mass) stomach masses compared to rats on Diet 4 and Diet 1. Rats on Diets 4 and 5, respectively had statistically significantly heavier ($P = 0.0001$) relative (% of body mass) small intestine mass compared to the relative (% of body mass) small intestine masses of rats on Diet 1. Rats on Diet 2 had statistically significantly ($P = 0.0001$) smaller relative (% of body mass) small intestine mass compared to those of rats on Diet 5. Diet 1 = 100% SBM as protein source; Diet 2 = 75% SBM + 25% XCSM as protein source; Diet 3 = 50% SBM + 50% XCSM as protein source, Diet 4 = 25% SBM + 75% XCSM as protein source; Diet 5 = 100% XCSM as protein source. Data presented as Mean±SD; n = 8.

Relative to body mass, graded dietary substitution of SBM with XCSM caused a significantly heavier mean stomach mass for rats on diet 5 compared to rats on diet 1. Rats on diet 5 also had significantly heavier small intestinal mass of rats on diet 5 compared to rats on diets 1, 2 and 3, respectively (Table 5.3). However, relative to the tibia length, dietary substitution of SBM with XCSM had no effect on stomach mass (Table 5.3). Similarly, relative to tibia length, dietary substitution of SBM with XCSM, narrowed the difference in intestinal mass [compared when relative to body mass (Table 5.3)] to only between rats on diet 5 and those on diet 1.

Table 5.4 shows the effect of a graded dietary substitution of SBM as a protein source with defatted *X. caffra* seed meal on the absolute and relative masses of other visceral organs in male Sprague Dawley rats weaned onto the control and test diets.

Table 5.4: Effect of a graded dietary substitution of soyabean meal (SBM) with defatted *X. caffra* seed meal (XCSM) on non-GIT visceral organ absolute and relative masses in male Sprague Dawley rats.

Visceral organ	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Significance level
Heart (g)	1.03±0.07[a]	1.18±0.09[ab]	1.18±0.18[ab]	1.26±0.07[b]	1.13±0.07[ab]	*
(% body mass)	0.37±0.03	0.40±0.02	0.41±0.13	0.43±0.02	0.43±0.03	n.s.
(g mm^{-1} tibia)	0.0298±0.0020[a]	0.0310±0.0029[ab]	0.0320±0.0039[ab]	0.0340±0.0017[b]	0.0311±0.0017[ab]	*
Thymus gland (g)	0.74±0.14	0.78±0.08	0.84±0.15	0.78±0.10	0.81±0.06	n.s.
(% body mass)	0.25±0.04	0.26±0.03	0.29±0.04	0.26±0.02	0.30±0.03	n.s.
(g mm^{-1} tibia)	0.0200±0.0035	0.0208±0.0021	0.0226±0.0035	0.0210±0.0074	0.0222 ±0.0020	n.s.
Pancreas (g)	1.27±0.28	1.49±0.28	1.36±0.27	1.26±0.18	1.37±0.30	n.s.

Table 5.4 continued

(% body mass)	0.43±0.09	0.50±0.10	0.48±0.10	0.43±0.05	0.44±0.20	n.s.
(g mm^{-1} tibia)	0.0343±0.0073	0.0397±0.0075	0.0367±0.0078	0.0340±0.0047	0.0331±0.0152	n.s.
Spleen (g)	0.85±0.19	0.83±0.05	0.85±0.15	0.88±0.11	0.81±0.08	n.s.
(% body mass)	0.29±0.05	0.28±0.02	0.30±0.03	0.30±0.04	0.30±0.03	n.s.
Visceral fat (g)	5.47±0.78	4.96±1.02	4.52±1.91	4.33±1.48	3.75±1.01	n.s.
(% body mass)	1.87±0.32	1.65±0.28	1.54±0.44	1.46±0.43	1.39±0.27	n.s.
(g mm^{-1} tibia)	0.1479±0.0228	0.1321±0.0266	0.1212±0.0469	0.1162±0.0384	0.1031±0.0268	n.s.
Kidneys (g)	2.14±0.19	2.13±0.19	2.16±0.36	2.15±0.25	2.16±0.71	n.s.
(% body mass)	$0.72±0.02^{a}$	$0.71±0.03^{a}$	$0.75±0.06^{ab}$	$0.73±0.06^{a}$	$0.81±0.03^{b}$	***
(g mm^{-1} tibia)	0.0578±0.0055	0.0568±0.0045	0.0582±0.0088	0.0580±0.0069	0.0593±0.0192	n.s.
Testes (g)	4.15±0.51	4.36±0.45	4.084±0.64	4.31±0.36	4.03±0.47	n.s.
(% body mass)	1.40±0.15	1.46±0.11	1.42±0.09	1.47±0.13	1.52±0.08	n.s.

Table 5.4 ontinued

(g mm^{-1} tibia)	0.1120±0.0150	0.1163±0.0125	0.1204±0.0273	0.1161±0.0085	0.1109±0.0113	n.s.
Epididymal fat pad (g)	2.06±0.17	1.86±0.15	1.76±0.56	1.76±0.49	1.68±0.23	n.s.
(% body mass)	0.70±0.09	0.63±0.06	0.61±0.13	0.60±0.14	0.63±0.05	n.s.

n.s. = not significant, $P > 0.05$. *$P \leq 0.05$. ***$P \leq 0.001$. [ab]Within column means with a different superscript are significantly different at $P < 0.05$. Rats on Diets 1, 2, 3 and 5 had statistically ($P > 0.05$) similar heart masses; rats on Diet 4 had significantly ($P = 0.0440$) heavier hearts compared to those on Diet 5. Rats on Diet 5 had statistically significantly heavier (relative to body mass) kidneys ($P =0.0007$) compared to rats on diets 1, 2 and 4, however relative to tibia length diet had no significant effect ($P>0.05$) on kidney mass.

Dietary substitution of SBM with XCSM had no effect on absolute and relative mass of the caecum, the thymus, spleen, testes, kidneys, pancreas, visceral fat and epididymal fat pad (Table 5.4). However dietary substitution of SBM with XCSM caused heavier hearts (both absolute and relative to tibia length) in rats on diet 4 (75% substitution of SBM CP with XCSM CP) compared to the hearts of rats on the control diet (Table 5.4).

5.8.4 Liver mass and metabolic substrates

Table 5.5 shows the effect of a graded dietary substitution of SBM as a protein source with defatted *X. caffra* seed meal on the on the liver mass and the liver metabolic substrate (glycogen and lipid) storage of the rats across dietary treatments

Table 5.5: Effect of a graded dietary substitution of soyabean meal (SBM) with defatted *X. caffra* seed meal (XCSM) on the absolute and relative mass of the liver and hepatic storage of metabolic substrates in male Sprague Dawley rats

Parameter	**Diet 1**	**Diet 2**	**Diet 3**	**Diet 4**	**Diet 5**	**Significance level**
Liver (g)	9.84±0.88	9.81±0.72	9.57±1.14	10.02±0.94	8.86±0.70	n.s.
(% body mass)	3.33±0.22	3.28±0.07	3.35±0.17	3.41±0.19	3.33±0.13	n.s.
(g mm^{-1} tibia)	0.2656±0.0234	0.2616±0.0182	0.2587±0.0233	0.2696±0.0231	0.2439±0.0176	n.s.
Liver glycogen (mmol L^{-1} homogenate						
as glucose equivalent)	3.28±0.74	3.09±0.96	2.88±0.92	2.56±0.70	2.53±0.74	n.s.
Liver lipid (%)	4.25±0.70	4.97±1.31	5.26±0.79	5.25±1.25	5.32±0.92	n.s.

n.s. = not significant, $P > 0.05$. There were no statistically significant differences ($P > 0.05$) in liver mass (absolute or relative) and liver metabolic substrate storage in rats across the dietary treatments. Diet 1 = 100% SBM as protein source; Diet 2 = 75% SBM + 25% XCSM as protein source; Diet 3 = 50% SBM + 50% XCSM as protein source, Diet 4 = 25% SBM + 75% XCSM as protein source; Diet 5 = 100% XCM as protein source. Data presented as Mean±SD; n = 8 for all diet for liver glycogen, n = 8, 7, 8, 7 and 8 for Diets 1, 2, 3, 4 and 5, respectively.

Graded dietary substitution of SBM with XCSM had no effect on the liver mass and the liver metabolic substrate (glycogen and lipid) storage of rats across dietary treatments (Table 5.5).

5.8.5 General health profile

Table 5.6 shows the effect of a graded dietary substitution of SBM as a protein source with defatted *X. caffra* seed meal on fasting circulating blood metablic substrates and serum markers of general health profile across dietary treatments.

Table 5.6: Effect of a graded dietary substitution of soyabean meal (SBM) with defatted *X. caffra* seed meal (XCSM) on packed cell volume, fasting blood metabolite concentration and serum markers of general health in male Sprague Dawley rats

Parameter	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Significance level
Packed cell volume (%)	48.00±1.85	44.88±2.75	45.71±2.14	47.13±2.30	45.13±3.00	n.s.
Fasting glucose (mmol L^{-1})	5.11±0.68	5.30±0.60	5.15±0.53	5.11±0.23	5.04±0.50	n.s.
Fasting triglycerides (mmol L^{-1})	1.32±0.21^{ab}	1.36±0.14^{a}	1.16±0.14^{ab}	1.09±0.16^{b}	1.24±0.25^{ab}	*
Cholesterol (mmol L^{-1})	1.16±0.35	1.31±0.21	1.43±0.38	1.23±0.16	1.25±0.24	n.s.
Urea (mmol L^{-1})	5.43±1.00	5.01±0.79	4.79±0.81	5.69±1.31	4.69±1.04	n.s.
Creatinine (mmol L^{-1})	46.25±7.23	49.38±7.41	58.43±6.75	59.63±13.54	51.38±11.01	n.s.
Phosphorus (mmol L^{-1})	2.53±0.34	2.67±0.36	2.97±0.29	3.00±0.50	2.95±0.41	n.s.
Calcium (mmol L^{-1})	2.46±0.18	2.51±0.17	2.66±0.22	2.54±0.19	2.58±0.0.14	n.s.

Table 5.6 continued

Total protein (g L^{-1})	51.38±5.85	55.75±1.58	56.43±5.80	56.63±4.00	53.00±2.27	n.s.
Albumin (g L^{-1})	28.13±2.64	28.50±2.78	30.00±4.16	28.63±2.33	27.75±1.98	n.s.
Globulin (g L^{-1})	23.25±3.81	27.13±2.59	26.43±5.38	28.00±2.98	25.13±3.68	n.s.
Alanine transaminase (U L^{-1})	55.88±7.28	56.00±10.42	64.71±19.59	58.13±9.79	61.00±6.23	n.s.
Alkaline phosphatase (U L^{-1})	362.88±62.54	368.00±54.65	391.14±43.19	388.38±61.19	439.50±73.69	n.s.
Total bilirubin (µmol L^{-1})	6.25±2.31	6.75±1.04	6.43±1.51	6.75±2.05	6.25±2.05	n.s.
Amylase (U L^{-1})	1375.50±285.83	1433.13±158.32	1474.86±211.09	1361.75±183.70	1386.00±198.71	n.s.

n.s. = not significant, $P > 0.05$. *$P \leq 0.05$. [ab]Within row means with different superscripts are significantly different at $P<0.05$. Rats on Diet 1 through to Diet 5 had similar fasting blood glucose concentration. Rats on Diet 4 had statistically significantly lower ($P = 0.0299$) fasting blood triglycerides compared to rats on Diet 2. Rats on Diet 1 through to Diet 5 had statistically ($P>0.05$) similar serum urea, creatinine, phosphorus, calcium, total protein, albumin, globulin, alanine transaminase, alkaline phosphatase, cholesterol and amylase concentrations. Diet 1 = 100% SBM as protein source; Diet 2 = 75% SBM + 25% XCSM as protein source; Diet 3 = 50% SBM + 50% XCSM as protein source, Diet 4 = 25% SBM + 75% XCSM as protein source; Diet 5 = 100% XCSM as protein source. Data presented as Mean±SD; n = 8.

While graded dietary substitution of SBM with defatted *X. caffra* seed meal had no effect on circulating blood blood glucose and cholesterol concentration in rats across dietary treatment, it (substitution of SBM with defatted *X. caffra* seed meal) significantly reduced the fasting blood triglyceride concentration of rats on Diet 4 compared to that of their counterparts on Diet 2 (Table 5.6). Graded dietary substitution of SBM with defatted *X. caffra* seed meal had no effect on PCV and serum markers of general health profile (Table 5.6).

5.9 Discussion

The discussion for this chapter has been partitioned into sections; specifically looking at the effects of dietary substitution of SBM with defatted *X. caffra* seed meal (XCSM) on: the growth performance, GIT viscera and other visceral organs, liver metabolic substrate storage and general health profile of growing male Sprague Dawley rats.

5.9.1 Effects of the diets on the general growth profile

The growth profile of the rats on the control diet (Diet 1) and those on various levels of SBM substitution with XCSM (on CP basis) followed a normal sigmoid pattern in agreement with growth profiles of various rat strains including the Sprague Dawley as observed by Poiley (1972). Although the mean body mass of rats on diet 5 significantly

($P < 0.05$) lagged behind for the first four weeks, they caught up such that at day 35 and at termination (day 38), their mean body mass was statistically similar ($P>0.05$) to their counterparts on the control diet and other test diets. In the digestibility and N balance trial a general increase (statistically insignificant) in feed (nutrient) intake with an increase in the concentration of XCSM in the diet was observed (Chapter 4; Table 4.3). In the growth performance trial male Sprague Dawley rats were weaned onto the control diet (Diet 1) test diets (Diets 2-5) and were fed *ad libitum*. Rats exhibit an altricial mode of GIT development (Guiloteau et al., 2010). Although GIT development accelerates at birth and at weaning in rats; at these stages the GIT is not physiologically efficient at extracting nutrients from the poorly tolerated adult-type diets (Guiloteau et al., 2010). The observed the lag in growth could be ascribed to two possibilities. Firstly, it could be that, initially, with an increase in the XCSM in the diets; the relatively less developed GITs of the weanling rats were not as efficient at extracting nutrients from the diet. Secondly, it could also speculatively be ascribed to a possible poor efficiency of energy utilization from extracted nutrients by rats on diet 5 during the initial phase of the growth trial. Compensatory growth follows a period of nutrient restriction, particularly energy restriction (Drouillard et al., 1991). It could therefore mean that during the period of a possible "efficiency-imposed-nutrient extraction restriction and or poor efficiency of energy utilization", the DM intake of rats on diet 5 did not meet their energy requirements, thus they could have been restricted. Once "adapted" to the diet, they then showed a growth rebound in line with the observations of Drouillard et al. (1991). The above deduction could have been less speculative had the efficiency of energy utilization and the feed intake of the rats on different dietary treatments been

measured. However, the design of the cages in which each rat was individually housed and the “meal form” of the diets (control and test diets) made it impossible to have an accurate measure of feed intake.

5.9.2 Effect of the diets on growth performance

5.9.2.1 *Body mass*

The similarity in mean induction body mass of the rats across the dietary treatments meant that the effect of initial body mass on experimental outcomes was possibly minimised. Fox et al. (1984) reported that male rats at 55-65 days of age weighed 176-200 g while van Zutphen et al. (2003) pointed out that adult male rats weigh 300-500 g. In a study with Sprague Dawley rats, Alemán, et al. (1998) observed that at 6-8 weeks the rats (both sexes) weighed 180±20 g. From the study by Len et al. (1996) male Sprague Dawley rats inducted into a study at 37 days of age, weighed about 280 g at 85 days of age. Schoeffner et al. (1999) noted that at 8 weeks of age Sprague Dawley rats had a mean body mass of 267.45±8.57. In the current study, at termination the rats were 59 days old (8 weeks and 3 days), and probably in the puberty stage of growth (van Zutphen et al., 2003), with a body mass range of 266.50±24.71 to 295.75±23.44 g across the dietary treatments. The observed mean termination body mass range in the current study across the dietary treatments was higher than the mean body mass reported by Fox et al. (1984) and by Alemán et al. (1998) but was in agreement with

the observations by Len et al. (1996) although rats in the experiment by Len et al. (1996) were older. Similarly results of the current study concurred with the observations of Schoeffner et al. (1999). Thus the mean termination body mass of the rats on both the control diet (Diet 1) and test diets (Diets 2-5) indicated that for their age they grew normally.

Differences in GIT fill and body hydration status makes full body mass an inaccurate measure of animal growth (Cameron, 2002). In the current study, dietary substitution of SBM with XCSM had no effect on both the termination body (full body mass) mass and empty carcass mass of the rats across dietary treatments. It has been observed that GIT and liver protein stores may and can increase independently of carcass lean mass during catch-up (compensatory) growth, thus increase in protein mass and increase in lean body mass are not synonymous (Carstens et al., 1991). Results of the current study seemed to negate the observation by Casterns et al. (1991) because the empty carcass mass (after removal of all visceral organs) of rats across dietary treatments was similar despite the observed decrease in the mass and density of the tibia and femur with increasing dietary XCSM. The similarity in empty carcass mass despite a decrease in the mass and density of the femur and tibia with increasing dietary XCSM seemed to suggest a deposition of lean tissue (muscle) during compensatory growth. The inference was given more authenticity due to the fact that fat mass (visceral fat) which can contribute to body mass (Owens et al. 1993) was statistically similar and showed a trend to decrease with increasing XCSM content in the diet.

Optimal lean growth is achieved when amino acids and energy are supplied in the diet at the appropriate ratio (Lawrence et al., 1994). The similarity in empty carcass mass (a possible pointer to lean deposition) of the rats across dietary treatments suggested similarity in the supply of amino acids and energy by the control and test diets. In the digestibility and N balance trial rats on both the control diet (Diet 1) and test diets (Diets 2-5) had a positive (statistically similar) N balance (Chapter 4, Table 4.3). A positive N balance is normally associated with the deposition of lean in growing animals, thus the observed growth of the rats on both the control diet and test diets in growth trial was premised on a positive N balance.

5.9.2.2 *Linear growth*

Compared to body mass, lengths of the tibia and femur are more accurate measures of growth in growing animals (Butler et al., 1956) as they (tibiae and femora) respond to growth hormone in a dose dependent manner (Cheeke and Hill, 1974). In the current study, the average lengths of the tibiae and femora of the rats across the dietary treatments were similar, thus it would appear that XCSM could substitute SBM as a protein source in the diet of growing male Sprague Dawley rats with no adverse effects on growth.

Inadequate energy intake has been observed to influence bone strength and mass by mechanisms including alteration in hormone profiles (Kalu et al., 1984), a reduction in

bone matrix content (Ndiaye et al., 1992) and reductions in bone mineral content (Lee et al., 1986). It could be speculated that the energy restriction (as supported by the initial lag in body mass and subsequent compensatory growth), possibly transiently, could have led to the observed decrease in bone mass and density with an increase in XCSM in the diet.

In the future, effects of XCSM on bone mineral and matrix composition and strength in growing animals should be interrogated.

5.9.3 Effect of the diets on visceral organs

5.9.3.1 *GIT visceral organs*

Scheoffner et al. (1999) noted that in 9-10 week-old Sprague Dawley rats, the mean stomach, small intestine and large intestine mass as 1.49±0.12 g (range: 1.29-1.70 g), 8.68±0.57g (range: 7.62-9.55 g) and 3.20±0.28 (range: 2.79-3.58 g), respectively. In the current study, the rats although they were slightly younger (8 weeks and 3 days old) at termination, the results showed stomach, small intestine and large intestine mass ranges of 1.63-1.78 g, 7.71-9.32 g and 1.78-2.00 g, respectively, values that are generally in agreement with the findings of Scheoffner et al. (1999). The relative similarities in the findings seemed to suggest that the substitution of SBM with defatted *X. caffra* seed

meal had no deleterious effect on post-weaning stomach, small and large intestine (GIT) growth and development.

At birth the GIT is developed to the extent that it can support enteral nutrition in the neonate (Trahair and Sangild, 1997) though adult diets are poorly tolerated in altricial compared to precocial mammalian neonates (Sangild, 2006, Guiloteau et al., 2010). In the current study, the rats were weaned onto the control diet (Diet 1: 0% substitution of SBM CP) and test diets (Diets 2 to 5) and thus were exposed to iso-energetic and iso-nitrogenous (solid) grower diets (Table 4.1) at the same age and for the same duration.

The length of the tibia is, relatively, a more accurate proxy of overall growth in growing animals (Cheeke and Hill, 1974). Relative to the length of the tibia, results of the current study showed that defatted *X. caffra* seed meal had trophic effects on the mass of the small intestine of rats on diet 5 when compared to the mass of the small intestine of rats on the control diet. The observed trophic effects of diet 5 on the small intestine mass could not be ascribed to either hyperplasia and or hypertrophy since no histological assays of the GIT organs were done. In the future, dietary effects of substituting SBM with XCSM, on the histology of GIT organs, particularly the small intestine, should be interrogated. Relative to the length of the tibia, my results showed similarities in the average masses of the stomach, large intestines and caeca of the rats across dietary treatments. The observed similarities in the masses of the three GIT organs suggested that substituting SBM with defatted *X. caffra* seed meal had no

deleterious effect on the growth and development of the organs. However the observed differences in the masses of the stomach and small intestines relative to body mass could be ascribed to the variability of body mass as a measure of overall growth. Body mass is affected by factors such as GIT fill and body hydration status thus is not an accurate proxy of growth in animals (Cameron, 2002). Additionally, the heavier stomach and small intestine (relative to body mass) of rats on diet 5 (100% substitution of SBM CP with XCSM CP) compared to the mean mass of the organs of rats on the control diet (0% substitution of SBM) (Table 5.3) could also be attributed to the relatively, though not statistically significant ($P > 0.05$), lower mean termination body mass of rats on diet 5 compared to those on diet 1.

The similarities in the stomach, large intestine and ceaca masses relative to tibia length in rats across dietary treatments could be a pointer of the similarity in the stimulatory effects of the diets on the post-weaning growth and development of the three GIT organs. The significantly heavier ($P \leq 0.05$) mean small intestine mass (relative to the tibia) of rats on diet 5 compared to those on the control diet could be a pointer to increased stimulatory effect of test diet 5 on the growth and development of the small intestine compared to the control diet.

The GIT organ cells are among the cells that retain the ability to divide post-natally (Bunting and Hawley, 2003) thus post-natal growth and development of the GIT involves cellular hyperplasia and hypertrophy (Trahair and Sangild, 1997). The

observed trophic affects of defatted *X. caffra* seed meal on the small intestine of the rats on diet 5 could have been due to either hypertrophy or hyperplasia. It is imperative that histological assays should be incorporated in any future studies in order to be able to explain possible trophic effects of test materials on any segment of the GIT.

5.9.3.2 *Other visceral organs*

Scheoffner et al. (1999) reported that in 9-10 week old Sprague Dawley rats, the mass of the heart, kidneys, spleen and liver relative to body mass were 0.45±0.04% (range: 0.41-0.50%), 0.80±0.06% (range: 0.72-0.90%), 0.24±0.03% (range: 0.20-0.31%) and 3.86±0.23% (range: 3.45-4.24%), respectively. In the current study, with 8-weeks and 3-day old male Sprague Dawley rats (at study termination), the corresponding organ relative mass ranges were 0.37-0.43%, 0.72-0.81%, 0.28-0.30% and 3.28-3.41% for the heart, kidneys, spleen and liver, respectively. Despite the observed relatively heavier hearts (absolute mass and relative to tibia length; $P = 0.0440$ and $P = 0.0346$, respectively) for rats on Diet 4 compared to those of their counterparts on Diet 1, the mean heart mass (relative to body mass) of rats on Diet 4 fell within the relative heart mass range reported by Scheoffner et al. (1999). The results of the current study concurred with the findings of Scheoffner et al. (1999), a possible pointer to normal heart, kidneys, spleen and liver growth and development on the test diets (Diets 2 to 5).

In the current study, the mass (both absolute and relative) of the thymus gland, testes, abdominal visceral fat and epididymal fat pad from the rats were similar across dietary treatments. The thymus gland is central to the maturation of the immune system and has a regulatory role on immunological reactivity (Miller, 1963), while the testes are critical to reproduction. The distribution of fat in the body has implications in the development of metabolic dysfunction and related systemic disease conditions (Lamarche, 1998; Freedland, 2004). In animal production, fat distribution affects meat quality. While results of the current study suggested that substitution of SBM with defatted *X. caffra* seed meal did not seem to affect the morphometry of the thymus gland, the testes, visceral and epididymal fat pads, their function could have been affected. Functional tests, for example, immunological reactivity, sperm counts, hormone (leptin and insulin) assays, and carcass (meat) quality tests (muscle fibre length, intramuscular fat content, muscle glycogen content) would have assisted shed more light on the dietary effects of defatted *X. caffra* seed meal on the function(s) of the respective visceral organs.

5.9.4 Effect of the diets on liver metabolic substrate storage

The liver is a vital organ that is central to major macronutrient (carbohydrates, protein and lipid) and xenobiotic metabolism (Björkholm et al., 2009). The vena portae and lymphatic system are afferent pathways via which nutrients and metabolites are transported from the intestines to the liver. The bile ducts constitute the efferent pathways through which molecules from the liver are transported to the intestine. Thus

the afferent and efferent pathways constitute a closed loop, the enterohepatic circulation system. The liver regulates and fine tunes homeostasis of blood glucose, lipids, cholesterol, bile acids and haem concentration (Gaw et al., 2006, Björkholm et al., 2009).

In the current study dietary substitution of SBM with defatted *X. caffra* seed meal did not result in significant differences in the liver glycogen and lipid content from rats on the control diet (Diet 1) and test diets (Diets 2 to 5). Further, there were no differences in the fasting blood glucose and serum cholesterol concentration in rats on the control diet and test diets. The serum triglyceride concentration from rats on test diet 4 was significantly lower ($P = 0.0299$) compared to that of rats on tst diet 2. The lower limit of the serum triglyceride of rats across the dietary treatments was lower than that reported by D'Angelo et al. (2005) in 12-week old male Sprague Dawley rats on a standard rat chow. However, the upper limit of the serum triglyceride concentration in the current study fell within the range reported by D'Angelo et al. (2005) despite age and dietary differences between the studies.

Similarities in liver glycogen and lipid content and similarities in fasting blood glucose and serum cholesterol concentration in rats on the control diet and those on the test diets suggested that defatted *X. caffra* seed meal did not alter the metabolism of carbohydrates and lipids in and by the liver.

5.9.5 Effect of the diets on the general health profile of the rats

5.9.5.1 *Markers of liver function*

Liver function tests (LFTs) do not assess quantitatively the capacity of the liver tissue to carry out its physiological functions but include a battery of measurements of blood components that provide a lead to the existence, extent and type of liver damage (Gaw et al., 2006). Gaw et al. (2006) contend that LFTs provide results for serum bilirubin, amino-transaminases [alanine transaminase/transferase (ALT) and aspartate transaminase/transferase (AST)] alkaline phosphatase (ALP) and albumin. Serum AST and ALT activities are widely used in clinical practice as a sensitive, non-specific index of acute hepatocyte (liver parenchyma cell) damage while increased ALP activity is used as an index (also non-specific) of cholestasis, be it intra-hepatic (from cells lining the bile canaliculi) or extra-hepatic (from bile duct cells) whilst hypoalbuminemia is a feature of advanced chronic liver disease (Richardson et al., 1999, Gaw et al., 2006).

Plant-associated toxins (PATs) produced by the plants per se or by endophytic and or saprophytic organisms growing on plants (Than et al., 2005) present a challenge to the utilization of non-conventional plant-derived feeds. PATs include phomopsins that are associated with lupin seed (Edgar, 1991), and pyrrolizidine alkaloids found in some plant species (Than et al., 2005) cause different types of liver damage. The pentacyclic triterpenoids (e.g. lantadenes from *Lantana camara*) cause hepatotoxicity and

cholestasis in ruminants and monogastrics (Sharma et al., 2007). Feeding of non-conventional plant-derived feeds containing phenols and condensed tannins has been reported to cause liver congestion (Mahgoub et al., 2008). Poisoning from PATs that affect the liver is associated with either an increase in the serum activity of ALT and AST or ALP from the damaged hepatocytes and or cholestasis, respectively.

In the current study, there were no differences in the serum activities of ALT and ALP as well as the concentrations of albumin and total protein from rats raised on the different diets suggesting that defatted *X. caffra* seed meal as a non-conventional plant-derived protein source did not cause hepatocyte, bile canaliculi (biliary) and bile duct cell damage neither did it compromise the liver's synthetic function. These observations seemed to suggest that the defatted *X. caffra* seed meal did not contain hepatotoxins.

In the current study total ALP activity was determined. ALP comes in different iso-enzymes: the liver and bone ALP iso-enzymes (Yorio et al., 2000). The separation of ALP into its iso-enzymes has added value in clinical investigations compared to when total ALP activity is measured (Yorio et al., 2000) thus in future dietary effects of defatted *X. caffra* seed meal on ALP iso-enzymes should be interrogated.

5.9.5.2 *Surrogate markers of kidney function*

Kidney function includes regulation of water, electrolyte and acid base balance and the excretion of urea, creatinine and uric acid (Gaw et al., 2006). Thus kidney malfunction affects the clinical biochemistry of animals. Although the assessment of glomerular filtration rate is the gold standard in the determination of overall kidney function (Shock-Kusch et al., 2011), serum creatinine and urea concentration can be used as convenient markers of glomerular function (Gaw et al., 2006). Creatinine, a waste product of muscle metabolism, is derived non-enzymatically from creatine (Burtis and Ashwood, 1994). In human beings under normal circumstances creatine turnover rates are constant and represent 1.6% of the creatine pool (Burtis and Ashwood, 1994). Muscle contains 98% of the total creatine pool, thus the quantity of creatinine produced and released from an individual can be computed when the total mass of muscle is taken into account (Burtis and Ashwood, 1994). The concentration of creatinine in the blood could be influenced by body mass, diet and analytical methods (Banfi, 2010). Total muscle is generally similar in animals of the same age, sex and species, thus with normal kidney function serum creatinine would be the same. Compromised kidney excretory function usually manifests itself with several signs among which include uraemia, increased serum creatinine and bilirubin concentrations.

The seeds and leaves of many leguminous species of the genus *Crotalaria* contain monocrotaline, a nephrotoxic pyrrolizidine alkaloid (Triniade de Medeiros et al., 1999)

which militates against their use as animal feed resources. Seeds and seed cake from toxic provenances of *Jatropha curcas* have been shown to elicit toxicity in mice with post mortem examination revealing pathology of major organs including the kidneys (Adam, 1974), thus seeds and seed cake from such provenances cannot be utilized in animal feeds despite their high nutritional potential. Research reporting toxicity of some plants to kidney function (Adam, 1974; Trindade de Medeiros et al., 1999) have made it imperative to screen any potential non-conventional plant feed resource for possible negative effects on kidney function.

In the current study, while no assaying of known nephrotoxic phytochemicals was done on the defatted *X. caffra* seed meal, the serum concentrations of urea and creatinine (convenient markers of glomerular function) and total bilirubin from the rats was similar across dietary treatments. This finding, despite the lack of histopathologic assessment of the kidneys from the rats, seemed to suggest that glomerular function of the kidneys of rats exposed to defatted *X. caffra* seed meal was not altered. According to Burtis and Ashwood (1994) muscle is the major source (98%) of creatine from which creatinine is derived non-enzymatically. The similarity in the termination and empty carcass masses of the rats across the dietary treatments suggested similarity in the muscle mass and hence the pool of creatine from which the creatinine was derived, hence possibly explaining the similarity in serum concentrations of creatinine from the rats on the different diets. It would also appear from the results of the current study that the defatted *X. caffra* seed meal may not have contained nephrotoxic or kidney damaging phytochemicals further supporting the potential of defatted *X. caffra* seed

meal to replace SBM as a protein source in grower feeds for rats (and probably other monogastric animals) without comprising the kidney's function.

5.9.5.3 *Effect of the diets on PCV*

Packed cell volume (PCV) and total serum proteins determinations provide baseline data for monitoring erythropoiesis and fluid balance (Shmon, 2003). Packed cell volume in rats ranges from 34 to 57% and is dependent on, among other factors, age (Kohn and Clifford, 2002). Balkaya et al. (2001) reported that the PCV range in Sprague Dawley rats (males and females) ranged from 40-49%. In the current study rats on the control diet (Diet 1) and test diets (Diet 2 to5) had a PCV range of 44.88±2.75 to 48.00±1.85%; findings that agreed with other reported research work (Balkaya et al., 2001, Kohn and Clifford, 2002). While there were no statistical differences in the PCV results of the rats across dietary treatments, determining other indices of erythrocytes [mean corpuscular volume (MCV), haemoglobin (Hb), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC)] would have opened a window of opportunity to evaluate the possible dietary effects defatted *X. caffra* seed meal on anaemias. The incidence, development and type of anaemia is influence by several factors among them diet. Vitamins and minerals are required to make red blood cells. In addition to iron, vitamin B_{12} and folate are required for the proper production of haemoglobin. Deficiency of any one of iron, folate and or vitamin B_{12} would lead to the development of some form of nutrition related anaemia.

The congruity between the range of PCV results of the rats in the current study and those of other research findings (Balkaya et al., 2001, Kohn and Clifford, 2002) and the similarity (P>0.05) in the PCV values of the rats raised on the control diet (Diet 1) and their counterparts raised on the test diets (Diet 2 to 5) suggested that SBM could be substituted with defatted *X. caffra* seed meal without affecting erythropoiesis. The similarity in total serum protein, globulin and albumin concentrations from rats on the control diet and their counterparts on test diets also suggested that SBM could be substituted with XCSM without negatively affecting fluid balance in growing male Sprague Dawley rats. In the future effects of dietary defatted *X. caffra* seed meal on other indices of ad blood cells have to be interrogated.

5.9.5.4 *Effect of diets on serum calcium and phosphorus*

Serum calcium and phosphorus concentrations have a bearing on dietary availability and absorption from the GIT, the homeostatic balance of the minerals as influenced by kidney function (Berndt and Kumar, 2007), bone mineral dynamics (Gaw et al., 2006), and the effect of regulatory factors. Hormones involved in calcium and phosphorus homeostasis alter the efficiency of phosphorus absorption (and calcium) in the small intestines and their reabsorption in the kidney tubule (Berndt and Kumar, 2009). Serum calcium and phosphorus concentrations have implications on bone mineral homeostasis.

In the current study the serum calcium and phosphorus concentrations of rats on the control diet (Diet 1) and test diets (Diets 2 to 5) were statistically similar; ranged from 2.46±0.18 to 2.66±0.22 mmol L^{-1} and 2.53±0.34 to 3.00±0.50 mmol L^{-1}, respectively. The similarity in the serum concentrations of the two minerals seemed to suggest that their dietary availability, absorption from the GIT, and homeostatic balance as influence by kidney function and bone mineral dynamics was not affected by the substitution of SBM with defatted *X. caffra* seed meal. Interestingly, dietary substitution of SBM with defatted *X. caffra* seed meal caused a significant trend of a decrease in both the mass and the density of the tibia and femur with an increasing dietary concentration of defatted *X. caffra* seed meal. Since the mineral and matrix composition of the tibiae and femora from the rats on the control diet and their counterparts on the test diets were not determined, it is difficult to ascribe the observed decrease in bone mass and density with an increase in dietary defatted *X. caffra* to either a decrease in bone mineral content and or bone matrix content. In future the effect of dietary *X. caffra* on bone composition should be interrogated. Despite differences in diets and laboratory methods, the serum calcium and phosphorus concentrations from rats raised on the control diet and their counterparts on test diets were in agreement of the serum and calcium and phosphorus concentration reported by Petterino and Argentino-Storino (2005) in male Sprague Dawley rats.

However, more importantly, the lack of significant differences in serum concentrations of the two mineral from rats on the control diet and their counterparts on the test diets suggested that dietary substitution of SBM with defatted *X. caffra* seed meal could be

effected without negatively affecting calcium and phosphorus absorption, homeostasis and possibly kidney function in growing male Sprague Dawley rats.

5.9.5.5 *Effect of diets on serum amylase activity*

Serum amylase activity is elevated in pancreatic and salivary disorders (Suehiro et al., 1984), pulmonary disease (Weiss et al., 1951, Otsuki et al., 1977) and renal failure (Hanafy et al., 1973), thus determination of serum amylase activity is used in the general assessment of the health status of animals. Dietary composition (ingredient and chemical composition) has been reported to influence the synthesis, storage and secretion of digestive enzymes by the exocrine pancreas (Bourdel, 1983, Schick et al., 1984, Sabb et al., 1986, Dubick et al., 1988). Additionally, the exocrine pancreas has been reported to adapt to dietary carbohydrate by increasing the synthesis and secretion of amylase (Johnson et al., 1977).

The lack of differences ($P>0.05$) in the serum amylase activity from rats on the control diet(Diet 1) and from rats on the test diets (Diets 2 to 5) could be interpreted to mean that defatted *X. caffra* seed meal could substitute SBM as a protein source in rat (and probably monogastric animal) diets without eliciting salivary and exocrine pancreatic gland disorders (Suehiro et al., 1984) and without causing overt pulmonary disease (Otsuki et al., 1977) and renal failure (Hanafy et al., 1973).

5.10 Conclusion

Based on results of the current study and reported literature values on growth performance in rats, it could be inferred that use of defatted *X. caffra* seed meal as a dietary protein source in grower rat diets in place of SBM could be done without compromising growth performance as measured by body mass and long bone length. Use of defatted *X. caffra* seed meal (XCSM) in place of SBM did not negatively affect post-weaning growth of the major viscera in growing male Sprague Dawley rats. XCSM could be used in place of SBM in grower diets of Sprague Dawley rats without adverse effects in their general health profile. Although *X. caffra* is widely distributed in SSA, for commercial exploitation of its seed meal as a feed resource (and possible exploitation of its oil), it would be necessary to work with plant production/breeding specialists and plant physiologists to establish commercially viable tree plantations.

CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

6.0 Conclusions on main study objectives

The current study's research objectives broadly sought to evaluate the potential of *Kigelia africana, Mumisops zeyheri, Terminalia sericea* and *Ximenia caffra* seeds as alternative protein sources in feeds. The evaluation was designed around four key stages: physical, chemical, *in vitro* and *in vivo* evaluation. The determination of the physical traits of the seeds constituted the first stage of the evaluation. The physical evaluation was followed by a chemical determination of the lipid yield of the seeds, a profiling of the fatty acid and vitamin E content of the seed oils and a determination of the nutrient (proximate, mineral, amino acid) content, fibre (NDF and ADF) content and phytate-phophate composition of the dehulled/shelled seeds. The physio-chemical characterization was followed by testing *in vitro* the biological activity of the seed oils using established cell lines. The last phase of the evaluation involved a two-stage *in vivo* determination of the dietary effects of the substitution of SBM with the most suitable (on the basis of physical, chemical and *in vitro* evaluation results) defatted IFBTs seed meal. In the first phase of the *in vivo* trial the dietary effects of substituting SBM with the most suitable defatted IFBTs seed meal (*X. caffra* seed meal) on apparent nutrient digestion and nitrogen balance in mature male Sprague Dawley rats were determined. In the second phase of the *in vivo* evaluation the dietary effects of substituting SBM with the most suitable defatted IFBTs seed meal (*X caffra* seed meal) on growth performance, GIT macroscopic morphometry, liver metabolic substrate storage and function and the general health profile in growing male Sprague Dawley were determined.

On the basis of the physical traits and chemical nutrient composition of the four IFBTs seeds it was concluded that:

i. K. africana and *X. caffra* seed qualified as potential protein sources in feeds. They were thus evaluated further.

ii. Due to the low CP content of *M. zeyheri* seed, it did not meet the criteria for potential protein concentrates in feeds. *M. zeyheri* seed however showed potential as an alternative energy source, thus it was investigated further.

iii. Chemically *T. sericea* seed showed tremendous potential as an alternative protein source in feeds, however it was not further investigated as its seed size (yield) was deemed uneconomically viable.

iv. The oil yield from *K. africana*, *M. zeyheri* and *X. caffra* seed surpassed the yield reported for conventional oilseed crops, thus the three IFBTs seeds could be exploited as potential plant oil sources. The oil (by-product) value could cover for the processing of the seeds into protein and or energy rich feed ingredients.

v. The fatty acid profile of the seeds showed a high concentration of oleic acid and essential fatty acids, thus oils from the seeds could be used as nutritional and health supplements in addition to other industrial /pharmaceutical uses.

The *in vitro* evaluations of *K. africana*, *M. zeyheri* and *X. caffra* seed oils showed that they were not toxic to Caco-2 and HEK-293 cells in culture, thus the respective oil seed cakes, with residual oils, could be used /evaluated as feed ingredients with no risk of deleterious effects on animals.

X. caffra seed that had shown the most potential (seed yield, processing ease, chemical composition and *in vitro* evaluation) was judged most suitable for *in vivo* evaluations. Based on the outcome of the digestibility and nitrogen balance trial using mature male Sprague Dawley rats, it was concluded that:

i. Defatted *X. caffra* seed meal could be used to substitute SBM as a protein source without negatively affecting DM (feed) and nutrient (OM, GE and CP) intake of mature male Sprague Dawley rats.

ii. Defatted *X. caffra* seed meal could be used as an alternative protein source to SBM with no deleterious effect on the apparent digestibility of DM, OM, CP and GE and on N retention in mature male Sprague Dawley rats.

Based on the outcomes of the growth, GIT macroscopic morphometry and general health profile trial using male Sprague Dawley rats weaned on defatted *X. caffra* seed meal based diets for five and half weeks, it was concluded that:

i. Defatted *X. caffra* seed meal could be used as a substitute to SBM with no negative effects on growth (body mass and linear growth) in weanling (growing) male Sprague Dawley rats.

ii. Defatted *X. caffra* seed meal could be used as a substitute to SBM with no adverse effects on packed cell volume, liver carbohydrate and lipid metabolism, liver function, pancreatic and kidney function and the general health profile of weanling (growing) male Sprague Dawley rats.

Thus based on the *in vivo* trials results in rats, defatted *X. caffra* seed meal could be considered for use as an alternative protein source in animal feeds for other monogastrics.

6.1 Recommendations for further studies

The study evaluated the potential of *K. africana*, *M. zeyheri* and *X. caffra* seeds as protein sources in a three pronged approach: physico-chemical, *in vitro* and *in vivo*. Although not exhaustive, the study was extensive. It is recommended that future studies focus on detailed macro- and micro-mineral and water soluble vitamin characterization of *K. africana*, *M. zeyheri* and *X. caffra* seed as that would give valuable data on the true nutritional value of the seeds. Additionally, future research should focus on the identification and quantification of potential anti-nutritional factors (commonly found in nuts, seeds and legumes) in the IFBTs seeds as it could help with seed processing,

for instance, to inactivate thermo-labile anti-nutritional factors, using moist heat treatment. Over and above having significant oil yield *K. africana* and *M. zeyheri* seed oils were shown to contain significant quantities of oleic acid (OA). OA is a physiologically important MUFA. Long term intake of olive oil (high OA content) has been reported to reduce high pressure in hypertensive patients (Alonso and Martinez-Gonzàlez, 2004) and the risk of developing hypertension (Ruiz-Gutièrrez et al., 1996). The potential use of *K. africana* and *M. zeyheri* seed oil as a dietary source of OA in the attenuation of hypertension and the development of cardiovascular disease requires further interrogation.

Full fat *K. africana* seed (hand dehulled) had a CP and lipid content of 35.74% and 49.22%, respectively; an indication that on defatting, the resultant seed meal could contain a CP content in excess of 60%. There is a need to develop, as a matter of urgency, technology to facilitate dehulling of *K. africana* seed and test in other animal species the potential of the defatted *K. africana* seed meal to replace SBM in feeds. The potential shown by *M. zeyheri* seed as a possible energy source needs to be further investigated *in vivo* in digestibility and growth performance trials where maize, the major source of energy in feeds, is substituted with *M. zeyheri* seed meal in a graded manner. The potential shown by defatted *X. caffra* seed meal as a substitute to SBM in mature and growing Sprague Dawley rats needs to be further interrogated in poultry, rabbitry, piggery and aquaculture. In further interrogating the potential of defatted *X. caffra* seed meal as a substitute to SBM in livestock, feed intake has to be determined in order to facilitate computation of feed conversion efficiency. Additionally, true total

tract (as opposed to apparent) and ileal digestibility of various nutritional components when defatted *X. caffra* seed meal is used as a protein source have to be determined.

From a livestock production perspective, it is recommended that future studies with defatted *X. caffra* seed meal focus on effects on production traits such as meat yield and carcass quality and milk yield and composition. From a physiological perspective, future studies could also interrogate the effect of defatted *X. caffra* seed meal as an alternative to SBM on serum anti-oxidant profiles, GIT regulatory peptides (hormones), GIT microscopic (histology) morphometry, and exocrine pancreatic function and on metabolism, that is, oxygen consumption and carbon dioxide production.

6.2 Study limitations

An attempt to identify the limitations to each of the components of the study has been made in the relevant chapters. However it is justified to note that one of the current study limitations was the failure to determine feed intake (due to cage design) in the growth performance, GIT macro-morphometry and general health trial. This (failure) made it impossible to compute and hence compare the feed conversion efficiency (FCE) of rats on the control diet (Diet 1: 0%substitution of SBM) and test diets (Diets 2 to 5: 25%, 50%, 75% and 100% substitution of SBM CP with XCSM CP). In livestock and aquaculture production FCE is a critical determinant of profitability thus it is generally an expectation that nutritional evaluation studies give an indication of FCE. Additionally, in the digestibility and N balance trial, the control diet was replicated

thrice while the test diets were each replicated four times thus creating an unbalanced experiment. Unequal replication of treatments results in heterogeneity of variance across treatments that impact on means and standard errors thus affecting the accuracy of results.

APPENDICES

Appendix 1: Animal Ethics Screening Committee Certificate

AESC 3

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2008/46/01

APPLICANT: Mr E Chivandi

SCHOOL: School of Physiology
DEPARTMENT:
LOCATION:

PROJECT TITLE: **In vitro and in-vivo chemical characterization of *Kigelia africana*, *Mimusops zeyheri*, *terminalia sericea* and *Ximenia caffra* nuts and nuts meals**

Number and Species

70 250-300g and 84 21 day M Sprague Dawley rats

Approval was given for the use of animals for the project described above at an AESC meeting held on 30.09.2008. This approval remains valid until 30.09.2010

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 to the following additional conditions:

Include a rationale for Co2 for blood collection

Signed: _____ (Chairperson, AESC) Date: 25/03/2009.

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: _____ (Registered Veterinarian) Date: 25/03/2009

cc: Supervisor
Director: CAS

Works 2009/lain0015/AESCCert.wps

CHAPTER 7: LIST OF REFERENCES

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