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In vitro and *in vivo* effects of sorghum condensed tannins encapsulated in kafirin microparticles on digestive amylases

By

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Dissertation

Submitted in partial fulfilment of the requirements for the degree

MSc Nutrition

In the

Department of Food Science

Faculty of Natural and Agricultural Sciences

University of Pretoria

South Africa

February 2014

DECLARATION

I hereby declare that this dissertation submitted at the University of Pretoria for the degree MSc Nutrition is my own work and has not previously been submitted by me for a degree at this or any other university or institution of higher education.

Malory Rowena Links
February 2015

DEDICATION

To my Abba Father to whom all the glory goes

My loving and supportive parents, Maxie and Stalin

My grandfather, Barney and late grandmother, Jean

Jeandré for the support, patience, understanding and love when I needed it most

ACKNOWLEDGEMENTS

I would like to acknowledge and sincerely thank the following people and institutions:

- My supervisor, Prof John R.N. Taylor for all his inputs, open door, positive criticisms and encouragement throughout the study. The principles learned from him will take me far and beyond academia.

- My co-supervisor Dr Janet Taylor, for her kind hearted and nurturing spirit. The encouragement and support she had showed will never depart from my heart.

- My co-supervisor Prof Marlena Kruger for her input in the *in vivo* study and completion of the dissertation.

- Mr C. van der Merwe and Mrs A. Buys for their assistance with microscopy.

- Prof Vinny Naidoo and Mrs Ilse Janse van Rensburg for their input in the design of the *in vivo* study, the practical execution and helping to make sense of the data.

- All the staff and students from the Department of Food Science that played a role in my study with their help in the practical component of the research and encouragement and guidance during the write-up.

- Christian Revival Church and Klaradyn cell group for constant prayers and encouragement.

- My Pretoria family and friends, whom have opened their homes and adopted me as a daughter during my studies.

- The following institutions for financial support: The National Research Foundation for the Masters Innovation Scholarship, University of Pretoria postgraduate bursary and the Technology for Human Resources for Industry Programme of South Africa for study funding.

iii

ABSTRACT

In vitro and *in vivo* effects of sorghum condensed tannins encapsulated in kafirin microparticles on digestive amylases

Malory Rowena Links

Supervisor: Prof J. R. N. Taylor

Co-supervisor: Prof M. C. Kruger

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The International Diabetes Federation reported that by 2035 the expected increase in diabetes in sub-Saharan Africa will be 109%, the highest rise in the world. Furthermore, almost a quarter of the world's adult population have the metabolic syndrome. One therapeutic approach for type 2 diabetes (T2D) treatment is to inhibit digestive amylases and thereby decrease post-prandial hyperglycaemia. Polyphenols like sorghum condensed tannins (SCT) inhibit α-amylase and α-glucosidase enzymes. However, orally administered SCT may have poor inhibitory action against amylases, due to non-specific protein binding. Research is required to determine how to effectively deliver SCT to the small intestine to inhibit digestive amylases and decrease post-prandial hyperglycaemia.

This study investigated the encapsulation of SCT in kafirin microparticles (KEMS) using aqueous ethanol and acetic acid coacervation methods. The inhibitory action of SCT on α-amylase and α-glucosidase were compared to acarbose (standard drug). The inhibition of SCT-KEMS after simulated gastrointestinal digestion was assessed. Electron microscopy was used to characterise microparticle morphology. A confirmatory *in vivo* test was performed using an oral starch tolerance test (OSTT) on healthy rats.

SCT were about 20 000 times more effective at inhibiting α-glucosidase ($IC_{50} = 0.4$ µg/ml) than acarbose ($IC_{50} = 8464.0$ µg/ml), while acarbose ($IC_{50} = 3.1$ µg/ml) inhibited α-amylase better than SCT ($IC_{50} = 554.5$ µg/ml). The aqueous ethanol method of encapsulating SCT resulted in higher encapsulation efficiency (48%) than the acetic acid method (25%). Electron microscopy and quantitative data showed that SCT-KEMS were hardly digested by pepsin and trypsin-chymotrypsin during simulated digestion. SCT-KEMS retained their inhibitory activity against both amylases after simulated digestion, while SCT alone lost

iv

most of their inhibitory activity. *In vivo* data showed that the SCT-KEMS treatment decreased the maximum blood glucose level of rats by on average 11.8% and the area under the curve by 9%, compared to the water control. Acarbose decreased the blood glucose spike of the rats by on average 18.5% compared to the control. SCT-KEMS and acarbose did not elevate serum insulin levels and actually decreased insulin secretion by 60% and 48%, respectively, compared to the control.

These findings indicate that KEMS are effective SCT encapsulating agents as they deliver the SCT to the small intestine. SCT-KEMS prevents hyperglycaemia by inhibiting digestive amylases and seems to substantially reduce insulin secretion when carbohydrate is consumed. Therefore, SCT-KEMS could be employed as a nutraceutical to inhibit digestive amylases and thereby attenuate post-prandial hyperglycaemia associated with the metabolic syndrome and T2D.

TABLE OF CONTENTS

vii

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1: INTRODUCTION

According to the International Diabetes Federation (IDF) there were 19.8 million cases of diabetes in Africa in 2013 and it was predicted that diabetes cases will increase to about 41.8 Million in 2035, resulting in an increase of 109 % (IDF, 2013). Furthermore, about a quarter of the world's adult population have the metabolic syndrome (IDF, 2013). Overnutrition, physical inactivity and urbanisation are some of the reasons that have led to the vast increase of these conditions in Africa (Candib, 2007).

Metabolic syndrome can be defined as a cluster of clinical and biological abnormalities, consisting of abdominal obesity and at least two of the following conditions; dyslipidaemia, reduced high-density lipoprotein (HDL) cholesterol, raised blood pressure and raised fasting blood glucose levels (IDF, 2006). Type 2 diabetes (T2D) is the most common form of diabetes and is characterised by decreased insulin secretion and insulin resistance (Moller, 2001). According to Kassi, Pervanidou, Kaltsas and Chrousos (2011) insulin resistance is one of the core manifestations of the metabolic syndrome signifying the close aetiology between the metabolic syndrome and T2D. Hyperglycaemia (high blood glucose) is commonly associated with metabolic syndrome and diabetes and is responsible for chronic complications such as retinopathy and nephropathy (Moller, 2001; Mbanya, Motala, Sobngwi, Assah and Enoru, 2010). However, access to appropriate health care in sub-Saharan Africa is limited due to inadequate healthcare systems, shortage of medical personnel and facilities and lack or unaffordability of medication (Diabetes Leadership Forum, 2010). Consequently, there is a need for inexpensive dietary approaches to prevent and alleviate the adverse health consequences of the metabolic syndrome and T2D in this region.

Sorghum is one of the major cereal crops in Africa and there is significant production in 41 African countries (FAOSTAT, 2011). Extracts of polyphenols from sorghum grain have been found to demonstrate *in vitro* inhibition of the main starch hydrolysing enzymes, α-amylase and α-glucosidase (Kim, Hyun and Kim, 2011) and *in vivo* anti-diabetic activity (Kim and Park, 2012). Additionally, sorghum rich in condensed tannin has been reported to reduce weight gain in animals (Al-Mamary, Molham, Abdulwali & Al-Obeidi, 2001; Awika and Rooney, 2004). Therefore, sorghum condensed tannin (SCT) extracts have potential to be utilized as a dietary means of preventing hyperglycaemia and hence could

1

be employed as a natural plant-based nutraceutical for metabolic syndrome and T2D attenuation.

However, oral administration of sorghum phenolic extracts has various limitations. SCT bind proteins strongly (Butler, Riedl, Lebryk and Blytt 1984), which leads to the sensation of astringency and unpleasant bitter tastes (Kobue-Lekalake, Taylor and de Kock, 2007). Also, SCT bind to proteins in a non-specific way, which could lead to the loss or reduction of enzyme-inhibition in the small intestine. Consequently, a suitable delivery system needs to be developed to ensure that SCT reach the target site (small intestine) with enough biological activity to effectively inhibit starch digesting enzymes. Kafirin, the sorghum prolamin storage protein, is notably hydrophobic and resistant to pepsin digestion (Belton, Delgadillo, Halford and Shewry, 2006). Additionally, it has been found that microparticles made from kafirin (KEMS) can encapsulate and release SCT during simulated digestion (Taylor, Taylor, Belton and Minnaar, 2009a).

Therefore, the focus of this study was to determine the potential of KEMS encapsulating SCT (SCT-KEMS) as a nutraceutical to provide targeted release of SCT to the small intestine by assessing their *in vitro* α-amylase and α-glucosidase inhibitory activity after simulated digestion and their *in vivo* effect on starch digestion and absorption.

CHAPTER 2: LITERATURE REVIEW

In this review the aetiology and pathophysiology of the metabolic syndrome and T2D will be discussed in relation to sub-Saharan Africa. Current treatments for these conditions will be evaluated. The potential health-promoting role of dietary polyphenols in general and condensed tannins specifically in terms of the metabolic syndrome and T2D will be conferred. Potential mechanisms of condensed tannins in preventing and alleviating the metabolic syndrome and T2D will shortly be reviewed. Sorghum as a source of condensed tannins will be considered and the possible anti-diabetic and anti-metabolic syndrome role of SCT briefly discussed. The need for a SCT delivery system is discussed briefly, while some microencapsulation methods specifically used for polyphenols will be considered. *In vitro* and *in vivo* methodologies typically used to test the anti-diabetic effects of natural compounds will also be reviewed briefly.

2.1 Aetiology and pathophysiology of metabolic syndrome and type 2 diabetes

2.1.1 Aetiology

The metabolic syndrome is a constellation of interrelated risk factors of metabolic origin (metabolic risk factors) that appear to directly promote the development of atherosclerotic cardiovascular disease (Eckel, Grundy and Zimmet, 2005). The main components of the condition include insulin resistance, hyperglycaemia, hyperinsulinaemia, abdominal obesity, hypertension and dyslipidaemia that include increased plasma triglycerides, decreased high density lipoprotein (HDL) cholesterol and increased low density lipoprotein (LDL) (Okafor, 2012). The aetiology of the metabolic syndrome is through a combination of environmental and genetic factors. Environmental factors include urbanisation, a sedentary lifestyle, consumption of high-energy, low nutrient fast foods, obesity and an increase in stress (IDF, 2006; Lambert, Straznicky, Lambert, Dixon and Schlaich, 2010). The metabolic syndrome is strongly associated with prediabetes or impaired glucose tolerance, a condition present prior to the onset of full blown diabetes, where blood glucose levels are higher than basal and coupled with insulin resistance (Batsis, Nieto-Martinez and Lopez-Jimenez, 2007).

Rapid changes in dietary habits are playing a major role in the development of the metabolic syndrome in sub-Saharan Africa. These dietary habits include decreased consumption of staple foods rich in starch and dietary fibre, increases in consumption of foods from animal origin which are rich in total fat and saturated fatty acids, decreased consumption of plant protein sources such as legumes, and increases in energy-dense snack foods, carbonated sweetened beverages, commercial alcoholic beverages, as well as added sugar, fats and oils in the preparation of food (Vorster, Kruger and Margetts, 2011). The effect of these diets have been demonstrated in studies that involve inducing obesity and metabolic syndrome in rats by providing them with high-fat, high-sugar and high-fat-high-sugar diets *ad libitum* (Hariri and Thibault, 2010; Pranprawit, Wolber, Heyes, Molan and Kruger, 2013). These rats developed signs of impaired glucose tolerance and insulin resistance. Additionally, as insulin resistance is one of the main causes of the metabolic syndrome (Kassi et al., 2011) it is also a major characteristic of and contributor to the development of T2D (reviewed by Ross, Gulve and Wang, 2004).

Type 2 diabetes is typically a polygenic disease that results from a complex interplay between genetic predisposition and environmental factors, i.e. diet, degree of physical activity and age (Ross et al., 2004). T2D accounts for 90-95% of diabetes in sub-Saharan Africa (IDF, 2013). Prevalence of T2D has been linked to the adoption of a so-called 'Western' lifestyle, with a high proportion of processed food, little exercise and a tendency to overweight and obesity (Diabetes Leadership Forum, 2010). Rapid urbanisation has been proposed as a major determinant of the rising burden of diabetes and other cardiovascular diseases (Mbanya et al., 2010). Sub-Saharan Africa is undergoing the fastest rate of urbanisation worldwide, with an average annual rate of change in number of urban dwellers of more than 3% (United Nations Population Fund, 2007), while at least one third of the sub-Saharan African population already reside in urban areas.

It has been found that genetics play an important role in the cause of T2D as there is a raised risk in developing the diseases amongst those with affected first-degree relatives (reviewed by Nolan, Damm and Prentki, 2011). In rarer cases, T2D can occur primarily as a result of β-cell aberrations, where patients have mutations in genes for glucokinase (enzyme that facilitates phosphorylation of glucose to glucose-6-phosphate) or a variety of transcription factors (reviewed by Ross et al., 2004 and Leahy, 2005). Studies from Sudan and rural South Africa showed that a family history of diabetes is an independent risk

factor for the disease (Mbanya et al., 2010). However, despite intensive research the identification of a set of specific type 2 "diabetogenes" has been elusive.

There is also strong evidence that there is a link between foetal growth restriction (due to maternal malnutrition) and diabetes development in adult life (Barker, 2004). In sub-Saharan Africa most areas still suffer from severe maternal and neonatal under-nutrition and poverty coupled with rapidly increasing rates of life-style related diseases (Vorster et al., 2011). Barker (2004) proposed that as foetal growth restriction takes place, most available energy goes to the development of key organs like the brain and heart at the cost of organs and cells such as the pancreas and β-cells, which are responsible for insulin secretion. As energy consumption increases in later life (with overconsumption) these cells fail to function normally and the person becomes insulin resistant which could then develop into T2D.

Minor factors that play a role in the aetiology of T2D are micronutrient imbalances of vitamin D, vitamin B12, folic acid and iron in particular (Yajnik, Deshpande, Jackson, Refsum, Rao, Fisher, Bhat, Naik, Coyaji, Joglekar, Joshi, Lubree, Deshpande, Rege, and Fall, 2008). This is particularly important as many parts of sub Saharan Africa still suffer from chronic malnutrition and micronutrient deficiencies (Abrahams, Mchiza and Steyn, 2011). There is also evidence that exposure to synthetic organic pollutants affects endocrine cells and increases the risk of developing T2D (Casals-Casas and Desvergne, 2011).

2.1.2 Pathophysiology

Although the pathophysiology of the metabolic syndrome is mainly unexplained, it is thought to be strongly related to insulin resistance (Motala, Mbanya and Ramaiya, 2009) and abdominal obesity (excessive fat deposits around abdominal area leading to larger waist circumference). Physical inactivity, aging, polycystic ovarian syndrome and sleep apnoea also have a role in its pathophysiology (Cornier, Dabelea, Hernandez, Lindstrom, Steig, Stob, Van Pelt, Wang, and Eckel, 2008).

Insulin resistance is a condition in which the skeletal muscle, liver and adipocytes decrease their responsiveness to normal circulating levels of insulin (reviewed by Sesti, 2006 and

Kohei, 2010). In healthy individuals insulin is secreted by the pancreatic β cells in response to increased circulating levels of glucose and amino acids after a meal (Sesti, 2006). The secreted insulin binds to the insulin receptor, initiating a signal transduction cascade that triggers the mitogen activated protein (MAP) kinase and phosphatidylinositol 3-kinase (PI-3K) signalling pathways (Sesti, 2006). The MAP kinase signalling pathway initiates cell growth, proliferation and gene expression while the PI-3K pathway signals for lipid and glycogen synthesis, cell survival and proliferation and glucose transporter 4 (GLUT)-4 exocytosis (Ross et al., 2004). GLUT-4 transporter proteins are integrated into the cell membrane allowing glucose to be transported into the cell (Figure 2.1) (Chhabra, 2014). In the insulin-resistant individual, the cell-surface receptor on skeletal muscle and adipose cells no longer binds the insulin receptor properly and/or responds to the insulin signal, resulting in reduced glucose uptake and high blood glucose levels (Hirabara, Gorjao, Vinolo, Rodrigues, Nachbar and Curi, 2012).
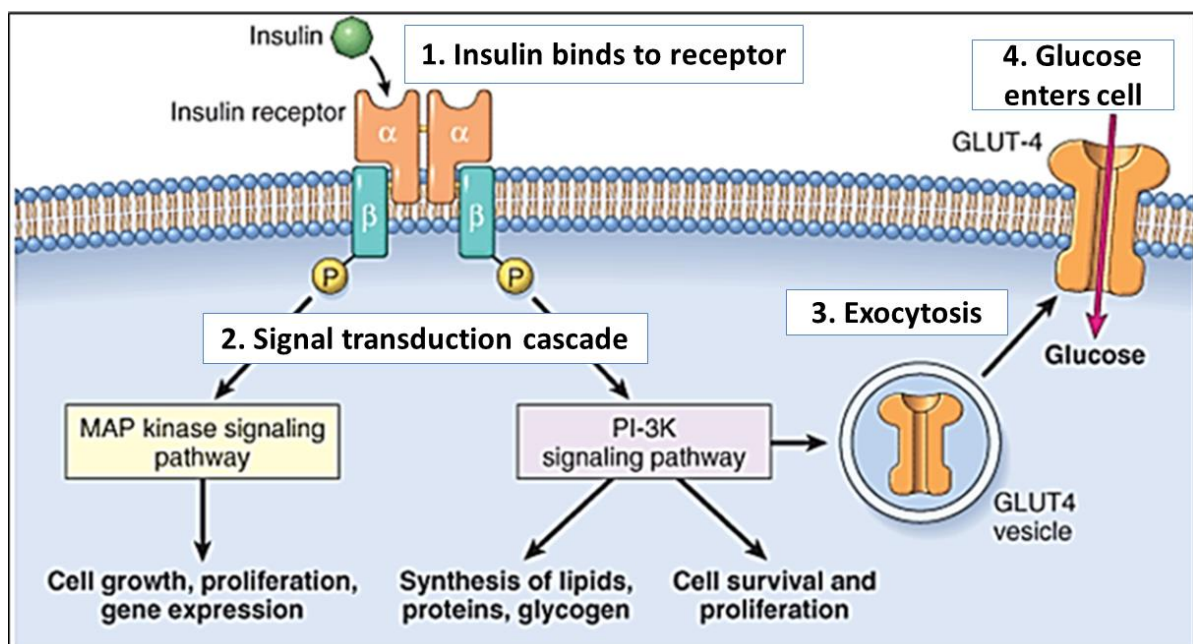
Figure 2.1: Mechanism of action of insulin. MAP-Mitogen activated kinase, PI-3K - phosphatidylinositol 3-kinase, GLUT-4, glucose transporter protein 4 (modified from Chhabra, 2014).

Abdominal obesity is believed to be an early step in the pathogenesis of the metabolic syndrome. According to Matsuzawa (2008) visceral adipose tissue secretes a variety of bioactive substances called adipocytokines. These adipocytokines include leptin, resistin, tumour necrosis factor α (TNFα), interleukin-6 (IL-6), and angiotensin II which induce insulin resistance, along with plasminogen activator inhibitor 1 (PAI-1), which is related to

6

thrombogenic vascular diseases. Opie (2007) explained the syndrome by observing abdominal adipose tissue as an endocrine organ as illustrated in Figure 2.2. First, the increased blood free fatty acids (FFA) inhibit the uptake of glucose by muscle and excess FFA and angiotensin II damage the pancreas. Although the pancreas manufactures extra insulin it is not enough to counter the hyperglycaemia, hence fasting hyperglycaemia persists.
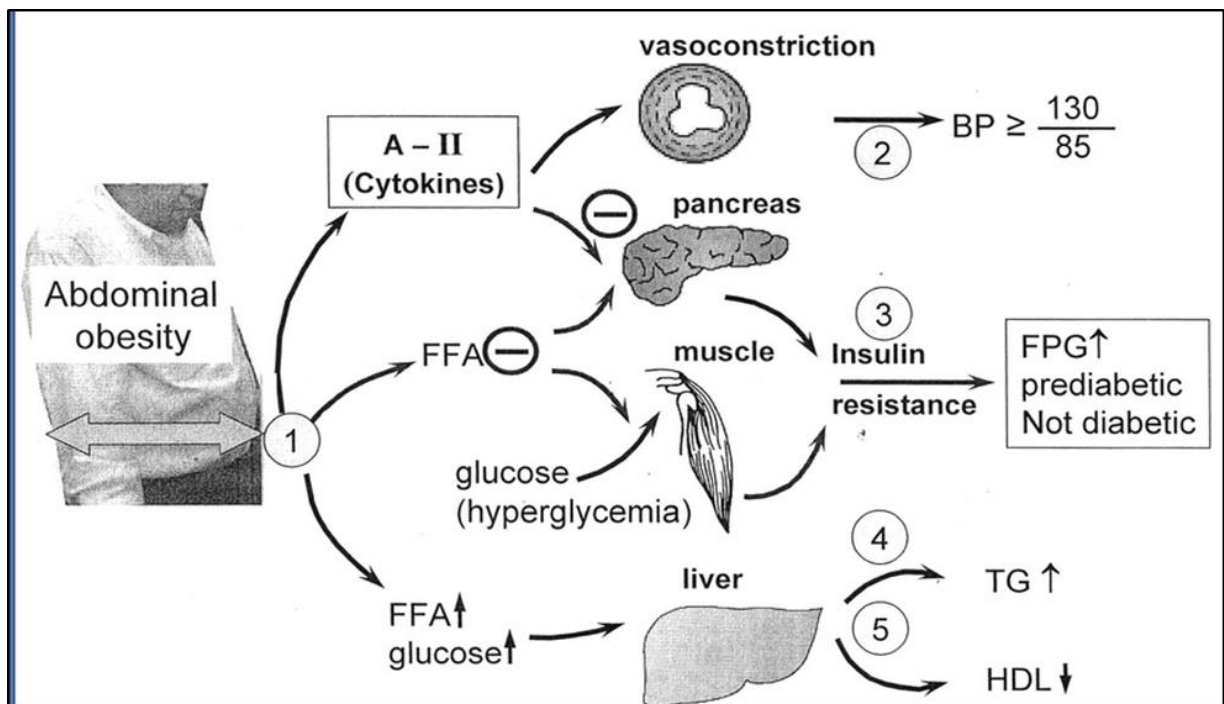
Figure 2.2: The proposed role of abdominal obesity in the pathophysiology of the metabolic syndrome. FFA-free fatty acids, BP-blood pressure, FPG-fasting plasma glucose; TG-triglycerides and HDL-high-density lipoproteins (from Opie, 2007).

Angiotensin II increases blood pressure through its vasoconstrictive effects. Tumour necrosis factor- α and other cytokines provoke inflammatory reactions that also reduce the efficacy of insulin and may promote hypertension. Hyperglycaemia and increased circulating FFA provide substrates for increased manufacture of triglycerides by the liver. Circulating triglycerides increase so that lipoproteins carry more triglycerides and less high-density lipoprotein.

A study in Cameroon found that among those diagnosed with the metabolic syndrome, abdominal obesity was more closely associated with the syndrome than insulin resistance (Fezeu, Balkau, Kengne Sobngwi and Mbanya, 2007). It was also found that although some people had abdominal obesity, they did not show characteristics of metabolic syndrome. It was proposed that the high level of physical activity among these individuals

greatly improved their metabolic profile. These observations indicate that the lack of physical activity plays a significant role in the development of metabolic syndrome and T2D.

Type 2 diabetes will develop when insulin resistance is associated with abnormal β-cell function (e.g. decreased insulin secretion), which is considered a key pathophysiologic abnormality (Ross et al., 2004). It has been observed that a progressive loss of the insulin-secretory capacity of β-cells appears to begin years before the clinical diagnosis and, given the presence of insulin resistance, results in a state of "relative" insulin deficiency leading to hyperglycaemia (Cefalu, 2007). Ross et al. (2004) explain the process in detail. During the initial stages of T2D, insulin simulated glucose transport in skeletal muscle is impaired. To compensate, pancreatic β-cells display augmented insulin secretion that results in hyperinsulinaemia. Then peripheral insulin resistance combined with early phase impairment of insulin secretion, results in hyperglycaemia. As pancreatic dysfunction deteriorates combined with the inability to compensate fully for the degree of insulin resistance, clinically overt T2D becomes present.

Chronic hyperglycaemia is another primary pathogenic event that leads to the development of T2D (Prentki and Nolan, 2006). Various organs play a crucial role in the pathophysiology of T2D as disorders of the pancreas, liver, skeletal muscle, adipose tissue, gut and central nervous system may lead to alteration of glucose homeostasis and thereby T2D (Scheen, 2003). The inability of β-cells to compensate for excess blood glucose, increased glucagon secretion, reduced incretin (gut hormones that stimulate insulin secretion) response, impaired expansion of subcutaneous adipose tissue, hypoadipocectineamia (reduced blood adiponectin levels), inflammation of adipose tissue, increased endogenous glucose production and the development of peripheral insulin resistance all contribute to the pathophysiology of T2D (Leahy, 2005; Nolan, et al., 2011).

DeFronzo (2009) developed the "omnious octet" model which proposes that in addition to the muscle, liver, and β-cells, the fat cells (accelerated lipolysis), gastrointestinal tract (incretin deficiency/resistance), α-cell (hyperglucagonaemia), kidney (increased glucose reabsorption), and brain (insulin resistance) play important roles in the development of glucose intolerance in type 2 diabetic individuals. Collectively, these eight "players" comprise the "ominous octet". The "ominous octet" dictates that the eight organs should be

targeted to reduce hyperglycaemia associated with metabolic diseases. He suggests that diabetes should be treated with multiple drugs and that treatment should be based upon reversal of known pathogenic abnormalities and that therapy must be started early to prevent/slow the progressive β-cell failure that already is well established in impaired glucose tolerance subjects. This "ominous octet" model is illustrated in Figure 2.3.
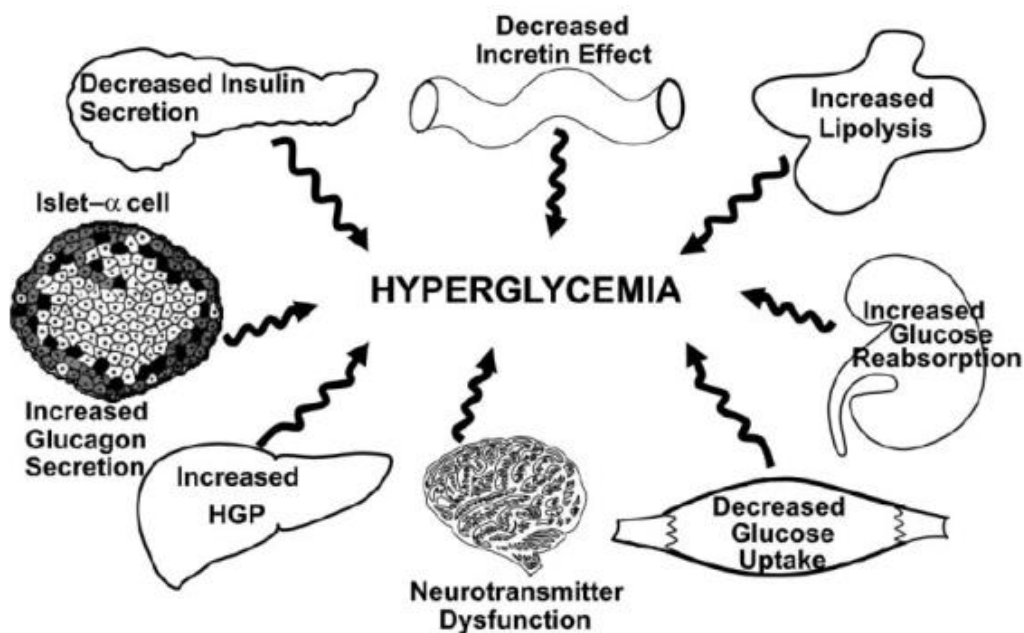
Figure 2.3: The "ominous octet" model that describes the metabolism abnormalities involved in the pathogenesis of type 2 diabetes (from DeFronzo, 2009). HGP − hepatic glucose production.

2.2 Treatment options for hyperglycaemia associated with metabolic syndrome and type 2 diabetes

2.2.1 Lifestyle adjustments

Lifestyle adjustments can help to control diabetes and attenuate its progression (Bazhan, Mirmiran, Mirghotbi, Vafaee, 2013). According to Ross et al. (2004) there has been remarkable progress in treatment of T2D with the use of lifestyle adjustments. Lifestyle adjustments such as increased physical activity and a healthy diet that includes wholegrains, low glycaemic index carbohydrates, fruit and vegetables, low fat dairy products, nuts and foods rich in monounsaturated fatty acids is usually the first line of treatment for metabolic syndrome and T2D.

9

Weight loss and exercise can enhance insulin sensitivity and glucose utilization but patient compliance is weak (Ross et al., 2004). Additionally, in some areas in sub-Saharan Africa these strategies are rather optimistic routes to follow in the face of weak healthcare systems and the high cost associated with the above-mentioned diet. As medical care in sub-Saharan Africa is generally severely limited, the diagnosis of these conditions is often at a very late stage or in severe cases not even detected, making treatment difficult (Mbanya et al., 2010).

2.2.2 Oral pharmacological agents

In the face of non-compliance with lifestyle interventions oral anti-diabetic medication is recommended. As it has been established that improved glycaemic control decreases the risk of development and progression of microvascular complications associated with T2D, most drug therapies aim to reduce hyperglycaemia (Ross et al., 2004). Drugs in this regard include: sulphonylureas, which increase insulin release from pancreatic islets; metformin, which acts to reduce hepatic glucose production; peroxisome proliferator-activated receptor-γ (PPARγ) agonists (thiazolidinediodes), which enhance insulin action and α-glucosidase inhibitors which interfere with gut starch digestion and glucose absorption (Moller, 2001).

However, a major concern with some of the treatments (sulphonylureas and thiazolidinediodes) is enhanced weight gain and hypoglycaemia, which could defeat the objective of treatment and lead to dire health consequences in the long term. Additionally, the drug strategies are not generally viable in regions like sub-Saharan Africa due to the lack of appropriate healthcare systems (Mbanya, et al., 2010). The above-mentioned drugs, their mechanisms and sites of action and adverse effects are summarised in Table 2.1.

The digestive enzymes α-amylase and α-glucosidase have been targeted as potential avenues for modulation of post-prandial hyperglycaemia through inhibition of the enzymatic hydrolysis of starch and dextrins to decrease post-prandial glucose absorption (Mccue, Kwon and Shetty, 2005). According to Ross et al. (2004) compounds that inhibit the digestive enzymes α-amylase and α-glucosidase are an interesting class of anti-diabetic agents as they do not target specific pathophysiological defects in T2D and have minimum

10

adverse effects (Ross et al., 2004). Pancreatic α-amylases are endoglucanases, which hydrolyse the internal α-1,4 glucosidic linkages in starch (polysaccharide) into trisaccharides and disaccharides (Shobana, Sreerama and Malleshi, 2009). Alpha-glucosidase is located in the brush border of the small intestine and is required for the final step in the hydrolysis of starches, dextrins and maltodextrins to glucose, which is absorbed (Mohan, Eskandari, and Pinto, 2013). Inhibitors of these enzymes delay, but do not prevent the absorption of ingested carbohydrates and reduce post-prandial insulin and glucose peaks (Wachters-Hagedoorn, Priebe, Heimweg, Heiner, Elzinga, Stellaard and Vonk, 2007). Patients with T2D demonstrate slow and unmatched insulin response following a meal (Ross et al., 2004). Thus, delaying glucose absorption helps to match the pancreatic insulin response and reduce post-prandial hyperglycaemia.

Table 2.1: Current therapeutic agents for T2D, their mechanisms of action, sites of action and adverse events (adapted from Moller, 2001 and Evans and Rushakoff, 2010)

Class	Drug (brand)	Mechanism of action	Site(s) of action	Adverse events
Sulphonylureas	Glibenclamide	Stimulate insulin secretion from the pancreas	Pancreatic β-cells	Hypoglycaemia and weight gain
Biguanides	Metformin	Decrease hepatic glucose output Increase peripheral glucose uptake	Liver and muscle cells	Nausea, vomiting, diarrhoea, flatulence and lactic acidosis
Thia-zolidinediones	Pioglitazone	Increase peripheral insulin sensitivity	Liver, fat and muscle cells	Weight gain, oedema and anaemia
Alpha-glucosidase inhibitors	Acarbose, Miglitol, Vogibose	Delay carbohydrate absorption	Small intestine	Flatulence, diarrhoea, abdominal pain

11

Acarbose is a tetrasaccharide of microbial origin that inhibits intestinal α-glucosidases and pancreatic α-amylase by reversibly binding to these enzymes (Wachters-Hagedoorn et al., 2007). A dose of 1–1.5 mg acarbose/kg body weight can reduce the post-prandial hyperglycaemia after ingestion of a carbohydrate-containing diet by 50% (Wehmeier and Piepersberg, 2004). The expected and intended mode of action of acarbose is to delay carbohydrate digestion and absorption without induction of malabsorption (Ross et al., 2004). Other anti-diabetic mechanisms of acarbose include increased glucagon-like peptide-1 secretion and decreased triglyceride, low density lipoprotein and glucagon secretion (Ma, 2014). The anti-diabetic mechanisms of action for acarbose are illustrated in Figure 2.4. Since the mechanism of action of α-glucosidase inhibitors is different from other oral anti-diabetic agents, their effects on glycaemic control are additive when used in combination with other oral anti-diabetic agents (Evans and Rushakoff, 2010).
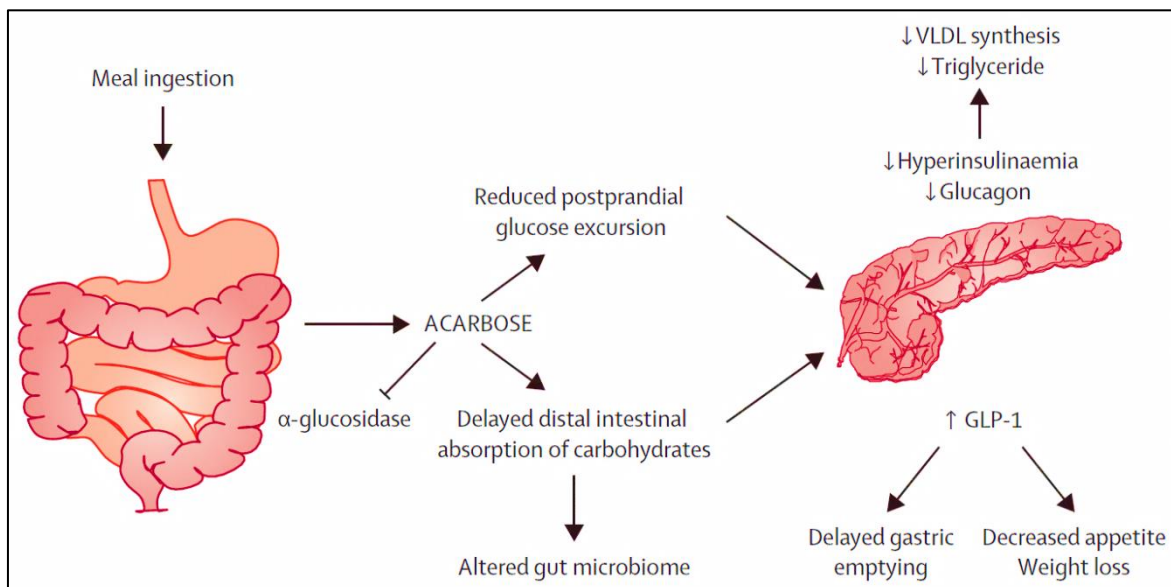
Figure 2.4: Acarbose mechanism of action in type 2 diabetes. VLDL- Very low density lipoprotein, GLP-1 – Glucagon-like peptide 1 (from Ma, 2014).

According to Ma (2014) the MARCH (Metformin and Acarbose in Chinese as the initial Hypoglycaemic treatment) trial that included 788 Chinese type 2 diabetic patients found that acarbose treatment was superior to metformin in controlling hyperinsulinaemia and markedly increased glucagon-like peptide-1 concentration. The authors suggested that as the main component of the Chinese diet is rice and the mean contribution of carbohydrates for energy amongst the patients was more than 65%, the success of acarbose treatment

could be related to high dietary carbohydrate content. The diets of the majority of people in sub-Saharan Africa also mainly consist of carbohydrates (Abrahams et al., 2011). Therefore, this type of anti-diabetic therapy could be particularly effective in this region.

The main drawbacks of acarbose are gastrointestinal disturbances such as diarrhoea, flatulence and abdominal pain (Chiasson, Josse, Hunt, Palmason, Rodge, Ross, Ryan, Tan and Wolever, 1994). This is due to the passing of unabsorbed carbohydrates into the large intestine where osmotic fluid retraction occurs and gastrointestinal flora respire the carbohydrates to produce gaseous products (Ross et al., 2004). These side effects can often be minimized by careful dose titration, and sometimes diminish with time (Evans and Rushakoff, 2010). However, a major advantage of α-glucosidase inhibitors is that they are not associated with hypoglycaemia or weight gain (Moller, 2001; Evans and Rushakoff, 2010).

2.2.3 Insulin

Insulin treatment is the only effective alternative when oral antidiabetic drugs can no longer achieve adequate glycaemic control in T2D (Bailey, 2000). Furthermore, insulin is increasingly being used as a first-line treatment for T2D and evidence is growing that early insulin treatment has a significant effect in delaying or preventing diabetic complications (Diabetes Leadership Forum, 2010). Exogenous insulin supresses glucose production and augments glucose utilization (Moller, 2001). One particular form of diabetes – ketosis-prone atypical diabetes – is mainly found in people of African origin. It involves severe hyperglycaemia and ketoacidosis but can be controlled with insulin treatment (Mbanya, et al., 2010). However, after almost a century of its discovery, insulin is still not available on an uninterrupted basis in many parts of the developing world especially Africa (IDF, 2013). Additionally, exogenous insulin treatment requires close observation by a physician as intensive and uncontrolled insulin treatment could lead to hypoglycaemia and weight gain and could aggravate the diabetic situation (Moller, 2001; Ross et al., 2004).

2.2.4 Nutraceuticals

The negative effects of anti-diabetic drugs and the costs associated with this kind of therapy are becoming increasingly evident and therefore natural approaches are being

13

actively researched. These approaches include the use of functional foods, plant extracts and nutraceuticals (reviewed by Perera and Li, 2012). According to Agriculture and Agri-Food Canada (2012) a nutraceutical is a product isolated or purified from foods that is generally sold in medicinal forms not generally associated with foods and is demonstrated to have a physiological benefit or provide protection against chronic disease. A review by Davì, Santilli, and Patrono (2010) on nutraceutical use for the metabolic syndrome and T2D has highlighted several nutraceuticals used in clinical practice to target the pathogenesis of diabetes, metabolic syndrome and their complications. These compounds include antioxidant vitamins such as vitamins C and E, vitamin D, conjugated linoleic acid, omega-3 fatty acids, minerals such as chromium and magnesium, α-lipoic acid, phytoestrogens, and dietary fibre. Additionally, among the known natural bioactive components and phytochemicals, polyphenols have recently become a focus because of their anti-hyperglycaemic effects, safety and non-observed side-effects (reviewed by Bahadoran, Mirmiran and Azizi, 2013). Therefore, plant phenolics may be appropriate nutraceuticals and supplementary treatments for various aspects of metabolic syndrome and diabetes.

2.3 Dietary polyphenols

Polyphenols are secondary plant metabolites that act as a defence against ultraviolet radiation, oxidants and pathogens (Bahadoran et al., 2013). Polyphenols are found in plant-based foods, such as fruits, vegetables, whole grains, cereals, legumes, tea, coffee and cocoa (Pandey and Rizvi, 2009). Polyphenols contain at least one aromatic ring with one or more hydroxyl groups in addition to other substituents, and they can be divided into several classes according to their chemical structures (reviewed by Xiao, Ni, Kai and Chen, 2013). Simple phenolic acids and flavonoids are two of the most important classes of dietary polyphenols (reviewed by Bravo, 1998). Flavonoids are especially important in the human diet and show significant and diverse health benefits (Lila, 2007).

The basic chemical structure of flavonoids consists of two aromatic rings joined by a three carbon link (Dykes and Rooney, 2006) as illustrated in Figure 2.5. The basic structure allows a multitude of substitutions on the benzene rings A and B (Figure 2.5) (Pandey and Rizvi, 2009). Flavonoids can be classified into subclasses of flavanols, flavanones, flavanols (including catechins), flavones, anthocyanidins and isoflavones (Hollman, 2004).
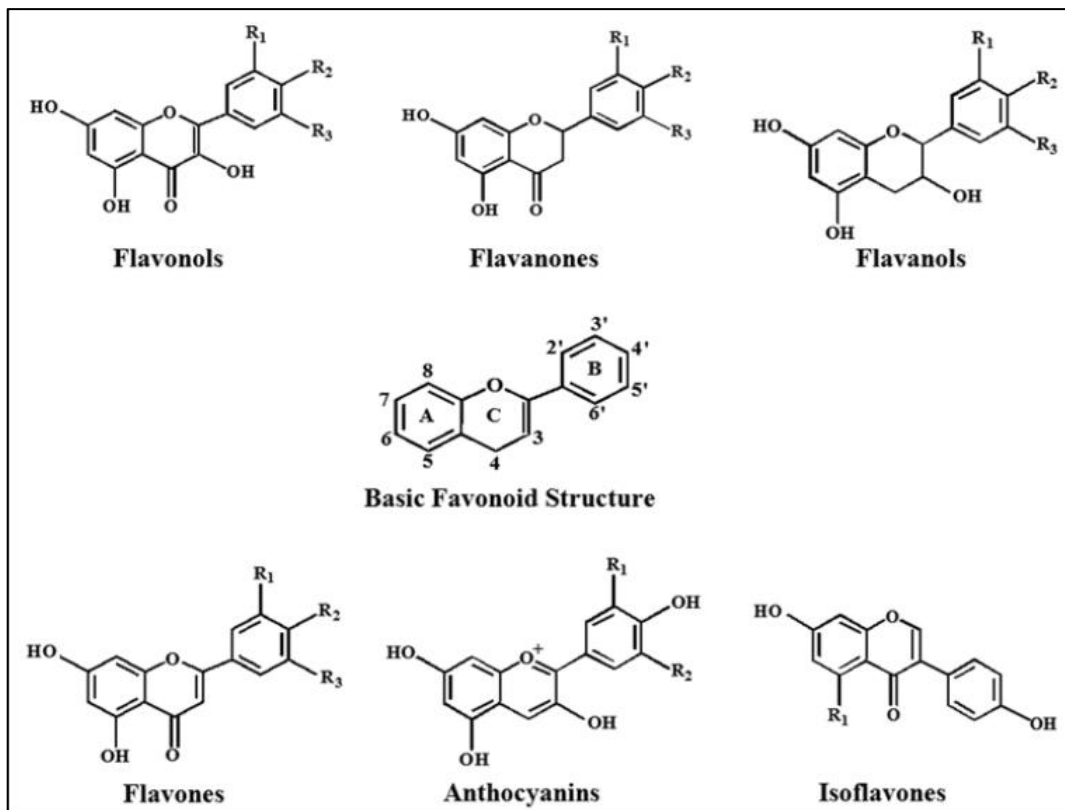
Figure 2.5: Basic structure and subclasses of flavonoids (from Pandey and Rizvi, 2009)

The majority of dietary polyphenols are metabolised by colonic microbiota before absorption, while only a small amount is absorbed directly from upper gastrointestinal tract (Selma, Espin and Thomas-Barberan, 2009). Most flavonoids, except for the subclass of catechins, are present in plants bound to sugars as *b*-glycosides. This structural feature determines whether the flavonoid can be absorbed from the small intestine or has to go to the colon before absorption can occur (reviewed by Hollman, 2004). Gut bacteria modulate polyphenols by various mechanisms including hydrolysis, ring-cleavage, reduction, decarboxylation and demethylation (reviewed by Hollman, 2004). The systemic effects of dietary polyphenols depend largely on the synergistic action that polyphenols may exert after entering circulation and are affected by other constituents present in the diet as well as endogenous factors (reviewed by Hanhineva, Törrönen, Bondia-Pons, Pekkinen, Kolehmainen, Mykkänen and Poutanen, 2010).

Epidemiological investigations strongly suggest that diets rich in foods with high contents of phytochemicals and polyphenolic compounds may be related to lower risk of diabetes and related metabolic diseases (reviewed by Bahadoran et al., 2013). Several biological activities and beneficial properties have been documented for dietary polyphenols. The

15

bioactive properties of polyphenols specifically related to metabolic disease include antioxidant and anti-inflammatory activity and modulation of important cell signalling pathways such as nuclear factor kappa-B, PI-3 kinase/protein kinase B (Akt) and MAP kinase (Han and Loa, 2007). Polyphenols have gained popularity in diabetes research due to their anti-hyperglycaemic effects, safety and non-observed side-effects (Bahadoran et al., 2013). The anti-hyperglycaemic effects of polyphenols are mainly attributed to reduced intestinal absorption of dietary carbohydrates, modulation of the enzymes involved in glucose metabolism, improvement of β-cell function and insulin action and stimulation of insulin secretion (reviewed by Bahadoran et al., 2013).

One of the most studied effects of polyphenols in relation to hyperglycaemia and T2D is their ability to inhibit digestive α-amylase and α-glucosidase – the two main enzymes involved in the conversion of dietary starch to absorbable glucose (Xiao and Högger, 2014). As mentioned, inhibition of these enzymes may be effective in retarding carbohydrate digestion and glucose absorption to suppress post-prandial hyperglycaemia (Ross et al., 2004). A variety of polyphenols have demonstrated α-amylase and α-glucosidase inhibitory activities *in vitro*. The inhibitory polyphenols include flavonoids (anthocyanins, catechins, flavanones, flavonols, flavones and isoflavones), phenolic acids and tannins (proanthocyanidins and ellagitannins) (Hanhineva et al., 2010). The important structure-activity relationship of polyphenols in inhibiting α-amylase and α-glucosidase has recently been extensively reviewed (Xiao et al., 2013; Xiao and Högger, 2014). In short, the hydroxylation (addition of –OH group) and galloylation (esterification with gallic acid) generally increases inhibitory activity of flavonoids against α-amylase and α-glucosidase, while hydrogenation (addition of hydrogen), methylation (addition of a methyl group) and glycosylation (addition of carbohydrate group) reduced inhibitory activity.

Condensed tannins (proanthocyanidins or procyanidins) are one of the main polyphenols in the human diet and are particularly important as they contribute to both the sensorial and nutritional quality of the diet (Rasmussen, Frederiksen, Struntze-Krogholm and Poulsen, 2005). Additionally, condensed tannins have recently received research interest for their positive effects on the metabolic syndrome and T2D (reviewed by Pinent, Cedó, Montagut, Blay and Ardévol, 2012). Therefore the following review sections will focus mainly on condensed tannin and their bioactivities related to metabolic syndrome and T2D.

2.3.1 Condensed tannins

Condensed tannins are polymers and oligomers of flavan-3-ol units (Gu, Kelm, Hammerstone, Beecher, Holden, Haytowitz, Gebhardt, and Prior, 2004). Condensed tannin structures may differ by the nature of the constitutive units, their degree of polymerization (varying between 2 and more than 100), and by the type of linkage (Cheynier, 2005). They are divided into several classes based on hydroxylation of the constitutive units and the nature of the linkages. Their most common constitutive units are (epi)-catechins and (epi)-gallocatechins, leading to procyanidin and prodelphinidin structures, respectively (Bahadoran et al., 2013). The complexity of the condensed tannin chemical structure and the difficulty in their quantification has prevented accurate estimation of their intake, which may range from tens to several hundred mg/day depending on the diet (reviewed by Santos-Buelga and Scalbert, 2000).

Research on the effect of gastric digestion on condensed tannins is limited. Nonetheless, Spencer, Chaudry, Pannala, Srai, Debnam and Rice-Evans (2000) showed that under simulated gastric conditions cocoa condensed tannins are hydrolysed to mixtures of epicatechin monomer and dimers. Conversely, *in vivo* rat studies showed that ingested grape seed condensed tannins were not depolymerised to monomeric and dimeric catechin units in the gastrointestinal tract as there was no catechin measured in the plasma or urine of rats after ingestion of the grape seed extract (Donovan, Lee, Manach, Rios, Morand, Scalbert and Rémésy (2002). Furthermore, Rios, Bennett, Lazarus, Rémésy, Schalbert and Williamson (2002) studied the effect of human gastric digestion on condensed tannins structure by providing healthy subjects with a condensed tannin-rich cocoa beverage and sampled their stomach contents using a nasogastric tube. High performance liquid chromatography (HPLC) of the stomach content showed that after consumption and gastric transit there was no change in the HPLC profile compared to the condensed tannins not subjected to digestion of the condensed tannins-rich cocoa beverage, suggesting that condensed tannins were stable in the gastric environment.

Concerning the absorption and bioavailability of condensed tannins, the most consistent finding is that condensed tannins are poorly absorbed, while monomeric flavonoids are absorbed relatively easily. For example, Jimenez-Ramsey, Rogler, Housley, Butler and

Elkin (1994) fed lyophilized radiolabelled (^{14}C) condensed tannins to chicks and analysed the distribution of ^{14}C in chick tissues and excreta. Their analysis suggested that ^{14}C condensed tannins from sorghum grain were not absorbed from the digestive tract of chicken, while the ^{14}C labelled non-tannin polyphenols was absorbed and distributed in various tissues. Additionally, *in vitro* studies with Caco-2 cells showed that only catechin dimers and trimers were able to pass across monolayers of these cells (Deprez, Mila, Huneau, Tome and Scalbert, 2001). Even though condensed tannins are poorly absorbed in the small intestine, once the condensed tannins reach the colon, bacteria in the colon extensively metabolise condensed tannins by breaking down the flavonoid ring structure into phenolic acids that can be absorbed and has been measured in plasma and urine (Hollman, 2004).

The most significant biological effect of tannins on human and animal nutrition is their ability to interact strongly with proteins (Le Bourvellec and Renard, 2012). The strong interaction between condensed tannin and protein has been the main reason for their ability to bind and thereby inhibit digestive enzymes (Gonçalves, Mateus and de Freitas, 2011). However, the potential of condensed tannins in the alleviation and prevention of metabolic syndrome and T2D does not solely rely on the ability to inhibit digestive enzymes. The following section reviews the possible mechanisms of condensed tannins to alleviate and prevent metabolic syndrome and T2D.

2.3.2 Potential mechanisms of condensed tannins in alleviating and preventing metabolic disease

The interactions of condensed tannins with molecules of biological significance have important nutritional and physiological consequences (Schofield, Mbugua and Pell, 2001). These effects are mainly observed with proteins, where condensed tannins form indigestible complexes and inhibit digestive enzymes (Taylor, Bean, Ioerger and Taylor, 2007; Gonçalves et al., 2011). There have been several *in vitro* studies on the effects of condensed tannin on digestive enzymes. For example, Barret, Ndou, Hughey, Straut, Howell, Dai and Kalentunc (2013) found that condensed tannins from cocoa, pomegranates, cranberries and grapes inhibited α-amylase to varying degrees. Moreover, a condensed tannin extract of grape seeds and skin at a concentration of 1 mg/mL has been found to inhibit 80% of lipase activity *in vitro* (Moreno, Ilic, Poulev, Brasaemle, Fried,

18

Raskin, 2003). Adisakwattana et al. (2010) studied the effects of grape seed extract (containing condensed tannins) on lipid digestion and absorption *in vivo* and found that acute administration of grape seed extract markedly suppressed the elevation of serum triglyceride and cholesterol in normal rats. Importantly, *in vivo* studies using grape seed extract indicate that they seem to lack toxicity (Tucci, Boyland, Halford, 2010) and hence could be used as a safe weight-loss strategy.

Additional to enzyme inhibition, condensed tannins can also modulate the expression and activity of other important metabolic molecules. Pinent, Blay, Blade, Salvado, Arola and Arswvol (2004) studied the effect of grape seed condensed tannins on streptozotocin-induced diabetic rats. They found that these condensed tannins increased glucose uptake in adipocytes and myotubes and acts through particular intracellular mediators described for the insulin signalling pathway such as the PI-3K and p38 MAPK.

Pinent et al (2004) stated that a suitable anti-diabetic agent should have insulin-like activity or it should bypass the defects in insulin action characterized by insulin resistance. A study by Montagut, Onnockx, Vaqué, Bladé, Blay, Fernández-Larrea, Pujadas, Salvadó, Arola, Pirson, Ardévol and Pinent (2010) showed that condensed tannins from grape seeds are able to increase the amount of insulin-sensitive glucose transporter, GLUT-4, in the plasma membrane. Their work was specifically on oligomers of grape-seed procyanidin extract (GSPE) and they found that oligomeric GSPE activate the insulin receptor by interacting with and inducing the phosphorylation of the insulin receptor and that this interaction leads to increased glucose uptake. These authors also showed that Protein Kinase B is required for GSPE-induced glucose uptake. Furthermore, they showed that GSPE phosphorylates proteins of the insulin signalling pathway differently than insulin does (Figure 2.1). Their results indicate to Akt, p44/42 and p38 MAPKs as key points for GSPE-activated signalling mechanisms.

A study by Kurimoto, Shibayama, Inoue, Soga, Takikawa, Ito, Nanba, Yoshida, Yamashita, Ashida, and Tsuda, (2013) showed that black soybean seed coat contains abundant levels of condensed tannins. Additionally, their study found that dietary black soybean seed coat extract ameliorates hyperglycaemia and insulin resistance via the activation of adenosine monophosphate-activated protein (AMP)-kinase (AMPK) in type 2 diabetic mice. AMPK was activated in the skeletal muscle and liver of diabetic mice fed

19

the soybean extract. This activation was accompanied by the up-regulation of GLUT-4 in skeletal muscle and the down-regulation of gluconeogenesis in the liver.

2.4 Condensed tannin sources in sub-Saharan Africa

Condensed tannins are found in several fruits and vegetables (reviewed by Santos-Buelga and Scalbert, 2000). However, the most significant source of condensed tannins in the diets of populations in sub-Saharan Africa comes from the consumption of cereals (Dykes and Rooney, 2006). As mentioned, sorghum is a major cereal in Africa and is produced in 41 African countries (FAOSTAT, 2011). Additionally, sorghum grain containing condensed tannins dominate production in hot and humid regions of Africa as they have resistance to grain moulds and birds allowing their successful production (Dykes and Rooney, 2006). Therefore, condensed tannin containing sorghum is a major food crop in eastern and southern Africa. Furthermore, the condensed tannin content (milligrams catechin equivalents (CE) per 100g) of high-tannin sorghum (approx. 780 mg CE/100g) is higher compared to the amounts found in a range of different plant foods including red kidney beans (570 mg CE/100g), cranberries (430 mg CE/100g), strawberries (150 mg CE/100g), red grape (64 mg CE/100g) and cereals like buckwheat (7 mg CE/100g) and barley (100 mg CE/100g) (Wu, Li, Xiang, Zhu, Lin, Wu, Li, Pandravada, Ridder, Bai, Wang, Trick, Bean, Tuinstra, Tesso and Yu, 2012). In view of the multiple health benefits associated with condensed tannins, obtaining condensed tannins from sorghum grains could be viable means of utilising their health benefits in the sub-Saharan African region that is burdened with a rapid rise in T2D and related complications. Therefore reviewing the phenolic composition and bioactive properties of SCT in relation to T2D and metabolic syndrome is essential and will be addressed briefly.

2.4.1 Sorghum condensed tannin composition and bioactive properties related to metabolic syndrome and type 2 diabetes

The phenols in sorghums comprise two major categories; phenolic acids and flavonoids. The phenolic acids are benzoic or cinnamic acid derivatives (Hahn, Faubion and Rooney, 1983), whereas the flavonoid group include condensed tannins and anthocyanins (Awika and Rooney, 2004). The condensed tannins found in sorghum are polymeric forms of mainly flavan-3-ols and flavan-4-ols and their derivatives (Figure 2.6). The degree of

20

polymerisation ranges from 2 to 10, and typically occurs via C4→C8 interflavan bonds, known as B-type linkages (Awika, 2011). There is a great diversity in sorghum tannin polymer composition. Catechin, epicatechin, gallocatechin, epigallocatechin, eridictyol and their glycosides are chain extenders or terminators (Krueger, Vestling and Reed, 2003) (Figure 2.6).
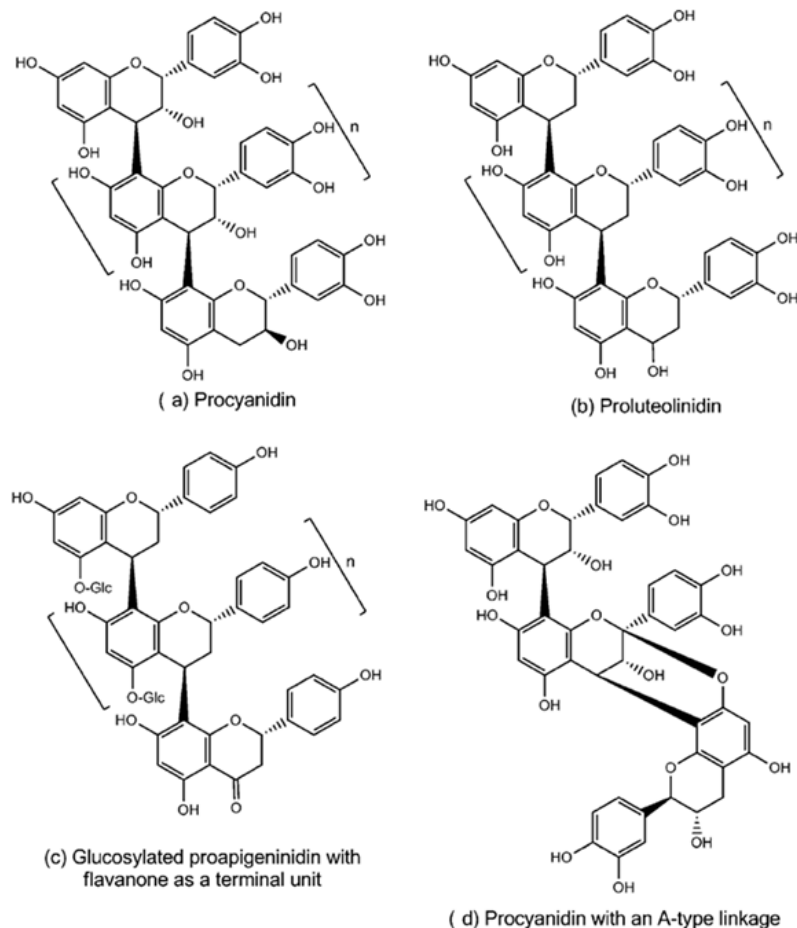
Figure 2.6: Some condensed tannin structures identified in sorghum (from Awika, 2011).
. Dimers, n = 0, otherwise n = 1 →10. Glc-glucose

SCT are well known for their ability to bind with proteins (Butler et al., 1984), which when orally ingested, leads to sensations of astringency in the mouth. SCT also decrease protein digestibility by their ability to bind and inhibit digestive enzymes (Taylor et al., 2007). The binding between SCT and proteins involves hydrogen bonding and hydrophobic interactions and SCT preferably bind to proteins rich in proline with an open structure and large molecular weights (Butler et al., 1984).

Sorghum phenolic extracts (including SCT) have been shown to reduce the absorption of nutrients by inhibition of lipids (Moreno et al., 2003), protein and carbohydrate digestive

21

enzymes (Al-Mamary et al., 2001; Hargrove, Greenspan, Hartle and Dowd, 2011). Although their enzyme inhibitory characteristics were previously deemed negative, SCT are currently a topic of great interest regarding human health in terms of reducing dietary caloric intake, increasing antioxidant status and regulating hyperglycaemia (Awika, 2011). For example, Barros, Awika and Rooney (2012) found that SCT interact with amylose and significantly increasing the resistant starch content of normal and high amylose starches which would subsequently lead to decreased digestibility of these carbohydrates. Also, Lemlioglu-Austin, Turner, McDonough and Rooney (2012) found that high condensed tannin containing sorghum bran extracts decreased maize starch porridge digestibility, estimated glycaemic index and increased the resistant starch content.

Further, Hargrove et al. (2011) found that inhibition of α-amylase by sorghum bran extracts showed that extracts high in condensed tannin were more effective at inhibiting α-amylase than the condensed tannin-free fractions. In fact, Gonçalves et al. (2011) found that α-amylase was inhibited by condensed tannin in a dose responsive manner *in vitro*. Ethanol extracts of sorghum polyphenols (containing condensed tannins) also inhibited α-glucosidase (better than acarbose) and substantially inhibited porcine and human α-amylase (Kim et al., 2011).

Sorghum phenolic extracts (condensed tannin content not specified) have also been shown to have *in vivo* efficacy in terms of hypoglycaemic activity in diabetic rats (Chung, Kim, Yeo, Kim, Seo and Moon, 2011). However, the mechanism by which they exert anti-diabetic effects was not clear. Upon investigation into gluconeogenesis as a possible mechanism, Kim and Park (2012) showed that the hypoglycaemic effect of their sorghum extract was related to hepatic gluconeogenesis but not stimulation of glucose uptake in skeletal muscle. They observed that the hypoglycaemic effect of their sorghum extract was similar to that of metformin (Owen, Doran and Halestrap, 2000).

The health benefits associated with sorghum phenolics indicated that SCT could be employed as a nutraceutical for the alleviation and prevention of metabolic syndrome and T2D related pathologies. However, the application of SCT as a nutraceutical against the metabolic syndrome and T2D appear to rely primarily on their ability to inhibit digestive amylase enzymes and thereby reduce post-prandial hyperglycaemia. However, for condensed tannins to have inhibitory effects against digestive enzymes, it is necessary that

they are protected against possible depolymerisation and binding with digestive proteins and enzymes not targeted for glycaemic control. As mentioned, depolymerisation can occur during the gastric phase of digestion (Spencer et al., 2000) and dimeric and trimeric tannins have weaker protein binding ability and can be more readily absorbed, thereby limiting their inhibition potential in the small intestine (Awika, Dykes, Gu, Rooney and Prior, 2003; Serrano, Puupponen-Pimiä, Dauer,, Aura and Saura-Calixto, 2009; Gonçalves et al., 2011). Therefore, the delivery of these molecules requires a delivery system that will ensure that the active molecular form is contained until the condensed tannin molecule reaches the site of action.

2.5 Condensed tannin delivery system

Janaswamy and Youngren (2012) state two important characteristics of an efficient nutraceutical delivery system are to preserve the active structural form of the nutraceutical until the time of delivery and effective delivery of the nutraceutical to the physiological target. An additional function of a condensed tannin delivery system should be to mask their adverse sensory characteristics. Condensed tannins in general are responsible for the sensation of astringency as they interact with the salivary proteins (Gonçalves et al., 2011) and SCT in particular has been described as being astringent and bitter (Kobue-Lekalake et al., 2009). Furthermore, SCT could bind to non-target enzymes and dietary proteins in the oral and gastric environment and could thereby lose their efficacy to bind and inhibit digestive amylases. Therefore, a SCT delivery should be able to protect SCT during the gastric phase of digestion, but "release" the SCT in the small intestine where it can exert its beneficial effect and be safe for human consumption. Taylor et al. (2009a) encapsulated SCT in kafirin microparticles and studied the release of antioxidants under simulated gastric conditions. They found that the SCT released approximately 50% of their total antioxidant activity after 4 hours of simulated digestion, indicating that with kafirin microparticle encapsulation some bioactivity of SCT could be retained. Hence, this type of system could be applied in the encapsulation of condensed tannins.

2.5.1 Microencapsulation

Microencapsulation can be defined as a technology of packaging solids, liquids or gaseous materials in miniature sealed capsules that can release their content at controlled rates

under specific conditions (reviewed by Desai and Park, 2005). The main objective of encapsulation, including microencapsulation is to protect the active ingredients from adverse environmental conditions and promote controlled release of the active substance (Shahidi and Han, 1993). Encapsulation of plant polyphenols has only recently commenced (reviewed by Fang and Bhandari, 2010) and there is currently only one published article involving the encapsulation of SCT (Taylor et al., 2009a). In contrast, encapsulation of polyphenols including condensed tannins isolated from tea, fruits and vegetables has been investigated using various encapsulation systems (Oidtmann, Schantz, Mäder, Baum, Berg, Betz, Kulozik, Leick, Rehage, Schwarz and Richling, 2012; Davidov-Pardo, Arozarena and Marín-Arroyo, 2013).

The type of wall material is critical when encapsulating phenolics as it affects the microparticle stability, process efficiency and the degree of protection of the active ingredient (Desai and Park, 2005). Generally, wall materials are composed of synthetic polymers or bio-based materials such as carbohydrates, fats, waxes and animal or plant derived proteins (Nesterenko, Alric, Silvestre and Durrieu, 2013). The use of plant proteins as wall-forming materials in microencapsulation reflects the current "Green" trend in the pharmaceutical, cosmetic and food industries as they are considered renewable and biodegradable resources (Nesterenko et al., 2013). Additionally, plant proteins may be less allergenic compared to animal derived proteins (Jenkins, Breiteneder and Mills, 2007).

However, the use of plant proteins has been very limited in encapsulation (Nesterenko et al., 2013). Notwithstanding, Parris, Cooke and Hicks (2005) encapsulated essential oils in zein (maize prolamin protein) nanospheres by phase separation and found that the particles appeared to have limited digestibility in the stomach, slow release in the small intestine, and more rapid release in the large intestine using an *in vitro* digestion model. They suggested that zein nanospheres could be useful for oral or injectable administration of biological materials. Furthermore, Taylor, Taylor, Belton and Minnaar, (2009b) prepared kafirin (sorghum prolamin protein) microparticles by phase separation. Due to the high proportion of proline residues found in kafirin protein (Belton et al., 2006), kafirin forms hydrogen bonds and hydrophobic interactions between peptide chains and condensed tannin that are resistant to gastric digestion. Additionally, these kafirin microparticles had many internal holes or vacuoles and the authors suggested that the larger internal surface area of kafirin microparticles may be advantageous for use as encapsulation agents.

The two main techniques used for encapsulation with plant proteins are spray-drying and coacervation (Nesterenko et al., 2013). Spray drying is a continuous process to convert a liquid into a solid powder of microparticles. The initial liquid (solution, emulsion or suspension) containing wall and core material is sprayed into a stream of heated air and the solvent evaporates to give instantaneous powder (Gouin, 2004). Microencapsulation by coacervation involves the precipitation of the wall forming materials around the active core by change in pH or temperature, or by the addition of a non-solvent or electrolyte compound (Desai and Park, 2005).

The two major morphologies produced for encapsulation by coacervation and spray-drying are microcapsules which have a core enveloped by a shell and aggregates which have many cores embedded in a matrix (Schrooyen, van der Meer and De Kruif, 2001). The morphological characteristics of polyphenol encapsulating microparticles prepared by spray drying and coacervation are illustrated in Figure 2.7. Particle size obtained by spray-drying is between 1 µm and 50 µm, while sizes obtained by coacervation can vary from nanometers to several hundred microns (Nesterenko et al., 2013). Spray drying and coacervation can result in high encapsulation efficiency, which is defined as the ratio between the percentage of active core encapsulated in the particles and the percentage of active core in the initial liquid (Nesterenko et al., 2013).
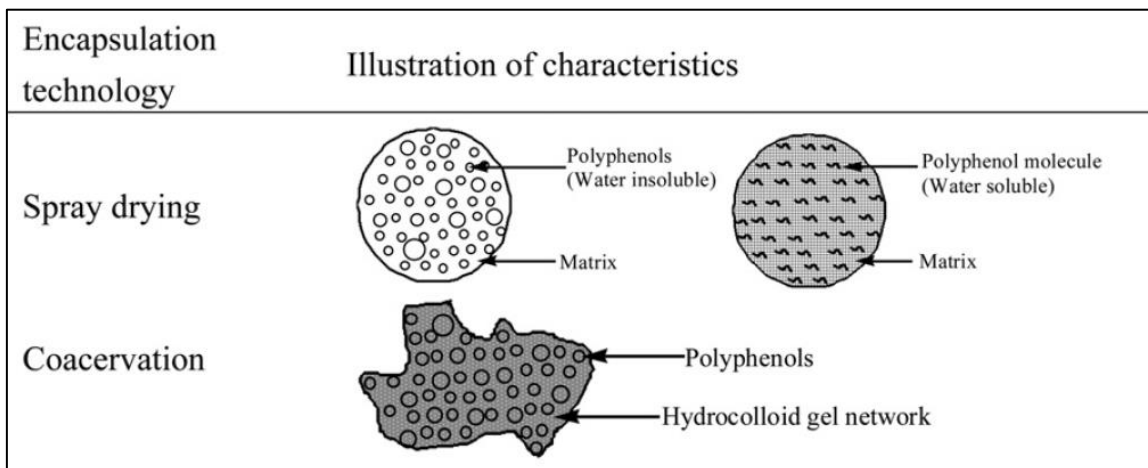
Figure 2.7: Characteristics of microcapsules produced by spray drying and coacervation (modified from Fang and Bhandari, 2010).

2.6 Methodologies for the assessment of plant extracts as anti-diabetic agents

The anti-diabetic effects of plant extracts can be studied *in vitro* using a variety of test systems or *in vivo* using animal models. *In vitro* tests can play a very important role in the evaluation of anti-diabetic activity of drugs as initial screening tools and they can provide useful information as to the mechanism of action of the therapeutic agent (Thorat, Patil, Limaye and Kadam, 2012). *In vivo* tests involve the use of several animal models to simulate the human diabetic condition and assess the effects of potential anti-diabetic compounds on disease development and pathology (Eddouks, Chattopadhyay and Zeggwagh, 2012).

2.6.1 *In vitro*

One fundamental anti-diabetic *in vitro* test is the inhibition of the digestive amylases, pancreatic α-amylase and α-glucosidase, as these are the main enzymes responsible for the hydrolysis of starch to glucose (Sales, Souza, Simeoni, Magalhães and Silveira, 2012). Digestive amylase inhibition can be determined using isolated enzymes and their substrates and assessing the hydrolysis of the substrate spectrophotochemically. For example, α-amylase inhibition can be assessed using soluble starch as substrate and 3,5-dinitrosalicylic acid (DNS) to detect reducing sugars after enzymatic digestion of starch (Kim et al., 2011). However, a much more specific assay is the Ceralpha method developed by McCleary and Sheehan (1987) which uses a defined oligosaccharide as substrate and is therefore specific for α-amylase (Tadera, Minami, Takamarsu and Matsuoka, 2006). The most commonly used α-glucosidase assay uses a specific maltoside, ρ-nitrophenyl-α-D-glucopyranoside as substrate (Mccue et al., 2005).

Testing the efficacy of newly developed nutraceutical delivery system *in vitro* also depends on the availability of digestion models that accurately simulate the complex physiochemical and physiological events that occur in the human gastrointestinal tract (Hur, Lim, Decker and McClements, 2011). During the past few years, several *in vitro* digestion models have been developed to test the biological effects of polyphenol ingestion under simulated gastrointestinal conditions (Serra, Macia, Romero, Valls, Blade, Arola and Motilva, 2010). Factors, such as sample characteristics, enzyme activity, ionic

composition, applied mechanical stresses and digestion times, have significant influences on the results of *in vitro* digestion methods (Hur et al., 2011).

To study the efficiency of a bioactive compound during digestion, different enzymes are usually added sequentially to simulate the different phases of the digestive process. It has been reported that using a single purified enzyme, rather than a complex biological mixture, may be advantageous because it facilitates the standardization of *in vitro* digestion models (Coles, Moughan, Darragh, 2005). For example, *in vitro* protein digestion models are often based on incubations with pepsin to simulate the stomach and then trypsin and chymotrypsin to simulate the small intestine (Almaas, Holm, Langsrud, Flengsrud and Vegarud, 2006) Additionally, Taylor et al. (2009a) assessed the efficiency of an bioactive polyphenol delivery system by determining the antioxidant release profiles of catechin and SCT during simulated pepsin followed by trypsin-chymotrypsin digestion. To accurately simulate starch digestion *in vitro* models generally firstly subject samples to protein digestion before α-amylase digestion is assessed (Weurding, Veldman, Veen, van der Aar, and Verstege, 2001). Mkandawire, Kaufman, Bean, Weller, Jackson and Rose (2013) used a multi-step *in vitro* starch digestion protocol to investigate the effects of tannins on starch digestion of sorghum flour. They firstly subjected samples to pepsin digestion before samples were digested with pancreatin and amyloglucosidase.

Positive *in vitro* α-amylase and α-glucosidase inhibitory action may not always correlate with *in vivo* actions (Ali, Atangwho, Kuar, Ahmad, Mahmud and Asmawi (2013). However, strong *in vitro* evidence of a bioactive compound's anti-diabetic activity under simulated digestive conditions warrants further investigations into the compounds ability as an anti-diabetic agent. Therefore, the next step is to confirm findings by using suitable *in vivo* methodologies.

2.6.2 *In vivo*

In vivo trials, using animals or humans, usually provide the most accurate results and are therefore used to confirm positive *in vitro* findings. Animal models have been used extensively to investigate the *in vivo* efficacy, mode of action and side effects of anti-diabetic plants (Eddouks et al., 2012). The most widely used animal models are small

rodents, which are less expensive to maintain compared to larger animals and generally show a more rapid onset of the diabetic condition (Ross et al., 2004; Eddouks et al., 2012).

For example, healthy non-diabetic rodents have been used to demonstrate hypoglycaemic activity of various natural compounds (Eddouks et al., 2012). For example, Ali et al. (2013) demonstrated that extract of *Phaleria macrocarpa* fruit pericarp can decrease post-prandial hyperglycaemia in healthy male Sprague Dawley rats. Additionally Jo, Ha, Moon, Lee, Jang and Kwon (2011) used the healthy Sprague Dawley rat model to demonstrate the hypoglycaemic effect of a phenolic rich water extract of Omija (*Schizandra chinensis*) on temporarily induced post-prandial hyperglycaemia. However, to study the effect of natural compounds on specific aspects of diabetes pathology, disease-specific rodent models are used.

As reviewed by Srinivasan and Ramarao (2007), diabetic rodent models can be obtained spontaneously, induced with chemicals or high-fat and/or high sugar diets and by surgical removal of parts of the pancreas. Furthermore, knockout and transgenic technology has made it possible to induce diabetes by deleting or overexpressing genes related to diabetic development (reviewed by Wang, Sun, Sun, Liu, Wang, Xu and Miao, 2013).

Many studies assessing the anti-diabetic effects of plant extracts have used rodents whose pancreatic insulin production has been chemically destroyed with alloxan or streptozocin (Deshmuk, Yadav, Badole, Bodhankar and Dhaneshwar, 2007). For instance, Kim and Park (2012) tested the effect of a polyphenol rich sorghum extract on hepatic gluconeogenesis of streptozotocin-induced diabetic Winstar rats. Chung et al. (2011) evaluated the anti-diabetic effects from phenolic extracts of three Korean sorghum varieties in normal and streptozotocin-induced diabetic rats. Furthermore, Kumar, Lakshman, Jayaveea, Shekar, Khan, Thippeswamy and Veerapur (2012) investigated the antidiabetic, antihyperlipidemic and antioxidant activities of methanolic extract of whole plant of *Amaranthus viridis Linn* in alloxan induced diabetic rats.

Although widely used, these chemically-induced diabetic models attract criticism because of the artificial way in which diabetes is induced (Houghton, Howes, Lee and Steventon, 2007). Additionally, it has been shown that rats with chemically induced diabetes that are exposed to prolonged blood sugar extremes, typically induced during experiments, can

28

lead to confusion, convulsions and eventually life-threatening diabetic coma (Matteucci and Giampietro, 2008).

In the attempt to naturally replicate the disease process as it occurs in humans, diet-induced diabetic rodent models are typically used to examine the anti-diabetic effects of natural compounds (Eddouks et al., 2012). Type 2 diabetes and the metabolic syndrome are closely associated with the overconsumption of a high-fat and/or high sugar diet (Preuss, 2009). Therefore, diet-induced diabetic rodent models are used to mimic human disease development. For example, Liu, Tzeng, Liou and Lan (2007) used a high-fructose induced insulin resistant rat model to demonstrate the effect of the flavonol myricetin on insulin sensitivity. Moreover, Pranprawit et al. (2013) showed that a high-fat high-sugar diet fed to rats over eight weeks induced several signs of the metabolic syndrome in Sprague Dawley rats. Similarly, Park, Lee, Chung and Park (2012) used a high-fat fed obese mice model to investigate the hypothesis that a sorghum phenolic extract exerts anti-diabetic effects

2.6.3 The glucose and starch tolerance test

The oral glucose tolerance test (OGTT) is a simple test that is widely used in clinical practice to diagnose insulin resistance and T2D (American Diabetes Association, 2013) by measuring the body's ability to dispose an oral glucose load into peripheral tissue (Ayala, Samuel, Morton, Obici, Croniger, Shulman, Wasserman and McGuinness, 2010). The protocol for carrying out an OGTT is relatively simple. Following an overnight fast, a glucose load is orally administered and blood glucose is measured over a span of 2 hours. A blood sample is taken prior to the glucose load to determine baseline blood glucose levels and then at 15 to 30-minute intervals following the glucose load for the duration of the experiment (Ayala et al. 2010). In some cases (mainly animal experiments), an intraperitoneal glucose tolerance test is used to assess glucose and insulin tolerance by injecting a glucose solution into the intraperitoneal cavity (Andrikopoulos, Blair, Deluca, Fam, and Proietto, 2008).

The OGTT has been widely used to assess the anti-diabetic effect of specifically plant extracts. For example, Kumar et al. (2012) used an OGTT to assess the effects of daily administrations (200-400 mg/kg) of polyphenol-rich *Amaranthus viridis* extract on the glucose tolerance of diabetic rats. Additionally, Park et al. (2012) used the OGTT to

evaluate the effects of long term (6 weeks) oral administrations of 0.5% and 1% sorghum phenolic extract on the glucose tolerance of high-fat fed mice. Whereas Kim and Park (2012) employed an intraperitoneal glucose tolerance test to assess the anti-diabetic effects of oral administration of 0.4 g/kg and 0.6 g/kg body weight sorghum phenolic extract over six weeks.

There are various versions of the test depending on the specific effect being investigated. For instance, to determine the effect of potential anti-diabetic compounds on hyperglycaemia induced by carbohydrate loads, the oral starch tolerance test (OSTT) has been used. Wolf, Humphrey, Hadley, Maharry, Garleb and Firkins (2002) used an OGTT, OSTT and a sucrose loading test to evaluate the effects of supplemental fructose on post-prandial glycaemia of rats. Additionally, an OSTT was used on streptozotocin-diabetic rats to determine the effects of long term (21 days) oral administration of fenugreek seed and balanites fruit extracts (1.5 g/kg body weight) on starch digestion and absorption *in vivo*. Also, Ali et al. (2013) investigated the effects of *Phaleria macrocarpa* fruit extracts on *in vivo* α-glucosidase and α-amylase activity by assessing starch digestion and absorption with the use of an OSTT. Futhermore, Itoh, Kita, Kurokawa, Kobayashi, Horio and Furuichi (2004) found that a condensed tannin containing Adzuki bean extract reduced hyperglycaemia after an OSTT on mice. However, it does not appear that the OSTT has been used to measure the inhibition of starch digestion as a potential anti-diabetic mechanism of sorghum phenolic extracts.

2.7 Conclusions

The metabolic syndrome and T2D are related major health issues in sub-Saharan Africa and there is a need for affordable and easily accessible means for their prevention and control. The challenge with currently used drugs is that they are not generally accessible to people in sub-Saharan Africa and their use can have adverse side effects. Dietary polyphenols can alleviate and prevent hyperglycaemia associated with T2D and metabolic syndrome and condensed tannins in particular have shown potential as a nutraceutical against metabolic syndrome and T2D. Sorghum is a good source of condensed tannins in sub-Saharan Africa and it has been shown that SCT could not only inhibit digestive enzymes and delay macromolecule absorption, but also demonstrate insulin-like effects. However, the majority of *in vitro* studies on the use of condensed tannins as anti-diabetic and anti-metabolic syndrome agents do not simulate the physiological environment appropriately. Additionally, there is limited *in vivo* research to confirm the enzyme inhibitory effects of SCT. Though it has been shown that oral dosage of condensed tannins reduces its efficacy, there has been a lack in studies on the effects of condensed tannin encapsulation on amylase enzyme activity after simulated digestion. Further, encapsulation of condensed tannin is necessary to avoid possible depolymerisation, non-specific protein binding and mask the adverse sensory qualities of SCT. Kafirin has potential as an encapsulation agent for SCT as it is a natural (food sourced) protein that is resistant to gastric digestion and readily binds SCT. Therefore, research is required to determine whether SCT encapsulated in kafirin microparticles can retain their ability to inhibit intestinal α-amylase and α-glucosidase after digestion and have potential as an anti-diabetic and anti-metabolic syndrome nutraceutical agent.

CHAPTER 3: HYPOTHESES AND OBJECTIVES

3.1. Hypotheses

3.1.1 Hypothesis 1

Encapsulation of sorghum condensed tannins (SCT) in kafirin microparticles (KEMS) will enable SCT to retain their inhibitory activity against α-amylase and α-glucosidase enzymes after simulated *in vitro* digestion compared to SCT alone (i.e. not encapsulated).

Spencer et al. (2000) demonstrated that condensed tannin oligomers were depolymerized to monomers and dimers in a simulated gastric environment. While Gonçalves et al. (2011) found that depolymerisation of condensed tannins decreased their inhibitory effect on α-amylase. Additionally, as SCT interact with proteins in a non-specific manner (Butler et al., 1984) this could lead to a reduction or complete loss of inhibitory action against α-amylase and α-glucosidase in the small intestine (active site) when SCT is administrated orally. Due to the high proportion of proline residues found in kafirin protein (Belton et al., 2006) kafirin forms hydrogen bonds and hydrophobic interactions between peptide chains and condensed tannin that are resistant to gastric digestion (Butler et al., 1984; Emmambux and Taylor, 2003; Taylor et al., 2007; Taylor et al., 2009b). Therefore, SCT encapsulation in KEMS will protect SCT against possible depolymerisation and non-specific protein binding that could occur during gastric digestion.

3.1.2 Hypothesis 2

Oral administration of SCT encapsulated in KEMS (SCT-KEMS) will decrease blood glucose and serum insulin levels *in vivo* compared to SCT alone and acarbose (standard α-glucosidase inhibitor) after an oral starch tolerance test (OSTT) on healthy rats.

Ali et al. (2013) showed that oral administrations of polyphenols extracted from *Phaleria macrocarpa* fruit pericarp reduced the peak blood glucose and area under the glucose tolerance curve (AUC) of healthy and diabetic rats after an OSTT. Additionally, Itoh et al. (2004) found that a hot water Adzuki bean extract known to contain condensed tannins reduced hyperglycaemia after an OSTT on mice. Sorghum phenolic extracts rich in

condensed tannin have also been found to decrease hyperglycaemia in diabetic rats and mice fed a high-fat diet (Park et al., 2012).

3.2 Objectives

3.2.1 Objective 1

To determine the inhibitory effects of SCT-KEMS on α-amylase and α-glucosidase activity after simulated digestion using an *in vitro* model gut system with the aim of assessing SCT-KEMS as a simple delivery vehicle for SCT to the small intestine to inhibit digestive amylases.

3.2.2 Objective 2

To determine the effect of oral administration of SCT-KEMS on blood glucose and serum insulin levels after an OSTT on healthy rats with the aim of demonstrating the potential of SCT-KEMS as a nutraceutical against post-prandial hyperglycaemia.

CHAPTER 4: RESEARCH

4.1 Encapsulation of sorghum condensed tannins in kafirin microparticles to inhibit digestive amylases

Published in part as Links, M. R., Taylor, J., Kruger, M. C., Taylor, J. R. 2015. Sorghum condensed tannins encapsulated in kafirin microparticles as a nutraceutical for inhibition of amylases during digestion to attenuate hyperglycaemia. Journal of Functional Foods 12, 55-63.

4.1.1 Abstract

Type 2 diabetes (T2D) prevelance is increasing rapidly in sub-Saharan Africa. An appropriate therapeutic approach is to use compounds capable of inhibiting intestinal carbohydrate digesting enzymes. A crude preparation of sorghum condensed tannins (SCT) was highly effective (approx. 20,000 times) at inhibiting α-glucosidase compared to acarbose, while acarbose was a better α-amylase inhihitor (approx. 180 times). Microparticles prepared from kafirin (KEMS) were investigated as an oral delivery system for SCT. SCT were encapsulated in KEMS (SCT-KEMS) using simple acetic acid and aqueous alcohol coacervation methods. The encapulation efficiency of SCT in the KEMS using the acetic acid method was approx 25% and the aqueous ethanol method 48%. Quantitative data and electron microscopy revealed that SCT-KEMS were digested to only a limited extent during simulated gastrointestinal digestion with pepsin and trypsin-chymotrypsin. Hence, SCT-KEMS retained their inhibitory activity against both amylases, throughout simulated gastrointestinal digestion, whereas unencapsulated SCT lost most of their inhibitory activity. Therefore, SCT-KEMS have potential as a nutraceutical to attenuate hyperglycaemia and control T2D development.

4.1.2 Introduction

Diabetes has emerged as one of the major non-communicable diseases in sub-Saharan Africa (Mbanya, Motala, Sobngwi, Assah and Enoru, 2010). The International Diabetes Federation (IDF) estimates that by 2035, 41.4 million African residents will suffer from diabetes (IDF, 2013). Type 2 diabetes (T2D) is the most common form of the condition and is characterised by decreased insulin secretion and insulin resistance (Moller, 2001). A reason for this rise in T2D is that the diets of populations in sub-Saharan Africa are becoming progressively "Westernized" (Diabetes Leadership Forum, 2010). Such diets are rich in foods containing high glycaemic index carbohydrates, leading to post-prandial hyperglycaemia (high blood glucose) (Preuss, 2009). Hyperglycaemia is most commonly associated with diabetes and is responsible for chronic complications such as retinopathy and nephropathy (Mbanya et al., 2010; Moller, 2001). Controlling the rate of glucose absorption in the small intestine is an important physiological target to delay, prevent and control the development of T2D (Moller, 2001; Ross, Gulve and Wang, 2004; Mbanya et al., 2010).

A therapeutic approach for decreasing post-prandial hyperglycaemia is to prevent glucose absorption by inhibiting carbohydrate digesting enzymes, specifically α-glucosidase and α-amylase (Shobana, Sreerama and Malleshi, 2009). However, drugs that are used for this purpose can have multiple undesirable side effects (Ross et al., 2004; Shobana et al., 2009). Drug therapies can also fail to significantly alter T2D development or prevent chronic complications (Moller, 2001). Further, in sub-Saharan Africa a specific problem is that the use of drug-based therapies is severely limited due to lack of funds, facilities and trained staff (Mbanya et al., 2010; Diabetes Leadership Forum, 2010). Thus, the development of nutraceutical type treatments for T2D using components from locally grown plants would be highly beneficial.

Tannins extracted from a number of plant foods can inhibit the starch digesting enzymes α-amylase and glucoamylase (Barrett, Ndou, Hughey, Straut, Howell, Dai and Kaletunc, 2013). Sorghum is one of the major cereal crops in Africa and there is significant production in 41 African countries (FAOSTAT, 2011). Extracts of polyphenols from sorghum grain have demonstrated *in vitro* inhibitory activity against α-amylase and α-glucosidase (Kim, Hyun and Kim, 2011) and *in vivo* anti-diabetic activity in diabetic rats

(Kim and Park, 2012). However, oral administration of such polyphenolic extracts has various limitations. Sorghum tannins (proanthocyanidins/procyanidins) bind proteins (Butler, Riedl, Lebryk, and Blytt 1984), which lead to the sensation of astringency and unpleasant bitter tastes (Kobue-Lekalake, Taylor and De Kock, 2007). Further, sorghum condensed tannins (SCT) bind to proteins in a non-specific way (Butler et al., 1984), which can lead to a reduction or complete loss of their enzyme inhibitory activity in the small intestine, where the major digestive amylases are secreted.

Thus, a suitable delivery system is needed to ensure that SCT reach the target site (small intestine) with enough biological activity to effectively inhibit carbohydrate digesting enzymes and thereby decrease hyperglycaemia. Zhao, Iyer, Flores, Donhowe and Kong (2013) demonstrated that by encapsulating tannic acid in calcium alginate microspheres, α-amylase could be substantially inhibited in the small intestine phase in a simulated gastrointestinal digestion. Kafirin, the sorghum prolamin storage protein, is notably hydrophobic and resistant to pepsin digestion (Belton, Delgadillo, Halford and Shewry, 2006). It has been found that microparticles made from kafirin (KEMS) can encapsulate and release SCT during simulated digestion (Taylor, Taylor, Belton and Minnaar, 2009a). The aim of this work was to determine the potential of encapsulating SCT in KEMS (SCT-KEMS) as a simple delivery vehicle for SCT to the small intestine to inhibit digestive amylases.

4.1.3 Materials and methods

4.1.3.1 Materials

Type III tannin sorghum, cultivar PAN 3860, was used to prepare SCT. For preparation of KEMS, total kafirin (82% protein (as is basis)) was extracted from condensed tannin-free, white pericarp, tan plant sorghum cultivar (Orbit) as described by Emmambux and Taylor (2003). Porcine α-amylase (EC 3.2.1.1) (>1000U/mg, where one unit will liberate 1.0 mg of maltose from starch in 3 minutes at pH 6.9 at 20 °C), yeast α-glucosidase (EC 3.2.1.20), (≥100 U/mg, where one unit will liberate 1.0 micromole of D-glucose from p-nitrophenyl α-D-glucoside per min at pH 6.8 at 37 °C), pepsin (EC 232.629.3) (800-2,500 U/mg, where 1 U will produce ΔA_{280}, of 0.001/min at pH 2.0 at 37 °C), trypsin (EC 232.650.8) (13,000-20,000 BAEE U/mg protein, where 1 U will produce ΔA_{253} of 0.001/min at pH 7.6 at 25 °C), chymotrypsin (EC 232.671.2) (83.9 U/mg, where 1 U will hydrolyse 1.0 μm of N-benzoyl-L-tyrosine ethyl ester/min at pH 7.8 at 25 °C), acarbose and ρ-nitrophenyl-α-D-glucopyranoside (PNPG) were obtained from Sigma-Aldrich (Johannesburg, South Africa). Amylase high range (HR) reagent (non-reducing-end blocked *p*-nitrophenyl maltoheptaoside) was obtained from Megazyme International (Wicklow, Ireland).

4.1.3.2 SCT preparation

Condensed tannins were extracted according to Price, Van Scoyoc and Butler (1978) with slight modifications. Sorghum bran was obtained by decorticating whole grain with a Tangential Abrasive Dehulling Device (TADD) (Venables Machine Works, Saskatoon, Canada). SCT were extracted from the bran (75 g) with 750 ml methanol for 20 min with constant stirring at ambient temp. (22 °C). The suspension was centrifuged at $4500 \times g$ for 10 min. The supernatant was collected and the solvent evaporated off at ambient temp. in a fume hood. The dried SCT were defatted with hexane, ground to a fine powder with a pestle and mortar and stored at -20 °C until analysis. The assayed condensed tannin content of the dried extract was 312 mg/100 mg (as is basis) catechin equivalents. This shows that this crude extract was very high in condensed tannins, notwithstanding the fact that catechin as a standard overestimates tannin content (Price et al., 1978).

4.1.3.3 KEMS preparation

Encapsulation of SCT in KEMS by coacervation using different solvents was investigated:

i) KEMS were prepared by coacervation from a solution of kafirin in glacial acetic acid, as previously described (Taylor et al., 2009a). Defatted kafirin (1.95 g, 82% protein) was dissolved in glacial acetic acid (5 g) with gentle stirring while the temperature was slowly raised to 30 °C to ensure full solvation. The solution was allowed to 'rest' for 16 h. SCT (500 mg) was dissolved in distilled water at 50 °C. The SCT solution was then added to the kafirin-acetic acid solution using a peristaltic pump (Watson-Marlow Bredel, Falmouth, UK) at a rate of 1.4 ml/min with stirring. Upon addition of water, KEMS formed. After washing with distilled water to remove unbound SCT, the KEMS were air dried, ground to a fine powder and stored at 10 °C until analysis.

ii) SCT-KEMS were formed from an aqueous ethanol solution, according to a coacervation procedure described by Liu, Sun, Wang, Zhang and Wang (2005) with some modifications. Kafirin (2.7 g) was separately dissolved in 70% (w/w) aqueous ethanol (15 ml) at 70 °C and SCT (500 mg) was also dissolved in 15 ml 70% (w/w) ethanol at 70 °C. The latter was added to the kafirin solution at a rate of 3.6 ml/min with constant stirring. To coacervate the KEMS, distilled water was then added to the kafirin-SCT solution at a rate of 3.6 ml/min with constant stirring to a total weight of 110 g. To prepare KEMS alone, kafirin was dissolved in aqueous ethanol and precipitated via coacervation with distilled water. The aqueous ethanol prepared KEMS were then treated as described for the acetic acid method.

4.1.3.4 Simulated gastrointestinal digestion

An *in vitro* protein digestibility assay was performed on SCT-KEMS, KEMS alone and SCT alone using a micro-scale protein digestion protocol by Taylor et al. (2009a). Twenty mg of SCT-KEMS (18 mg for KEMS alone to make up for bound SCT) and 2 mg SCT alone were incubated with 3.7 mg pepsin in 1.75 ml sodium citrate buffer, pH 2 at 37 °C. Pepsin digestion was assayed after 10 and 120 min incubation. Pepsin digested samples were centrifuged and supernatants and pellets were retained separately and immediately frozen at -20 °C for determination of α-amylase and α-glucosidase inhibitory activity. Pellets samples used for protein determination were washed once with distilled water, centrifuged and the supernatant removed and discarded. Pellets were dried in a forced draft oven at 100 °C. The dried material was transferred to Dumas combustion foil cups for protein determination. Additional samples were digested with pepsin for 120 min,

centrifuged and the supernatants removed. The pellets were further digested with a mixture of trypsin (3.9 mg) and chymotrypsin (4.6 mg) in 1.75 ml 0.05 M phosphate buffer, pH 6.9 at 37 °C. Trypsin digestion was assayed after 10 and 120 min incubation and further treated as for the pepsin digested samples.

4.1.3.5 Tannin content

Tannin content was measured by the Vanillin HCl assay of Price et al. (1978). Reagent blanks that corrected for colour of the SCT were included. Catechin was used as a standard.

4.1.3.6 Protein content

Protein (N x 6.25) was determined by a Dumas standard combustion Method 46–30 (AACC, 2000).

4.1.3.7 Binding and encapsulation efficiency

Percentage SCT binding was estimated from the decrease in protein content of the KEMS. Encapsulation efficiency was calculated as the amount of SCT bound to KEMS, divided by the amount added initially and expressed as a percentage.

4.1.3.8 Alpha-amylase inhibition

An adaptation of the method of Tadera, Minami, Takamarsu and Matsuoka (2006) was used in conjunction with the Ceralpha α-amylase assay (Megazyme International). Incubation was at 37 °C for 20 min. Alpha-amylase inhibitory activity of SCT alone and the pellets and supernatants of KEMS alone and SCT-KEMS were separately assayed after pepsin and after trypsin-chymotrypsin digestion. The whole pellet after digestion or 50 μl of the supernatant was reacted with either 200 μl for the pellet or 100 μl for the supernatant of α-amylase (0.1 mg/ml) and (0.1 mg/ml) of 10 mM amylase HR reagent. It was observed that the high pH of the α-amylase stopping reagent, 1% tri-sodium phosphate (pH 11), caused the encapsulated SCT to separate from microparticles. Therefore, after incubation KEMS alone and SCT-KEMS were removed from the reaction mixture right before addition of the stopping reagent. The absorbance of released non-reducing-end blocked p-nitrophenyl maltoheptaoside (BPNPG7) was measured at 400 nm. Inhibition percentage was calculated as (A-B)/A x 100, where A is the amount of product in the absence of SCT extract/microparticles and B is the amount in the presence of the SCT

extract/microparticles. The standard inhibitor acarbose (Ross et al., 2004) (1 mM) was assayed as a standard reference. Solution without sample was used as a control. Solution without substrate was used as a blank to correct for background absorbance.

4.1.3.9 Alpha-glucosidase inhibition

An adaptation of the method by Kim et al. (2011) was used. Alpha-glucosidase inhibitory activity of SCT alone and the pellets and supernatants of KEMS alone and SCT-KEMS samples were assayed separately after pepsin and after trypsin-chymotrypsin digestion as for α-amylase inhibition. Reaction mixtures consisting of 100 µl α-glucosidase (0.04 mg/ml) in 0.1 M phosphate buffer (pH 6.9) and SCT alone, KEMS alone, SCT-KEMS (whole pellet and 100 µl of supernatant) were pre-incubated at 37 °C. The substrate ρ-nitrophenyl-α-D-glucopyranoside (10 mM) (100 µl) in phosphate buffer (pH 6.9) was then added. Incubation was for 20 min and the reaction was stopped using 1 M Na_2CO_3 (pH 11). The high pH of the α-glucosidase stopping reagent also caused the encapsulated SCT to separate from microparticles. Therefore, after incubation KEMS alone and SCT-KEMS were removed from the reaction mixture before addition of the stopping reagent. The absorbance of the liberated ρ-nitrophenol was measured at 400 nm. Acarbose (1 mM) was assayed as a standard reference. Inhibition percentage was calculated as for α-amylase inhibition. Solution without sample was used as a control. Solution without substrate was used as a blank.

4.1.3.10 Determination of IC_{50}

IC_{50} is defined as the concentration of inhibitor required to inhibit 50% of enzyme activity (Lacroix and Li-Chan, 2013). Alpha-glucosidase and α-amylase activities were assayed (as described) in the presence of SCT alone and acarbose at various concentrations. Inhibition percentage was plotted against SCT and acarbose concentrations and IC_{50} was determined by linear regression.

4.1.3.11 Microscopy

Suspensions of KEMS and SCT were prepared for scanning electron microscopy (SEM) and transmission electron microscopy (TEM) as described by (Taylor et al., 2009a). For SEM, samples were chemically dried using hexamethylenediamine (HDMA) and viewed using a JEOL JSM-840 SEM (Tokyo, Japan). High resolution SEM (50 000x) was with a

JEOL 6000F (Tokyo, Japan). TEM was performed using a JEOL TEM-2100F Field Emission Electron Microscope.

4.1.3.12 Statistical analysis

Data was analysed by one-way analysis of variance (ANOVA). All experiments were repeated at least once and expressed as means $\pm$ 1 standard deviation (SD). The mean differences were assessed by Fisher's Least Significant Difference (LSD) test using Statistica software version 10 (StatSoft, Tulsa, OK). Samples were considered significantly different when $p < 0.05$.

4.1.4 Results and discussion

4.1.4.1 Inhibition of amylases by SCT

SCT were effective inhibitors of α-amylase (IC_{50} = 554.4 µg/ml) and α-glucosidase (IC_{50} = 0.4 µg/ml) with essentially linear dose responses (r = 0.97) and (r = 0.93), respectively (Figure 4.1.1). Acarbose was a more powerful α-amylase inhibitor (IC_{50} = 3.1 µg/ml) than SCT, with approx. 180 times higher inhibitory activity. However, SCT had a far higher (approx. 20,000 times) α-glucosidase inhibitory effect than acarbose (IC_{50} = 8464.0 µg/ml).

SCTs in type III sorghum consist of polymerised flavan-3-ol and flavan-3,4-diol units linked by C4-C8 interflavan bonds with (-)-epicatechin as extension units and catechin as terminal units (reviewed by Dykes and Rooney, 2006). A proanthocyanidin-rich extract from sorghum bran similar to that used in this study was found to contain approx. 23% monomers, 18% dimers, 15% trimers, 11% tetramers, 4% pentamers, 3% hexamers and 26% were unresolved polymeric proanthocyanidins (Wu, Huang, Qin, Meng, Zou, Zhu and Ren, 2011). Proanthocyanidin polymers show much stronger inhibitory activity against α-amylase than oligomers (reviewed by Xiao, Ni, Kai and Chen, 2013), whereas proanthocyanidin oligomers show greater inhibitory activity than polymers against α-glucosidase (Lee, Cho, Tanaka and Yokozawa, 2007). Proteins rich in proline bind more condensed tannins than other proteins (Spencer, Cai, Gaffney, Goulding, Magnolato, Lilley and Haslam, 1988). Neither α-amylase nor α-glucosidase have particularly high proline content, approx. 4 % and 5 %, respectively (Brayer, Luo and Withers, 1995). Importantly, however, it has long been known that SCT bind to proteins in a non-specific way (Butler et al., 1984). Notwithstanding this, there is a direct relationship between sorghum tannin content and amylase inhibition (Adetunji, Khoza, de Kock and Taylor, 2013).

Kim et al. (2011) previously investigated the α-amylase and α-glucosidase inhibition of 70% ethanol phenolic extracts from tannin sorghum, while Hargrove, Greenspan, Hartle and Dowd (2011) studied the effects of 50% methanol extracts from Sumac (tannin) and black (non-tannin) sorghum varieties on α-amylase inhibition. The SCT (100% methanol extract from sorghum) used in this study was a stronger α-glucosidase inhibitor than the sorghum phenolic extract of Kim et al. (2011) (IC_{50} = 0.4 µg/ml versus 1.1-1.4 µg/ml), using a similar inhibition assay. However, the sorghum phenolic extracts of Kim et al.

43

(2011) (lowest IC_{50} = 2.9 µg/ml) and Hargrove et al. (2011) (1.4 µg/ml) were apparently far stronger α-amylase inhibitors than the SCT used in this study (554.5 µg/ml). The α-amylase inhibitory data are, however, not directly comparable. These authors used a non-specific α-amylase assay (starch substrate) as opposed to the specific α-amylase maltoheptaoside substrate (McCleary and Sheehan, 1987) used in this study.

The particularly low IC_{50} of the SCT against α-glucosidase indicates that they have the potential to control post-prandial hyperglycaemia associated with T2D. However, due to the challenges with the oral administration of SCT, as described, KEMS were investigated as a delivery system for SCT to the small intestine.
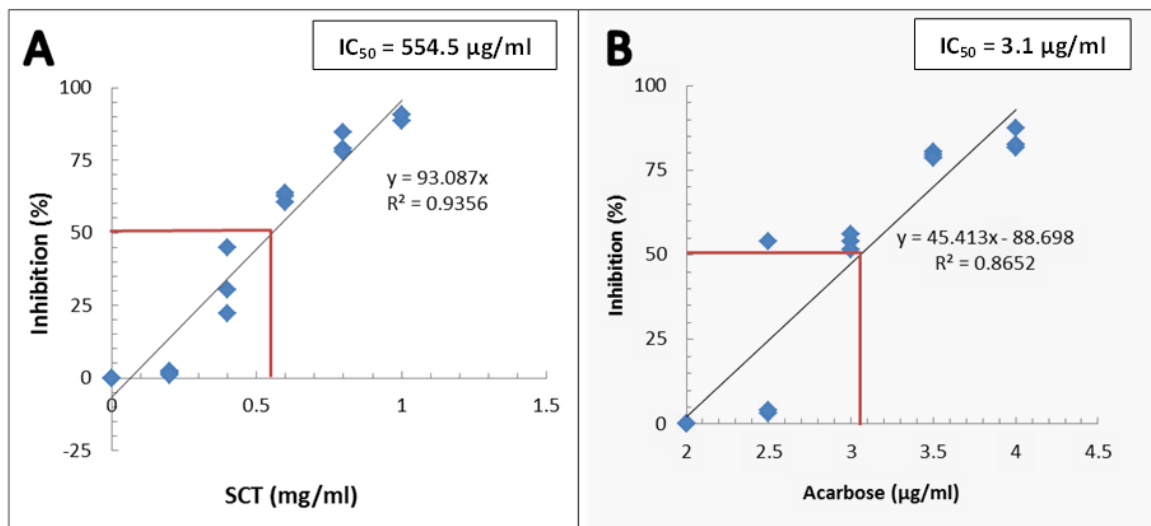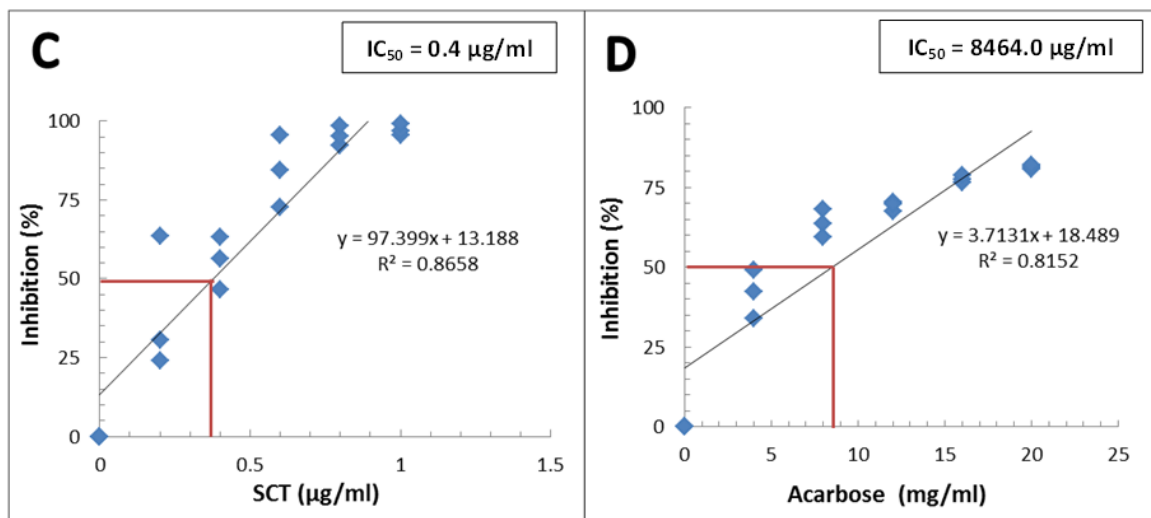
Figure 4.1.1: Dose dependent inhibition of α-amylase and α-glucosidase by SCT and acarbose. Intercept indicates IC_{50}. Inhibition by SCT (A, C) and inhibition by acarbose (B, D).

44

4.1.4.2 SCT encapsulation

The aqueous ethanol method of preparing SCT-KEMS was more effective at encapsulating SCT than the acetic acid method, with 11.22% bound as opposed to 7.94% (Table 4.1.1). Further, in terms of encapsulation efficiency, the aqueous ethanol prepared SCT-KEMS had an encapsulation efficiency of almost double (48%) that of the acetic acid prepared SCT-KEMS (25%). Encapsulation efficiency of SCT-KEMS prepared by the aqueous ethanol method was similar to that obtained by other workers who encapsulated tea catechins in chitosan-tripolyphosphate nanoparticles (Hu, Pan, Sun, Hou, Ye, Hu and Zeng, 2008) and tannic acid in calcium alginate microspheres (Zhao et al., 2013) using coacervation.

Table 4.1.1: The effects of method of KEMS preparation and sorghum condensed tannins (SCT) encapsulation on the protein content of KEMS

Material	KEMS protein content [1](g/100 g, as is)	KEMS SCT content (g/100 g of total)	SCT encapsulation efficiency[2] (%)
Acetic acid prepared KEMS	[3]80.6[d4] ± 0.3	NA	NA
Acetic acid prepared SCT-KEMS	74.2[c] ± 0.1	7.94[a] ± 0.4	24.8[a] ± 1.5
Aqueous ethanol prepared KEMS	80.2[d] ± 0.6	NA	NA
Aqueous ethanol prepared SCT-KEMS	71.2[b] ± 0.4	11.22[b] ± 0.9	48.4[b] ± 4.9
SCT alone	1.8[a] ± 0.0	NA	NA

[1]N x 6.25
[2]Encapsulation efficiency = mg SCT bound/mg SCT initially added $\times$ 100
[3]Mean ± standard deviation (n=3)
[4]Values with different superscripts in the same column differ significantly (p<0.05)
NA – Not applicable

The improved encapsulation efficiency of the aqueous ethanol prepared KEMS was probably related to the higher coacervation rate used to prepare the aqueous ethanol KEMS (3.6 ml/min), compared to the acetic acid prepared KEMS (only 1.4 ml/min). A faster coacervation rate leads to higher encapsulation efficiency as diffusion of the encapsulated

substance is limited (Yeo and Park, 2004). Additionally, SCT were dissolved in aqueous ethanol separately before being added to the kafirin-ethanol mixture. Therefore, hydrogen bonding and hydrophobic interactions between SCT and kafirin (Butler et al., 1984) could occur before coacervation, leading to higher encapsulation efficiency. In the acetic acid method, binding and coacervation had to occur simultaneously, probably limiting SCT binding and encapsulation efficiency.

KEMS prepared by the acetic acid method were spherical, porous and ranged in size from 1 to 10 μm (Figure 4.1.2 A, B) as previously observed (Taylor et al., 2009a). SCT binding did not affect microparticle size or structure (Figure 4.1.2 A, C), but appeared to join some KEMS together (Figure 4.1.2 D). The aqueous ethanol method of preparing KEMS had a profound effect on their morphology. These KEMS were aggregated into clumps of about 100 μm (Figure 4.1.2 E, G). However, at higher magnification (50000×) the aqueous ethanol prepared microparticles appear to be composed of individual spheres aggregated into larger clumps (Figure 4.1.3). Parris, Cooke and Hicks (2005) studied the morphology of zein nanoparticles prepared in a similar way and observed irregular shaped agglomerates at lower (500×) SEM magnification, but individual nanospheres at a higher (50000×) magnification. However, these researchers did not provide an explanation for the observed morphology. SCT encapsulation in aqueous ethanol prepared KEMS did not affect the size or structure of these KEMS (Figure 4.1.2 G, H).

SCT are strongly osmiophilic and they appear as darkly stained material in TEM with osmium tetroxide staining (Morrall, Liebenberg and Glennie, 1981). TEM showed with the acetic acid prepared KEMS the majority of the SCT bound around the outside the KEMS (Figure 4.1.2 D). In contrast, the SCT were embedded inside the aqueous ethanol prepared KEMS (Figure 4.1.2 H). Hydrophobic interactions could explain why SCT bound mostly to the outside of the acetic acid prepared KEMS. Insoluble complexes are formed when excess tannins bind protein and lead to the formation of hydrophobic outer layers on the complex surface (Cannas, 2013).
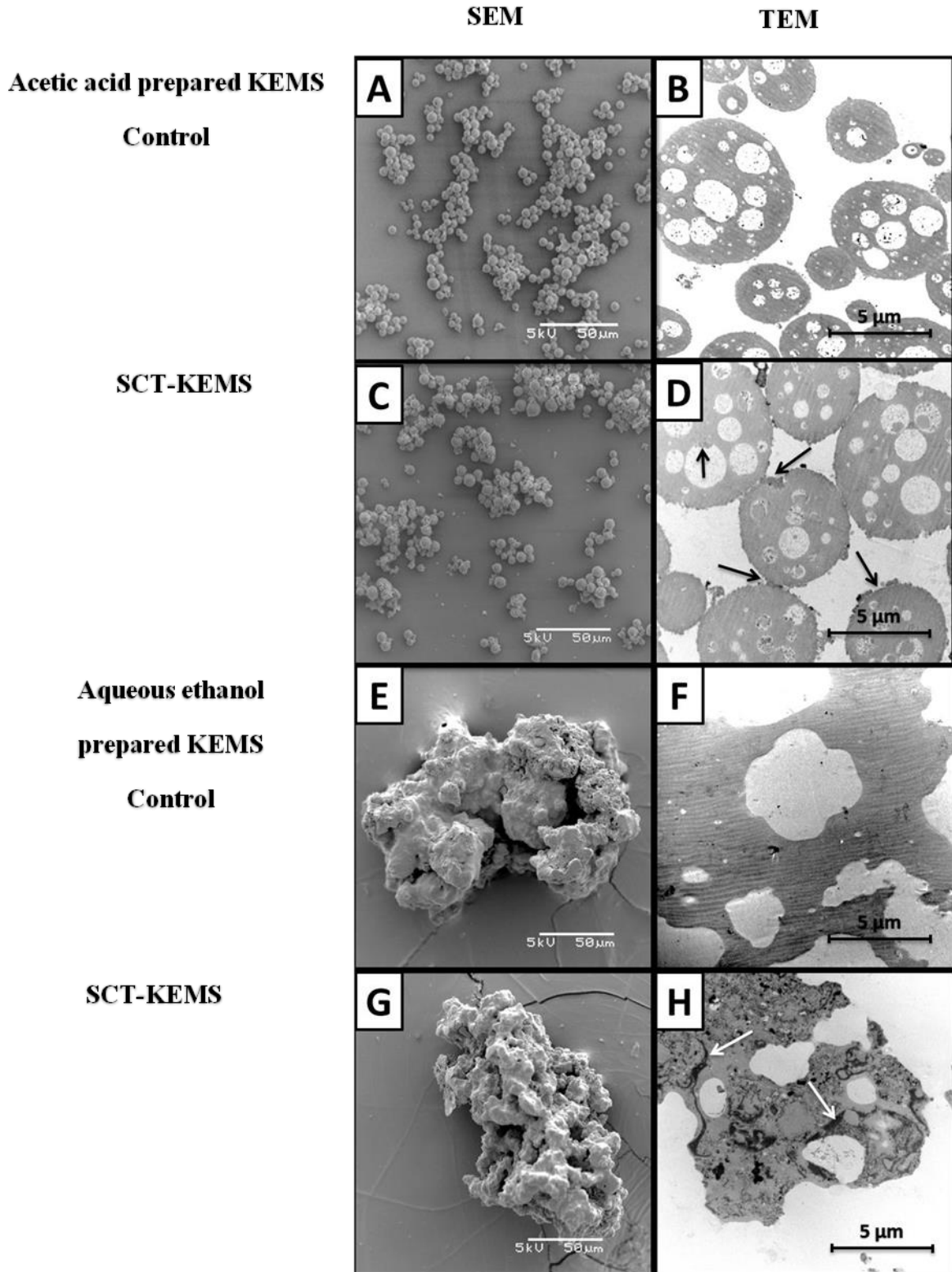
Figure 4.1.2: SEM and TEM showing the effect of method of preparation of KEMS and SCT encapsulation on KEMS morphology. KEMS prepared by acetic acid (A-D) and aqueous ethanol (E-H) methods. KEMS (A, B, E and F) and SCT-KEMS (C, D, G and H). Arrows indicate bound tannins.

47
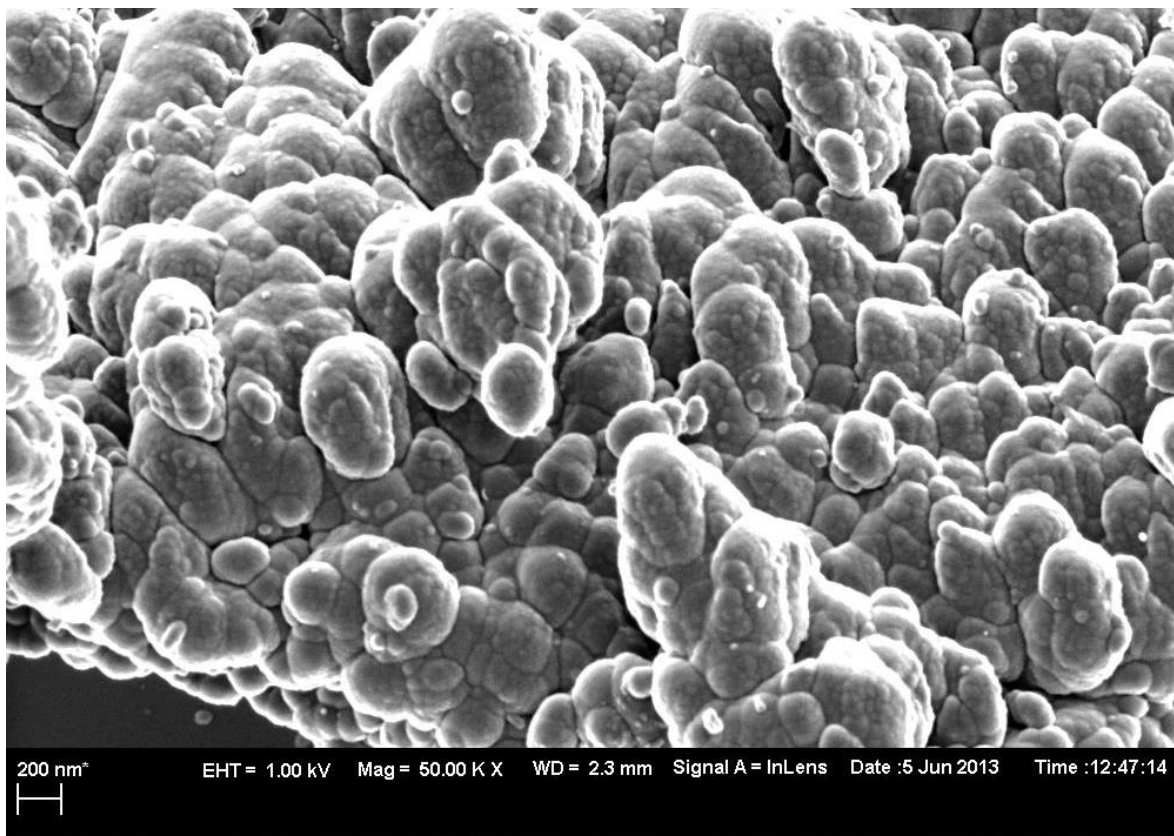
Figure 4.1.3: High resolution SEM of aqueous ethanol prepared KEMS encapsulating SCT showing individual spherical microparticles packed tightly into agglomerates.

The acetic acid method required that the SCT were dissolved in the water used to coacervate the kafirin protein. As coacervation and binding had to occur simultaneously, effective binding was limited. With the aqueous ethanol method, the SCT were completely dissolved in the solvent then mixed with the kafirin. The faster precipitation rate, mentioned earlier, could also explain why more SCT were encapsulated in the aqueous ethanol prepared KEMS compared to the acetic acid prepared KEMS. As the aqueous ethanol method of preparing KEMS was more effective at encapsulation SCT, trypsin-chymotrypsin digestibility and inhibition by KEMS were performed on the aqueous ethanol prepared KEMS only.

4.1.4.3 Simulated gastrointestinal digestion of KEMS

The method of preparation and encapsulation of SCT influenced the digestibility of the KEMS. Initially (10 minutes) the KEMS showed similar digestion patterns, in spite of their preparation method (Table 4.1.2). However, after 120 minutes of digestion the acetic acid

prepared KEMS showed a higher digestibility. Encapsulation of SCT decreased pepsin digestibility of the acetic acid and aqueous ethanol prepared KEMS. SCT did not show any pepsin digestibility as their protein content actually increased. This was most probably due to the SCT binding with the pepsin enzymes during the pepsin digestion phase (Butler et al., 1984) and the subsequent increase in their measurable protein content.

Table 4.1.2: The effects of method of kafirin microparticle (KEMS) preparation and encapsulation of sorghum condensed tannins (SCT) on *in vitro* pepsin and trypsin-chymotrypsin digestibility of acetic acid and aqueous ethanol prepared KEMS

Material	Pepsin digestibility (%)	
	10 min	120 min
Acetic acid prepared KEMS	[1]8.7[b2A3] ± 1.0	51.1[dB] ± 0.5
Acetic acid prepared SCT-KEMS	3.0[aA] ± 1.0	16.4[bB] ± 1.3
Aqueous ethanol prepared KEMS	7.2[bA] ± 1.8	33.2[cB] ± 1.6
Aqueous ethanol prepared SCT-KEMS	3.1[aA] ± 1.6	7.0[aB] ± 1.6
SCT alone	< 0	< 0
	Trypsin-chymotrypsin digestibility (%)	
	10 min	120 min
Aqueous ethanol prepared SCT-KEMS	6.1[aA] ± 1.2 (6.6)[4]	11.5[aB] ± 0.8 (12.4)
Aqueous ethanol prepared KEMS	25.6[bA] ± 1.5 (38.3)	39.2[bB] ± 0.4(58.7)
SCT alone	< 0	< 0

[1]Mean ± standard deviation (n=3)
[2]Values with different lower case superscripts in the same column differ significantly ($p < 0.05$)
[3]Values with different capital letter superscripts in the same row differ significantly ($p < 0.05$)
[4]Values in brackets represent calculated total digestion

Pepsin digestion of acetic acid prepared KEMS were clearly illustrated by electron microscopy (Figure 4.1.4 C and D). Even though SCT encapsulation decreased the pepsin digestibility of the acetic acid KEMS, some degradation could be observed on their surfaces (Figure 4.1.4 G). Digestion appeared to take place on the surface and from the inside of the KEMS. The vacuoles in the KEMS probably provided a channel through which the enzymes penetrated and digested the KEMS from within. Some of the KEMS were almost completely digested, while other showed very little digestion (Figure 4.1.4 A, C and D).

49
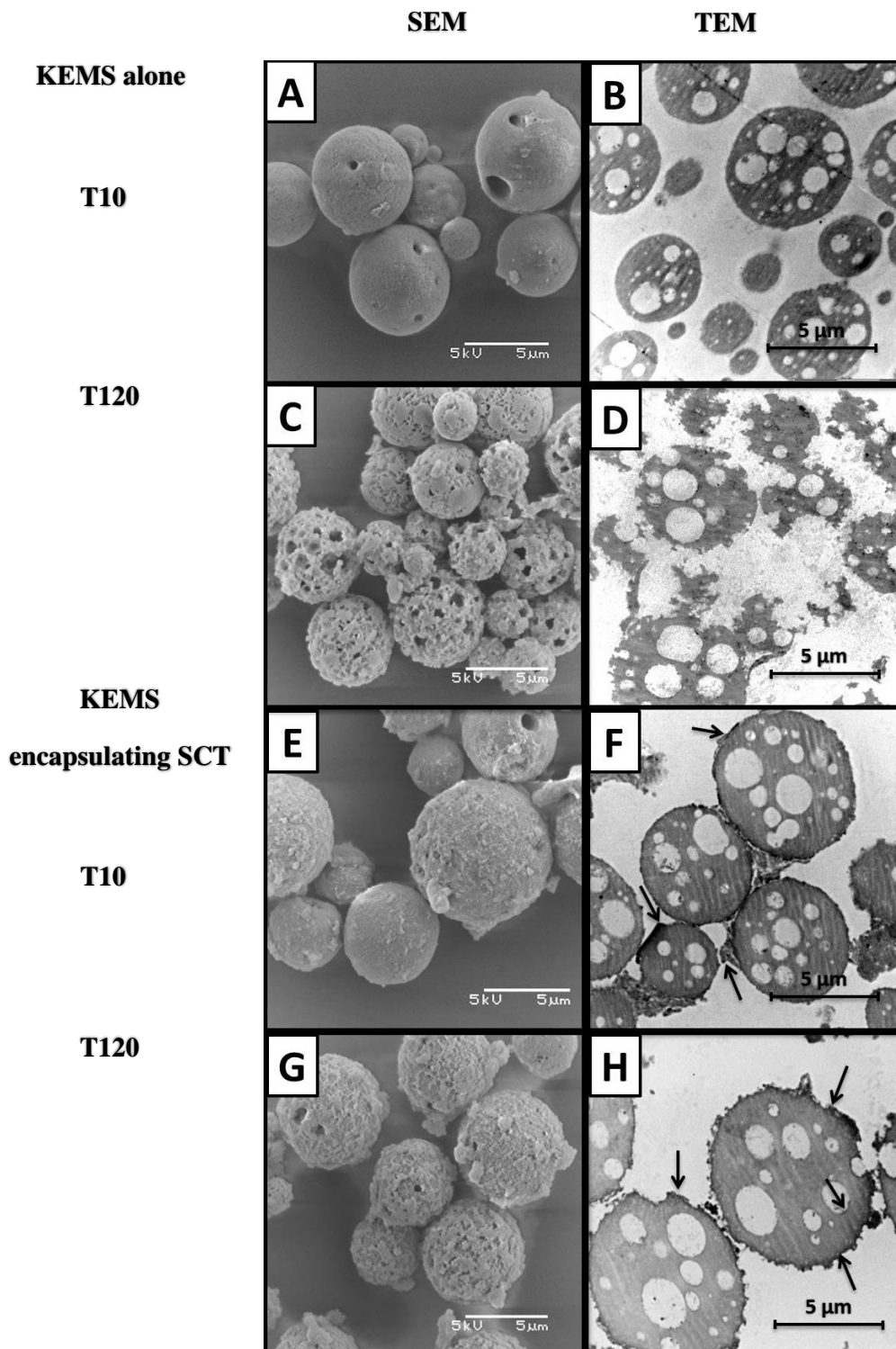
Figure 4.1.4: SEM and TEM showing the effect of encapsulation of SCT on the morphology of acetic acid prepared KEMS after *in vitro* pepsin digestion. KEMS alone (A-D) and SCT-KEMS (E-H) after 10 (A, B, E and F) and 120 (C, D, G and H) minutes of digestion. Arrows indicate bound tannins

50

Digestibility of aqueous ethanol prepared KEMS were lower than acetic acid prepared KEMS (Table 4.1.2). However, degradation of the aqueous ethanol KEMS alone was illustrated by electron microscopy (Figure 4.1.5 A-D). Digestion appeared to take place mainly on the surface of these KEMS as the initial smooth surface (Figure 4.1.5 A) was eroded after 120 minutes of pepsin digestion (Figure 4.1.5 C). Encapsulation of SCT resulted in very little digestion of the aqueous ethanol KEMS (Figure 4.1.5 E, F, G and H) and there was essentially no change on the surface (Figure 4.1.5 E and G) or the internal (Figure 4.1.5 F and H) morphology of these KEMS.

The reduced surface area of the aqueous ethanol KEMS relative to the acetic acid KEMS (Figure 4.1.2 A and E) was probably the main reason for aqueous ethanol's decreased pepsin digestibility. Furthermore, aqueous ethanol-kafirin solution was heated to 70°C before coacervation and it is known that wet heat decreases sorghum protein digestibility due to increased disulphide crosslinking (Duodu, Taylor, Belton and Hamaker, 2003). Encapsulation of SCT resulted in very little digestion over the digestion period. These results were not surprising as SCT are known to bind protein and render it indigestible (Emmambux and Taylor, 2003) presumably by their ability to bind and inhibit digestive enzymes and/or reduce enzyme accessibility (Taylor et al., 2009a). Sorghum tannins have also been shown to associate strongly with sorghum prolamins, resulting in indigestible complexes after pepsin digestibility (Butler et al., 1984).

SCT encapsulation also decreased trypsin-chymotrypsin digestibility of the KEMS (Table 4.1.2). However, after 120 minutes of trypsin-chymotrypsin digestion SCT-KEMS indicated some signs of corrosion along the edges of these KEMS (Figure 4.1.6 H). After protease digestion, there was essentially no change in the morphology of the SCT-KEMS (Figure 4.1.6, E-H). The low digestibility of the SCT-KEMS was most probably due to characteristics of the condensed tannins mentioned previously (Taylor et al., 2009a). The low trypsin-chymotrypsin digestibility of the KEMS alone could be attributed to the relatively small surface area (Figure 4.1.2 E) and substrate affinity of the enzymes. Trypsin and chymotrypsin are endopeptidases which cleave internal peptide bonds (Belitz, Grosch and Schieberle, 2009). As pepsin digestibility was relatively low, most of the proteins were left intact, thus limiting the amount of internal peptide bonds to cleave.
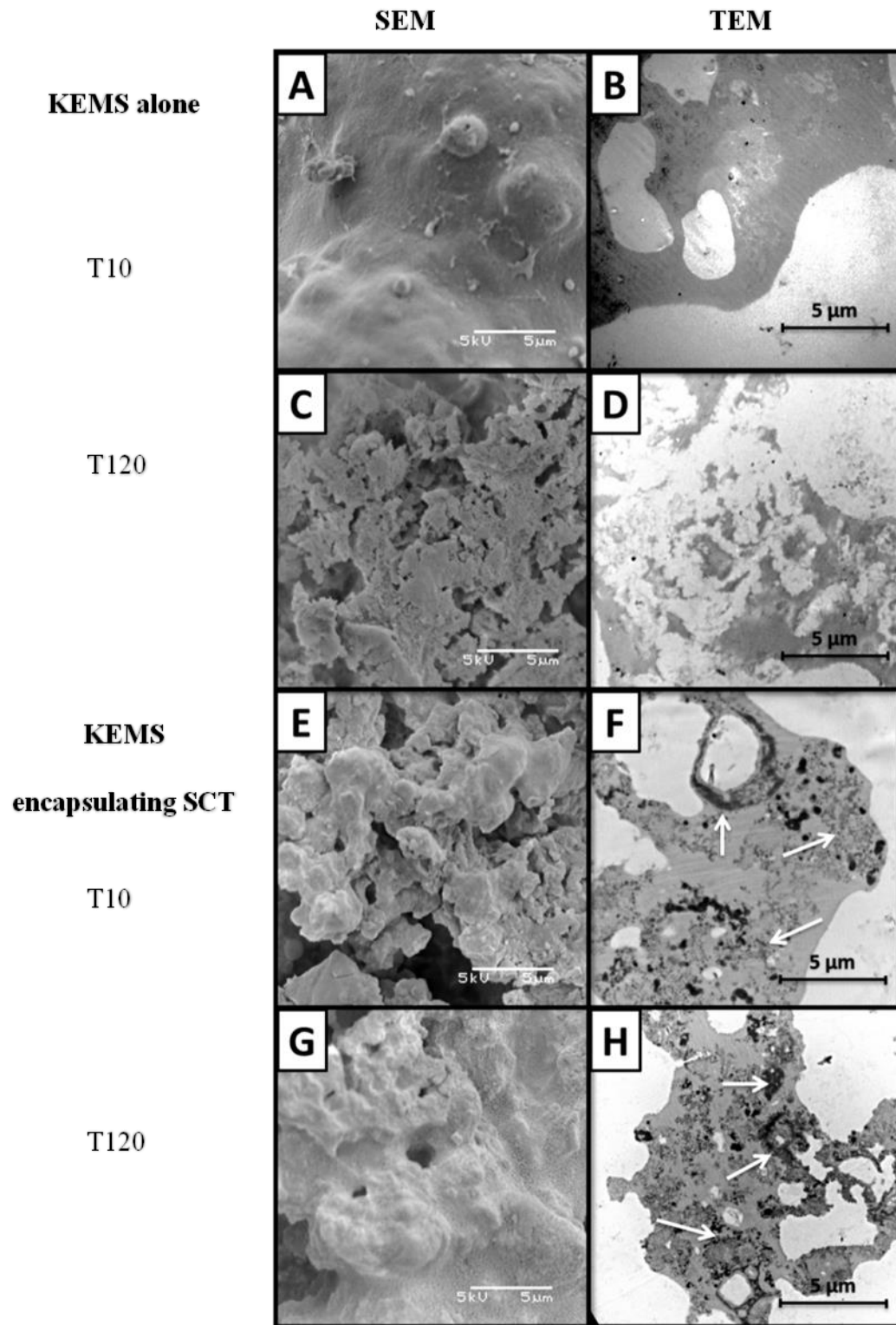
Figure 4.1.5: SEM and TEM showing the effect of SCT encapsulation on the morphology of aqueous ethanol prepared KEMS after *in vitro* pepsin digestion. KEMS (A-D) and SCT-KEMS (E-H) after 10 (A, B, E and F) and 120 (C, D, G and H) minutes of digestion. Arrows indicate bound tannins.
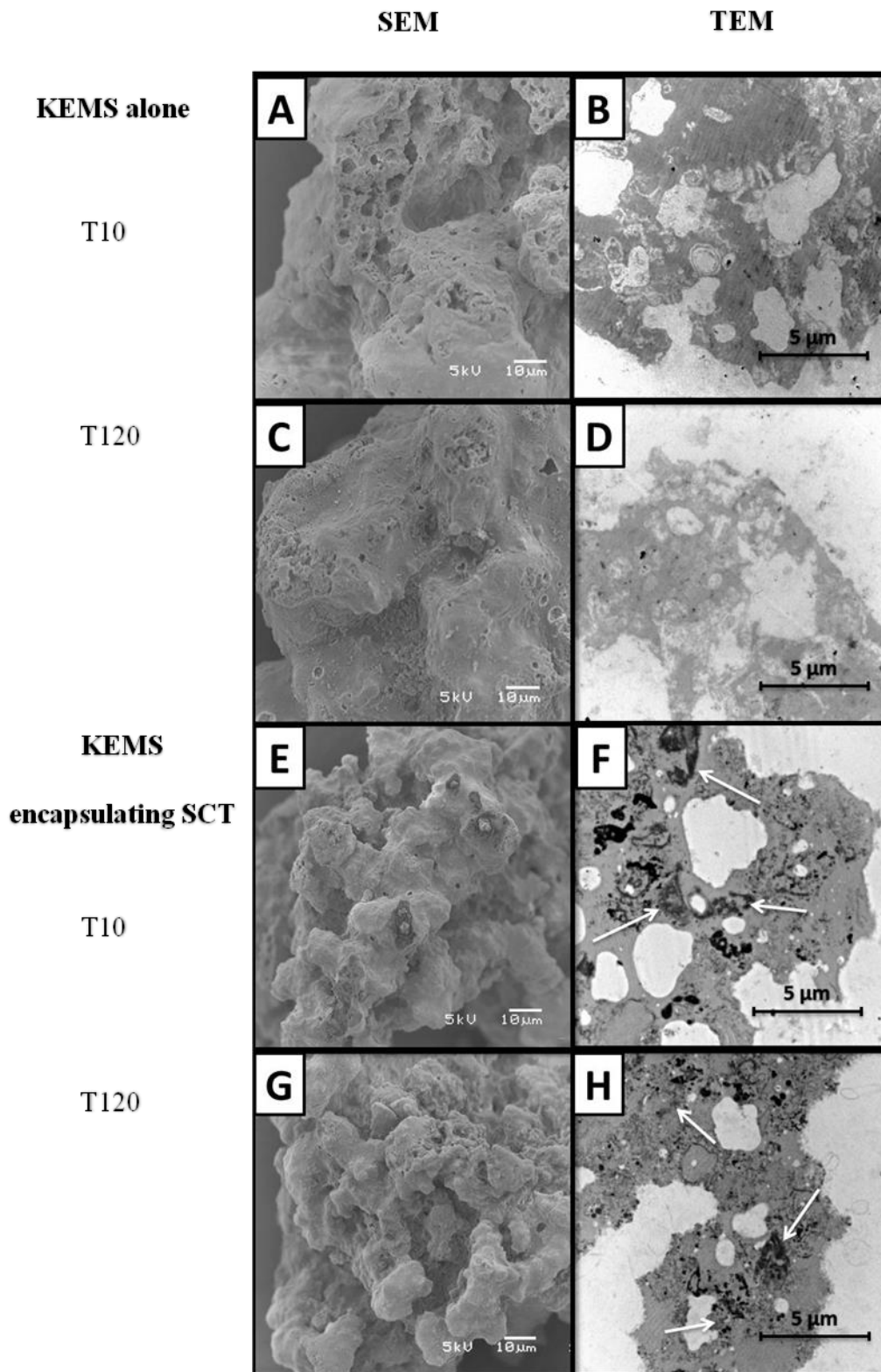
Figure 4.1.6: SEM and TEM showing the effect of SCT encapsulation on the morphology of aqueous ethanol KEMS after *in vitro* pepsin followed by trypsin-chymotrypsin digestion. SEM (A, C, E and G) and TEM (B, D, F and H) of KEMS (A-D) and SCT-KEMS (E-H) after 10 (A, B, E and F) and 120 (C, D, G and H) minutes of digestion. Arrows indicate encapsulated tannins.

4.1.4.4 Simulated gastrointestinal digestion of SCT

Prior to protease digestion, unencapsulated SCT showed a clustered, grainy morphology (Figure 4.1.7 A and D). However, after protease digestion they exhibited a honeycomb-like structure (Figure 4.1.7 B, C, E and F). Butler et al. (1984) found that enzymes were inhibited by SCT and that they (SCT) interact with proteins mainly by means of hydrogen bonding and non-polar hydrophobic interactions leading to polyphenol-protein bridges. There are multiple schematic representations illustrating the binding of polyphenols to protein similar to the honeycomb-like structure (Charlton, Baxter, Khan, Moir, Haslam, Davies and Williamson, 2002; Haslam, 2007).
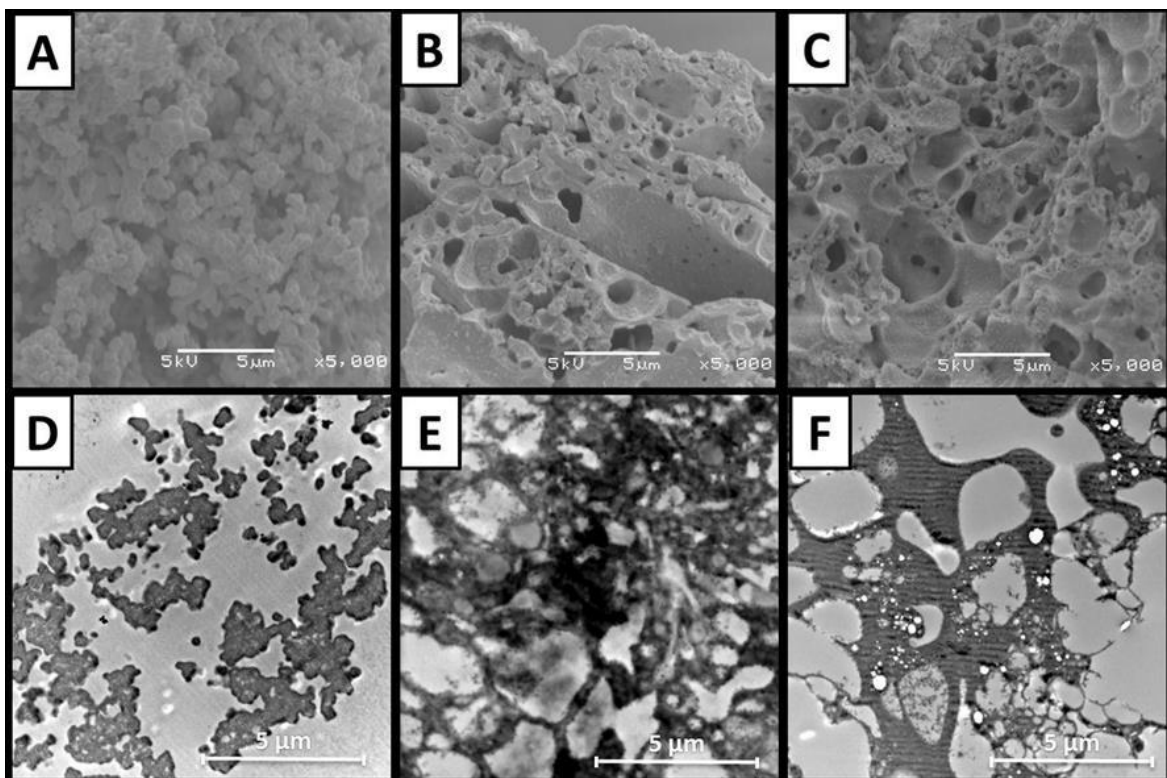
Figure 4.1.7: Electron microscopy of sorghum condensed tannins (A, D) subjected to *in vitro* pepsin (B, E) followed by trypsin and chymotrypsin treatment (C, F). SEM (A-C) and TEM (D-F).

4.1.4.5 Amylase inhibition by KEMS

To determine whether SCT encapsulated in KEMS retained amylase inhibitory activity after simulated gastrointestinal digestion, the α-amylase and α-glucosidase inhibition of pepsin and pepsin followed by trypsin–chymotrypsin digested SCT-KEMS were

investigated. Interestingly, the KEMS alone inhibited both α-amylase and α-glucosidase (Table 4.1.3). Encapsulation of SCT increased the inhibitory activity of KEMS against both enzymes. The increase in inhibition of α-glucosidase due to the encapsulated SCT was substantial up to the end of the pepsin digestion stage, between 92% and 212%, whereas the increase in inhibition against α-amylase up to the end of pepsin digestion was much lower, between 5% and 39%. This is in line with the much lower IC_{50} of SCT against α-glucosidase than α-amylase (Figure 4.1.1 C). With the subsequent trypsin–chymotrypsin digestion step, as the α-glucosidase inhibitory activity of the KEMS alone was so high > 96%, there was little increase in α-glucosidase inhibition as a result of encapsulating the SCT. However, the encapsulated SCT increased α-amylase inhibition by 17–23% compared to the KEMS alone.

Pepsin and trypsin–chymotrypsin digestion of KEMS increased inhibitory activity of both KEMS alone and SCT-KEMS against both amylase enzymes. The increase in inhibitory activity of the SCT-KEMS is presumably because the digestion of the KEMS by the proteases (Figure 4.1.6) partially exposed the encapsulated SCT and enabled them to bind and inhibit more amylase enzymes. The inhibitory activity of KEMS alone was not expected. Clearly, other factors were involved in the α-amylase and α-glucosidase inhibition. The kafirin protein used to prepare KEMS did not exhibit any inhibitory activity (results not shown). However, kafirin undergoes conformation changes when formed into microparticles (Taylor, Taylor, Belton and Minnaar, 2009b). Therefore, it can be surmised that conformational changes caused the amylase enzymes to adsorb to KEMS surface and be inhibited.

In line with the IC_{50} data, the SCT alone did not inhibit the α-amylase during pepsin digestion, but completely inhibited the α-glucosidase (Table 4.1.3). This observation is in agreement with the notion that α-amylase and α-glucosidase are both inhibited by plant polyphenols but that the size and structure of the polyphenols determine the degree of inhibition (McDougall, Shpiro, Dobson, Smith, Blake and Stewart, 2005; Lo Piparo, Scheib, Frei, Williamson, Grigorov and Chou, 2008).With the SCT alone, there were minimal α-amylase inhibition and no α-glucosidase inhibition after trypsin–chymotrypsin digestion. This suggests that SCT alone were prevented from inhibiting the amylases due to the non-specific binding of the protease proteins to the SCT. This binding would have taken place because the SCT were not encapsulated in the KEMS.

55

Table 4.1.3: Effect of encapsulation of sorghum condensed tannins (SCT) on the inhibitory action of kafirin microparticles (KEMS) and SCT alone on α-amylase and α-glucosidase after treatment with pepsin followed by trypsin and chymotrypsin

Enzyme treatment	Digestion time (min)	Material	Enzyme inhibition (%)	
			α-amylase	**α-glucosidase**
None	**0**	**KEMS alone**	[1]42.1[b2] ± 1.4	30.1[b] ± 0.2
		SCT-KEMS	58.7[b] ± 1.5 (39%)[3]	63.8[e] ± 1.8 (112%)
Pepsin	**10**	**KEMS alone**	84.7[fg] ± 0.9	12.9[a] ± 1.0
		SCT-KEMS	88.7[h] ± 2.3 (5%)	40.3[c] ± 1.6 (212%)
		SCT alone	< 0	100.6[f] ± 0.2
	120	**KEMS alone**	81.1[de] ± 0.4	29.9[b] ± 1.0
		SCT-KEMS	87.4[gh] ± 1.0 (8%)	57.3[d] ± 3.6 (92%)
		SCT alone	< 0	100.7[f] ± 0.2
Trypsin-Chymotrypsin	**10**	**KEMS alone**	67.6[c] ± 0.9	96.9[f] ± 0.3
		SCT-KEMS	83.3[ef] ± 0.2 (23%)	97.4[f] ± 1.2 (<1%)
		SCT alone	12.8[a] ± 4.4	< 0
	120	**KEMS alone**	67.6[c] ± 0.4	99.6[f] ± 0.3
		SCT-KEMS	78.8[d] ± 1.0 (17%)	100.5[f] ± 0.2 (1%)
		SCT alone	7.0[a] ± 3.8	< 0

[1]Mean ± standard deviation (n=2)
[2]Values with different superscripts in the same column are significantly different ($p < 0.05$)
[3]Increase in inhibition due to SCT

The loss of inhibitory activity after simulated digestion of SCT (Table 4.1.3) emphasised the importance of encapsulation for SCT delivery. Inhibitory activity of SCT-KEMS throughout simulated protease digestion suggests that encapsulating SCT in KEMS may effectively reduce carbohydrate digestion in the small intestine that could lead to improved glycaemic response in persons suffering from hyperglycaemia associated with T2D.

Table 4.1.4: Effect of encapsulation of sorghum condensed tannins (SCT) on the inhibitory action of KEMS digest supernatants on α-amylase and α-glucosidase activity after treatment with pepsin followed by trypsin and chymotrypsin

Enzyme treatment	Digestion time (min)	Supernatants material	Enzyme inhibition (%)	
			α-amylase	α-glucosidase*
Pepsin	10	SCT-KEMS	< 0	< 0
		KEMS alone	< 0	< 0
	120	SCT-KEMS	< 0	< 0
		KEMS alone	< 0	< 0
Trypsin-chymotrypsin*	10	SCT-KEMS	< 0	< 0
		KEMS alone	$1.6^a \pm 6.7$	< 0
	120	SCT-KEMS	< 0	< 0
		KEMS alone	< 0	< 0

* Results after heat treatment of supernatant material
[1]Mean ± standard deviation (n=3)
[2]Values with different superscripts in the same column are significantly different ($p<0.05$)

There was essentially no inhibitory activity of the supernatants of KEMS after simulated digestion (Table 4.1.4). These results suggest that either there was no release of SCT during the course of digestion or that released SCT bound the protease enzymes in a way that rendered them unable to inhibit the α-amylase or α-glucosidase enzymes. It is known that tannins bind proteins and render them indigestible (Butler et al., 1984). Additionally, Zhao et al. (2013) found that during simulated digestion tannic acid interacted with dietary proteins in a non-specific way that involved multiple sites. They also found that even after encapsulation of tannic acid in calcium alginate beads, after simulated digestion the inhibitory activity against α-amylase was decreased.

4.1.4.6 Conclusions

The findings indicate that ethanol extracts of SCT from sorghum bran and their encapsulation in microparticles made from sorghum kafirin protein using a simple alcoholic coacervation technique should substantially reduce carbohydrate digestion in the small intestine when SCT-KEMS are orally administrated. This nutraceutical type

treatment could enable improved post-prandial blood glucose tolerance in persons suffering from hyperglycaemia associated with T2D. Research is now required to determine the effect of the SCT-KEMS on glycaemic response and insulin secretion *in vivo*.

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4.2: *In vivo* rat model assessment of the efficacy of kafirin encapsulated sorghum condensed tannins as an anti-hyperglycaemic agent

4.2.1 Abstract

In vitro assays showed that sorghum condensed tannins (SCT) could survive gastric digestion and inhibit digestive amylases when encapsulated in kafirin microparticles (KEMS). This study investigated SCT-KEMS as a potential anti-hyperglycaemic agent *in vivo*. Oral starch tolerance tests (OSTT) using maltodextrin were performed on healthy rats. The OSTT was repeated three times over seven weeks. The first OSTT showed that SCT-KEMS treatment decreased the maximum blood glucose level by on average 11.8% and the AUC by 9%, compared to the water control. Acarbose (drug standard) decreased the blood glucose spike by on average 19% compared to the control. SCT-KEMS and acarbose did not elevate serum insulin levels and actually decreased insulin secretion by 60% and 48%, respectively, compared to the control. The second and third OSTT did not indicate clear differences, probably due to stress responses. SCT-KEMS can decrease blood glucose levels and prevent elevation of serum insulin in healthy rats after an OSTT. KEMS are effective SCT encapsulating agents as they deliver the SCT to the small intestine and appear to mask the bitterness and astringency of SCT. Therefore, encapsulating SCT in KEMS has potential as a nutraceutical for the management of hyperglycaemia associated with T2D.

4.2.2 Introduction

Hyperglycaemia is characteristic of metabolic syndrome and diabetes mellitus (Ross, Gulve and Wang, 2004). Type 2 diabetes (T2D) is primarily caused by insulin deficiency and/or insulin resistance (Pinent, Blay, Blade, Salvado, Arola and Ardevol, 2004). Control of hyperglycaemia in patients with T2D can attenuate the development of chronic complications (Moller, 2001) and "spare" insulin secreting β-cells by decreasing the amount of insulin needed to maintain normal blood glucose levels (Ross et al., 2004).

One of the therapeutic approaches for preventing post-prandial hyperglycaemia is to reduce absorption of carbohydrates after food intake (Shobana, Sreerama and Malleshi, 2009). Drugs used in this regard include acarbose and miglitol that suppress the digestion of starch and absorption of glucose in the gut by the inhibition of starch hydrolysing enzymes (Lacroix and Li-Chan, 2014). These treatments can have various side effects and limited success in attenuating T2D (Moller, 2001). The use of drug-based therapies in sub-Saharan African countries that suffer from inadequate healthcare systems, shortage of medical personnel and lack of, or unaffordability of medication has an even lower success rate (Diabetes Leadership Forum, 2010).

This study (Chapter 4.1) has shown that condensed tannins isolated from sorghum bran (SCT) strongly inhibited α-amylase and inhibited α-glucosidase *in vitro* at a far lower concentration than acarbose. SCT extracts were encapsulated in microparticles made from kafirin (KEMS), the sorghum prolamin protein. SCT-KEMS were subjected to simulated gastrointestinal digestion and it was found that SCT-KEMS could retain inhibitory activity against both α-glucosidase and α-amylase.

These findings suggest that SCT-KEMS may have potential as a nutraceutical to attenuate hyperglycaemia by inhibiting carbohydrate hydrolysing enzymes in the small intestine. However, positive α-glucosidase inhibition *in vitro* does not always correlate to positive *in vivo* action (Ye, Shen and Xie, 2002). Therefore it is necessary to confirm *in vitro* findings using *in vivo* methods. Rodents are commonly used models for T2D research and most anti-diabetic agents have been developed on the basis of primary efficacy in rodent models (Ross et al., 2004). To prove in principle the anti-hyperglycaemic effects of SCT, a healthy rat model was used.

To confirm the amylase inhibitory effects of SCT *in vivo*, it is necessary to demonstrate suppression of starch digestion and glucose absorption (Gad, El-Sawalhi, Ismail and El-Tanbouly, 2006). The oral glucose tolerance test (OGTT) mainly provides information on the activity of insulin by measuring the uptake of glucose (Ayala, Samuel, Morton, Obici, Croniger, Shulman, Wasserman and McGuinness, 2010). However, the oral starch tolerance test (OSTT) can be used as an indirect measure of digestive amylase activity by assessing the blood glucose levels after an oral starch load (Ali, Atangwho, Kuar, Ahmad, Mahmud and Asmawi, 2013).

Therefore the present proof of concept study was performed with the aim of determining: (1) the efficacy of SCT-KEMS versus acarbose in regulating blood glucose levels in healthy, normoglycaemic rats after an OSTT, and (2) whether SCT-KEMS administration will result in a decreased insulin response after an OSTT.

4.2.3 Materials and methods

4.2.3.1 Materials

Maltodextrin (419699, Dextrose Equivalent 13-17) and acarbose were purchased from Sigma-Aldrich (Johannesburg, South Africa). Enzyme-linked Immunosorbent Assay (ELISA) (EZRMI-13K) kit for rat/mouse serum insulin determination was from Merck Millipore (Johannesburg, South Africa).

4.2.3.2 Preparation of sorghum condensed tannins

SCT extract were prepared as described in Chapter 4.1.

4.2.3.3 Preparation of kafirin microparticles

KEMS were prepared as described in Chapter 4.1 with some modifications. The encapsulation efficiency was improved by the addition of more SCT per weight of kafirin (ratio-1:0.75). This resulted in the SCT-KEMS containing 29% SCT compared to the previous 11% (Table 4.1.1). The 29% binding was similar to that found by Emmambux and Taylor (2003) for SCT binding to kafirin protein.

4.2.3.4 Transmission electron microscopy (TEM)

The improvement in encapsulation efficiency was confirmed using TEM. Preparation for TEM was done as described in Chapter 4.1.

4.2.3.5 Animals

Thirty healthy, adult (15 week) male Sprague Dawley rats (260-350 g) were acclimatised to the lab environment for one week prior to the start of the study. The rats were housed in pairs in Type 3 rat cages (1291H, Eurostandard). The rats had *ad libitum* access to water and standard rodent pallets (Epol, Pretoria, South Africa). The study lasted for seven weeks and body weight was determined weekly. The study was approved by the University of Pretoria's Animal Ethics Committee (Project EC039-14) and rats were handled and maintained according to the guidelines set out by the University of Pretoria's Biomedical Research Centre.

4.2.3.6 Oral starch tolerance test (OSTT)

The OSTT were performed according to Wolf, Humphrey, Hadley, Maharry, Garleb and Firkins (2002) and Ali et al. (2013) with some modifications. Rats were randomly assigned in five groups with six rats per group. Rats were fasted for 12 hours prior to each OSTT. The treatments were SCT-KEMS (400 mg/kg body weight, containing 116 mg/kg SCT), KEMS alone (284 mg/kg) and SCT alone (116 mg/kg). The SCT-KEMS dosage was selected based on the maximum amount that could pass through a 1 ml syringe while suspended in 0.75 ml of water. KEMS alone and SCT alone dosages were the same as their amounts in the SCT-KEMS. Acarbose was used as the positive control (10 mg/kg, as used by Ali et al. (2013)), and distilled water (vehicle) as the negative control (0.75 ml). All treatments were administered orally with a syringe. Maltodextrin solutions (4 g/kg body weight) were prepared directly before each OSTT to an approx. total volume of 1.5 ml. High performance liquid chromatography (HPLC) analysis showed that the maltodextrin product was purely dextrins and did not contain glucose. Fifteen min after treatment administration, rats were administered maltodextrin orally via a syringe. Blood was collected via tail venipuncture. Blood glucose was measured before (0 min) and at 15, 30, 45, 60, 75, 90, 105 and 120 min after maltodextrin administration using a OneTouch Select glucometer (Lifescan, Johannesburg, South Africa). The OSTT was repeated (Experiment 2). It was performed a third time with a maltodextrin dose of 6 g/kg and a volume of approx. 3 ml (Experiment 3). There was a two week recovery period between experiments.

Blood glucose data were used to determine the blood glucose spike 15 min after maltodextrin administration, maximum blood glucose reached over analysed period and area under blood glucose response curve (AUC). AUC was calculated using: $AUC_{glucose}$ = mmol/L × h (Ali et al., 2013).

4.2.3.7 Serum insulin

Blood (0.2 ml) was collected for insulin measurement only before (0 min) and 120 min after the OSTT to limit pain and stress. Blood was allowed to clot for approx. 30 min and then centrifuged at 2600 × **g** for 15 min to obtain serum. Serum was frozen at -20°C until analysis. Insulin was determined using the solid phase-conjugated sandwich ELISA kit and analysed at least in triplicate (three replicates) for each rat in Experiment 1.

4.2.3.8 Statistical analysis

Results presented are means ± 1 standard deviation (SD). Mean differences among the five groups for blood glucose at each time interval, blood glucose at 15 min, maximum blood glucose, AUC and serum insulin were analysed by one-way analysis of variance (ANOVA) and Fisher's least significant difference test using Statistica software version 12 (StatSoft, Tulsa, OK). Basal blood glucose data differed significantly ($p < 0.05$) between groups and therefore blood glucose data was analysed by normalising to percentage of basal. Outliers were removed from data sets using Dixon's Q-test (Dean and Dixon, 1951).

4.2.4 Results and discussion

4.2.4.1 Improvement of encapsulation efficiency
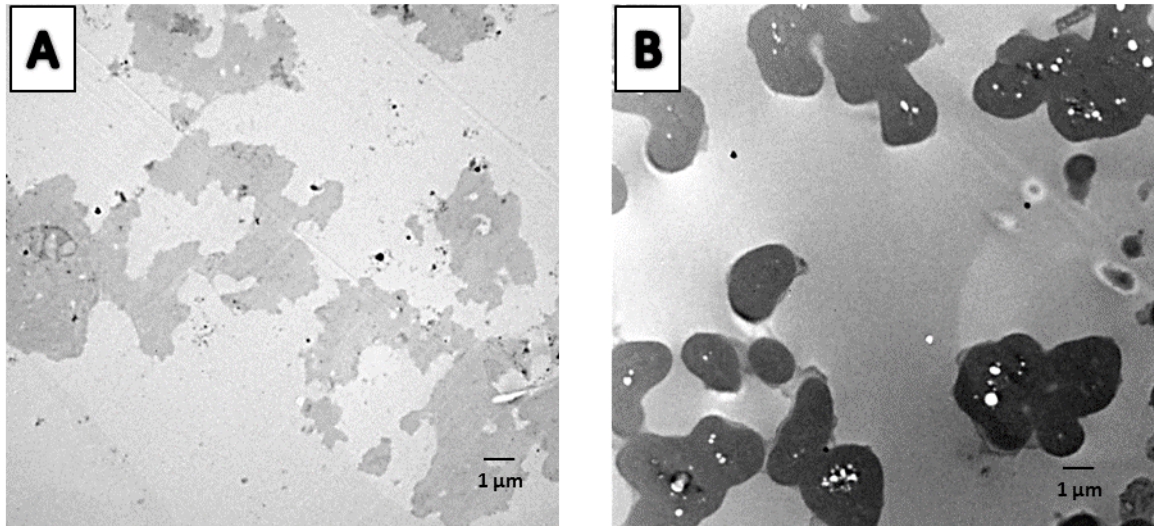
Figure 4.2.1: TEM of aqueous ethanol prepared kafirin microparticles (KEMS). A: KEMS alone. B: KEMS encapsulating sorghum condensed tannin (SCT-KEMS).

TEM confirmed the higher encapsulation efficiency (73%) of SCT-KEMS prepared in this study compared to those prepared in Chapter 4.1. The much darker colour of the SCT-KEMS (Figure 4.2.1 B) compared to the KEMS alone (Figure 4.2.1 A) suggests that some KEMS were saturated with SCT. Taylor, Taylor, Belton and Minnaar (2009a) showed that SCT-KEMS appear darkly stained due to the osmiophillic nature of condensed tannins. Therefore, with the improvement of encapsulation, SCT-KEMS showed almost complete darkening, not just dark spots within SCT-KEMS at an encapsulation efficiency of 49% (Figure 4.1.2 H).

4.2.4.2 Blood glucose response

The effect of encapsulating SCT in KEMS on the masking of their bitter and astringent taste was not specifically assessed. However, it was observed that in all three OSTT the rats took in the SCT-KEMS much more willingly than the SCT alone. Therefore, it can be assumed that the encapsulation masked the known bitter and astringent taste of SCT (Kobue-Lekalake, Taylor and De Kock, 2007). Fang and Bhandari (2010) state an important function of a polyphenol encapsulation agent is to mask the astringent taste

associated with most polyphenols. Therefore, the observed masking property of KEMS is highly desirable for their application as an encapsulation agent for SCT.

Concerning Experiment 1, the blood glucose response curves (Figure 4.2.2) showed that SCT-KEMS, acarbose and KEMS alone were effective in decreasing the incremental rise in blood glucose level (Figure 4.2.2 A). After 60 min, only the SCT-KEMS treatment showed a significant reduction ($p<0.05$) in blood glucose level compared to the water control and SCT alone treatment. Condensed tannins are known to inhibit the activity of digestive enzymes and thereby delay starch digestion (Kim, Hyun and Kim, 2011). The SCT released from the KEMS during digestion were probably able to inhibit intestinal amylases, decrease starch digestion and thereby reduced the amount of glucose available for absorption. There was an unexpected sharp decrease in blood glucose level for the water control treatment group between 15 and 45 min after OSTT. This decrease was probably due to the proportional rise in insulin level (see Figure 4.2.8). The normalised blood glucose response curves (Figure 4.2.2 B) showed that only acarbose and SCT-KEMS did not cause a glucose spike.

Blood glucose levels 15 min after maltodextrin administration (Figure 4.2.3 A) showed that acarbose reduced ($p<0.05$) the blood glucose spike by on average 19% compared to the water control. There was no difference between the SCT-KEMS, KEMS alone and the water control. SCT alone showed the highest ($p<0.05$) blood glucose level of all the treatments. SCT-KEMS treatment prevented a glucose spike and decreased ($p<0.05$) the maximum blood glucose level by on average 11.8% compared to the water control. The KEMS alone treatment also decreased ($p<0.05$) the maximum blood glucose, by 9.4% compared to the control. SCT-KEMS decreased ($p<0.05$) the total AUC, by on average 9% compared to the water control. There was no difference between the AUC of the water control and acarbose treatments, while SCT alone had the largest AUC of all the treatments.

Normalised blood glucose data (Figure 4.2.3 B) showed that only acarbose significantly ($p<0.05$) lowered the blood glucose spike after maltodextrin administration. There was no difference between treatments for the maximum blood glucose level. The KEMS alone and SCT alone treatments had a larger ($p<0.05$) AUC compared to the water control and SCT-KEMS. Similar to findings of this study, Chung, Kim, Yeo, Kim, Seo and Moon (2011)

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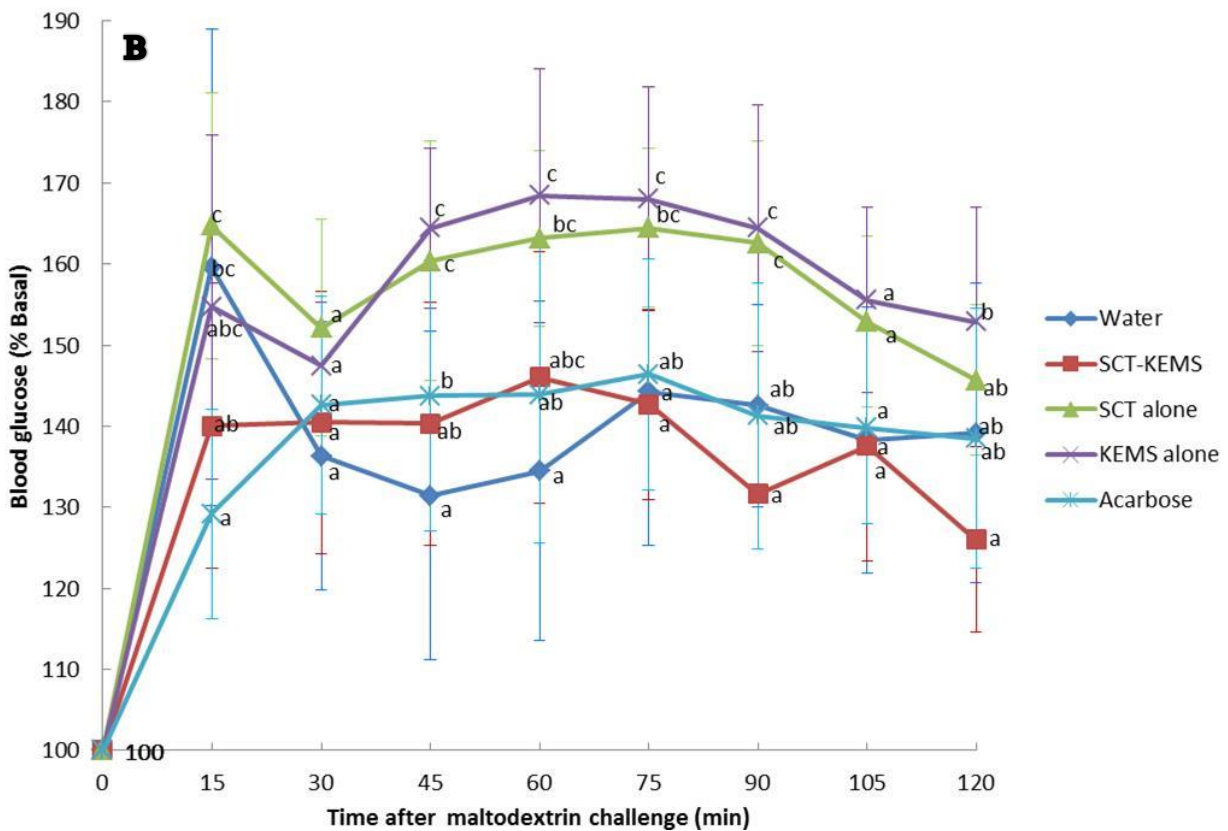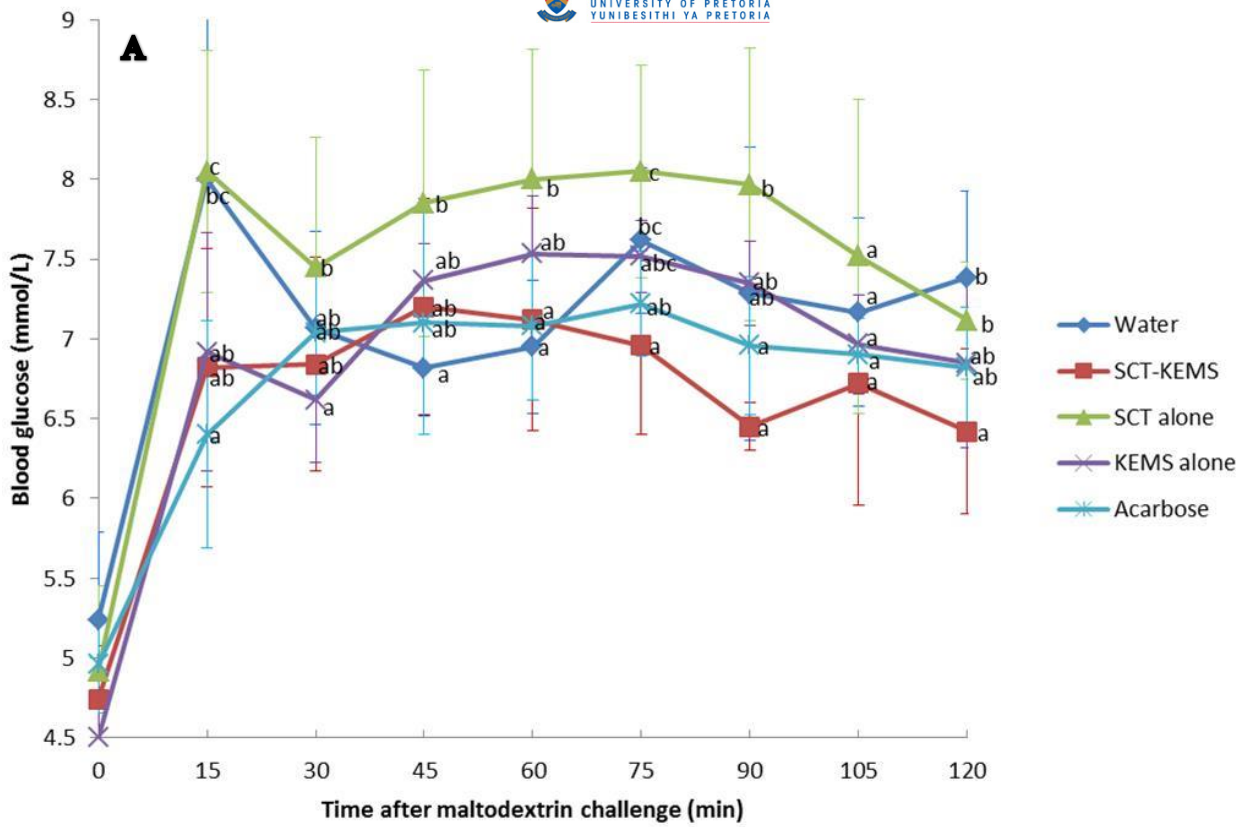
Figure 4.2.2: Blood glucose response curve after oral maltodextrin challenge, Experiment 1. A: Blood glucose response of rats treated with acarbose, SCT-KEMS, KEMS alone, SCT alone and the control, water. B: Data normalised to time 0. Results expressed as mean ± SD, n=6 rats (SCT-KEMS, n=5 rats). Values with different letters differ significantly at each time point (p<0.05)
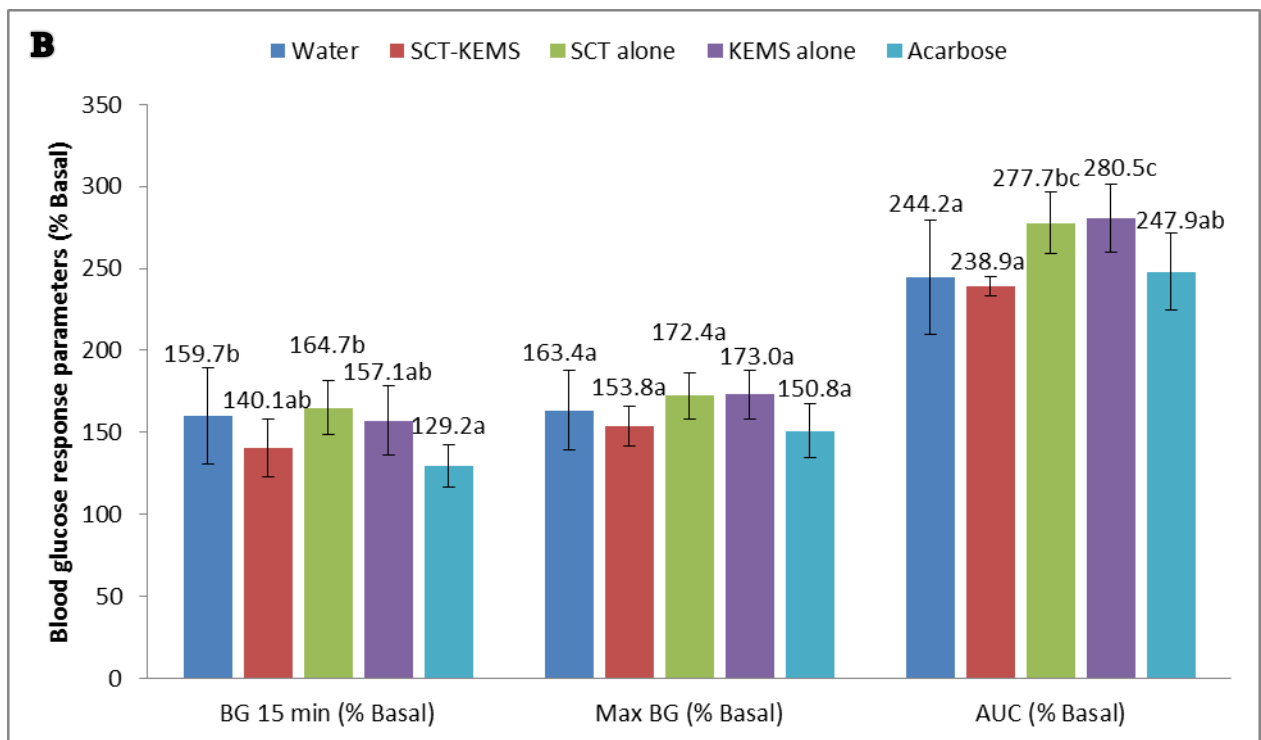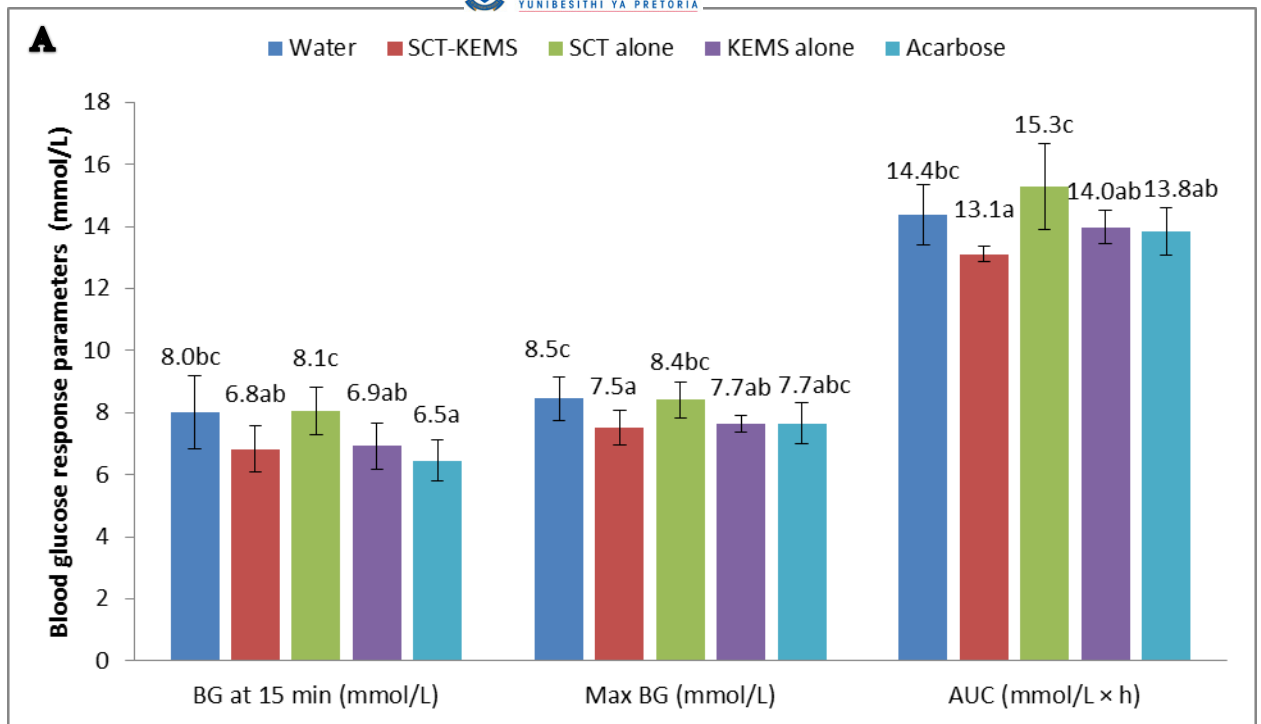
Figure 4.2.3: Blood glucose (BG) response after maltodextrin challenge, Experiment 1. A: The BG at 15 min, maximum BG over analysed time period and area under blood glucose curve (AUC) of rats treated with SCT-KEMS, SCT alone, KEMS alone, acarbose or the water control. B: Data normalised to time 0. Results expressed are mean ± SD, n = 6 (SCT-KEMS, n=5). Values with different letters differ significantly (p<0.05).

found that sorghum extracts rich in tannins suspended in acacia gum at dosages of 250-500 mg/kg of body weight decreased the serum glucose concentration of streptozotocin-induced diabetic rats fed a high-fat diet. Additionally, Kim and Park (2012) found that oral gavage administration of 600 mg/kg of sorghum extract by decreased blood glucose levels after an intraperitoneal glucose tolerance test on streptozotocin-induced diabetic rats. However, the dosages used by these researchers were not directly comparable to the ones used in this study as they did not quantify the tannins in their extracts. Furthermore, these researchers used diseased rodent models and did not assess the inhibition of digestive amylases as a mechanism for the observed anti-hyperglycaemic effects.

Blood glucose data from Experiments 2 and 3 revealed that there were generally no differences between treatments when the OSTT was repeated. During Experiment 2 and 3 it was observed that the rats acted rather agitated. Compared to the first OSTT, their movements were more rapid; they escaped the handler's grip multiple times and shrieked when their tails were pricked. Hence, it can be assumed that repetition of the OSTT caused rats to remember the stress associated with an OSTT. Stress in rats leads to the upregulation of corticosterone, a steroid hormone known to influence carbohydrate metabolism (Balcombe, Barnard and Sandusky, 2004; Ayala et al., 2010). Thus, it is probable that the results of Experiments 2 and 3 are not particularly meaningful.

Although Experiment 2 was a repetition of Experiment 1, blood glucose levels for all treatments in Experiments 2 (Figure 4.2.4 A) and 3 (Figure 4.2.6 A) were marginally lower than those of Experiment 1 (Figure 4.2.2 A). Experiment 2 showed that acarbose treatment had the lowest blood glucose levels throughout the OSTT, particularly at 90 min (p<0.05). There were essentially no differences between the SCK-KEMS, KEMS alone and the water control. However, at 90 min, the blood glucose levels after KEMS alone and acarbose treatment were significantly (p<0.05) lower than the water control. SCT alone again showed the highest blood glucose level. When the glucose response curves were normalised for Experiment 2 (Figure 4.2.4 B), SCT-KEMS showed the lowest blood glucose levels, particularly at 90 min. The blood glucose response of Experiment 3 (Figure 4.2.6 A) showed that only acarbose had a spike at 15 min, which was not observed in Experiments 1 and 2. The normalised glucose response curve for Experiment 3 (Figure 4.2.6 B) showed that acarbose treatment had the lowest blood glucose levels, especially at 30 min after the OSTT.

In Experiment 2 only acarbose decreased blood glucose at 15 min compared to the control (Figure 4.2.5 A). The maximum blood glucose levels did not differ among the SCT-KEMS, KEMS alone and acarbose treatments and were considerably lower than those found in Experiment 1 (Figure 4.2.3 A). The AUC for acarbose treatment was the smallest ($p < 0.05$) compared to the water and SCT alone. The normalised data of Experiment 2 (Figure 4.2.5 B) showed the acarbose treatment reduced ($p < 0.05$) the blood glucose at 15 min compared to the water. The maximum blood glucose level and AUC showed no differences between treatments. In Experiment 3, there was no differences between the treatments for blood glucose level at 15 min (Figure 4.2.7 A) .The maximum blood glucose level after SCT-alone treatment was higher ($p < 0.05$) and the AUC larger than SCT-KEMS, KEMS alone and acarbose treatments. Normalised blood glucose data of Experiment 3 (Figure 4.2.7 B) showed that maximum blood glucose and AUC for SCT alone was significantly higher ($p < 0.05$) than the SCT-KEMS and acarbose treatments.

Surprisingly, SCT alone showed the highest blood glucose levels in all three OSTT experiments. The *in vitro* results showed that SCT alone (unencapsulated) lost most of their inhibitory action once subjected to simulated gastrointestinal digestion (Table 4.1.3)**.** Therefore, it was expected that SCT alone would not reduce blood glucose levels. However, it was not expected that the SCT would increase the blood glucose levels higher than those of the control. In fact, similar studies have found that orally administered plant polyphenolic extracts reduced blood glucose levels during OSTT on healthy rats (Jo, Ha, Moon, Lee, Jang and Kwon, 2011; Ali et al., 2013).

The high blood glucose level after SCT alone administration can, in part, be attributed to the bitterness and astringency of SCT (Kobue-Lekalake et al., 2007) that might have caused stress when administered to the rats. As mentioned, stress can lead to increased corticosterone secretion which in turn is known to stimulate gluconeogenesis (Cawley, 2012). Additionally, stress can also increase salivary α-amylase secretion and result in digested starch (glucose) in the small intestine before pancreatic starch digestion (Nater and Rohleder, 2009). In previous studies involving sorghum phenolic extracts, extracts were administered to rats via gavage (Chung, Kim, Yeo, Kim, Seo and, Moon, 2011; Kim and Park, 2012) and this is probably why the elevating effect of SCT on blood glucose has not previously been reported.
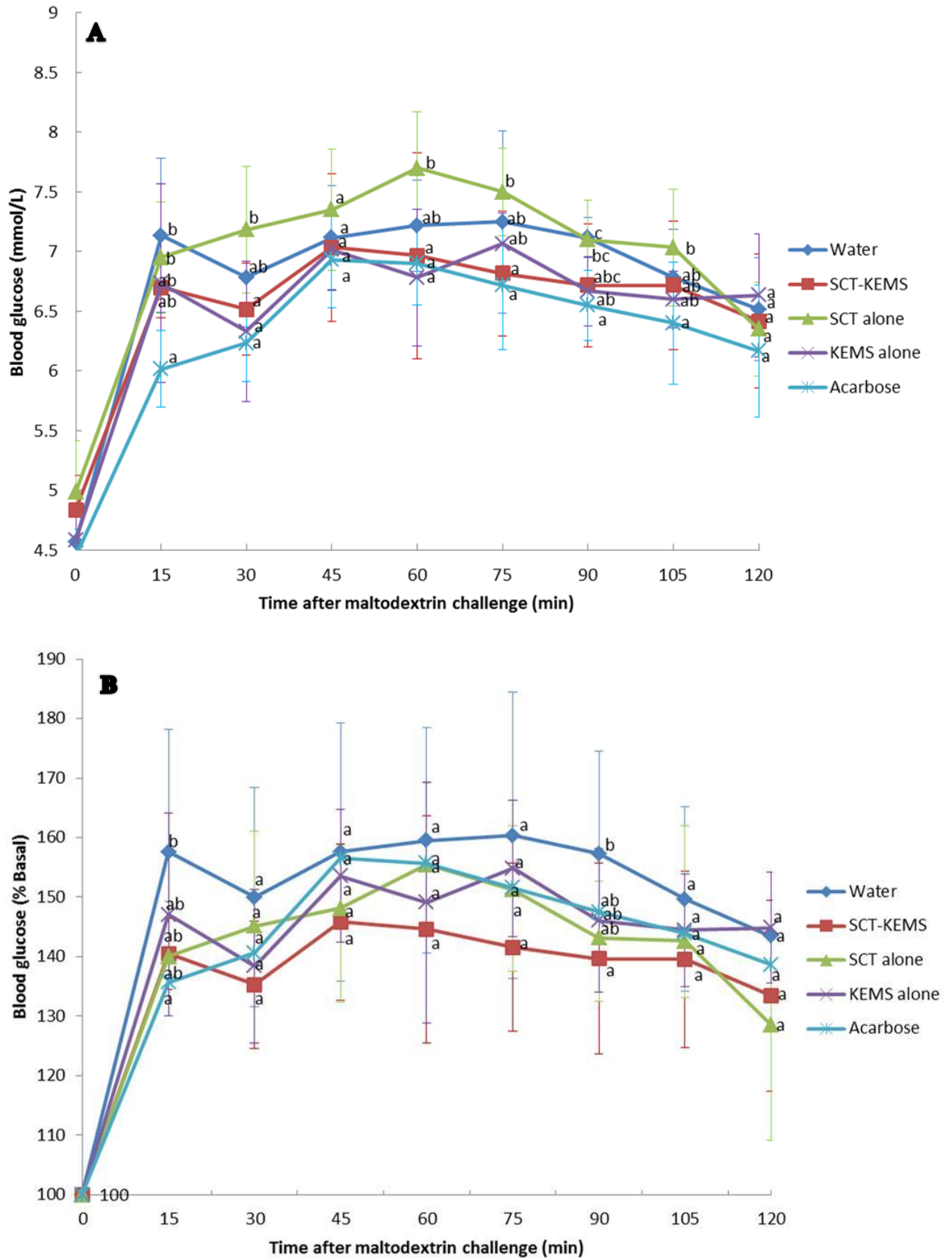
Figure 4.2.4: Blood glucose response curve after oral maltodextrin challenge, Experiment 2. A: Blood glucose response of rats treated with acarbose, SCT-KEMS, KEMS alone, SCT alone and the control, water. B: Data normalised to time 0. Results expressed as mean ± SD, n=6 rats. Values with different letters differ significantly at each time point ($p<0.05$).

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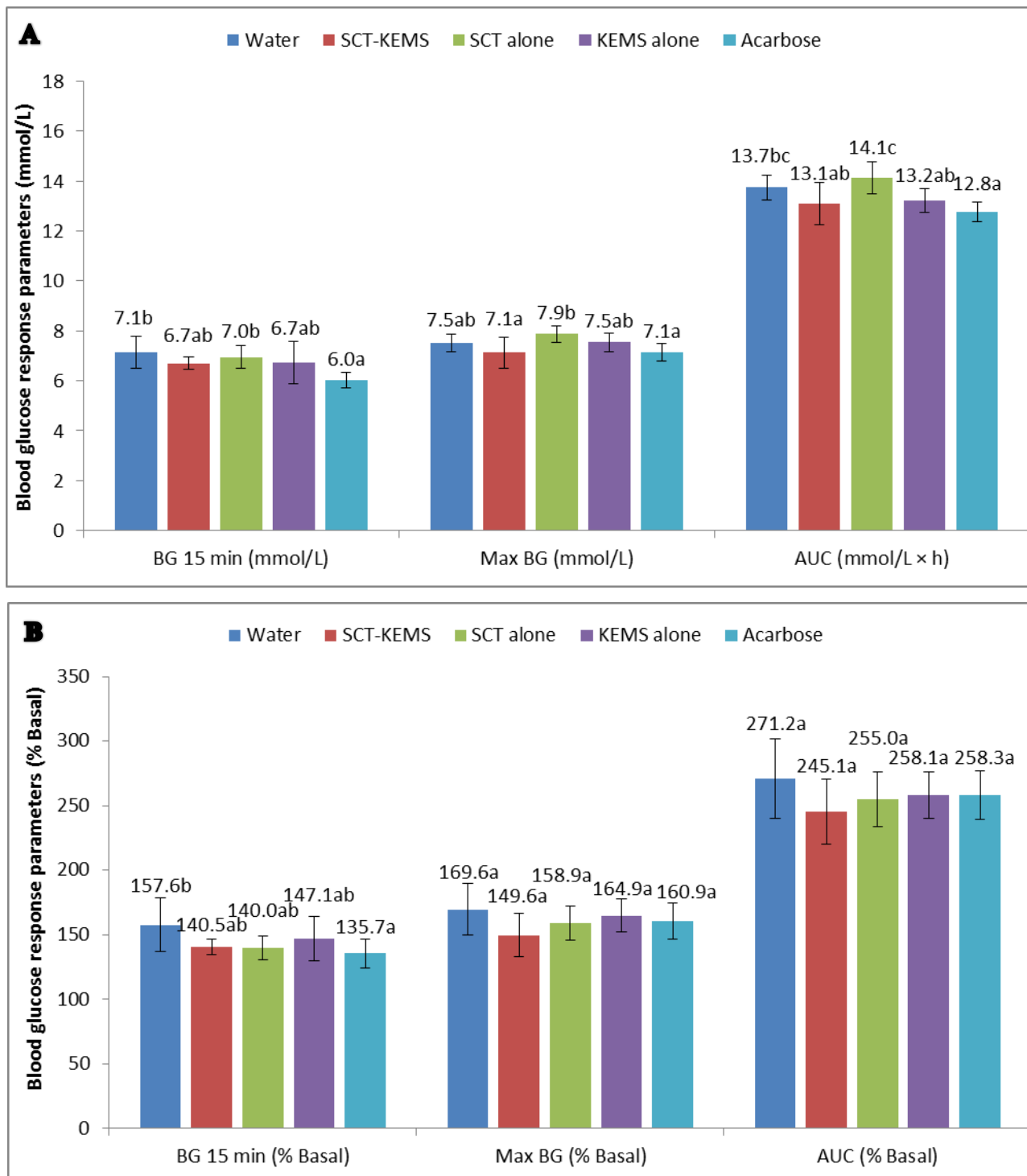
Figure 4.2.5: Blood glucose (BG) response after maltodextrin challenge, Experiment 2. A: The BG at 15 min, maximum BG over analysed time period and area under blood glucose curve (AUC) of rats treated with SCT-KEMS, SCT alone, KEMS alone, acarbose or the water control. B: Data normalised to time 0. Results expressed are mean ± SD, n = 6. Values with different letters differ significantly ($p<0.05$).
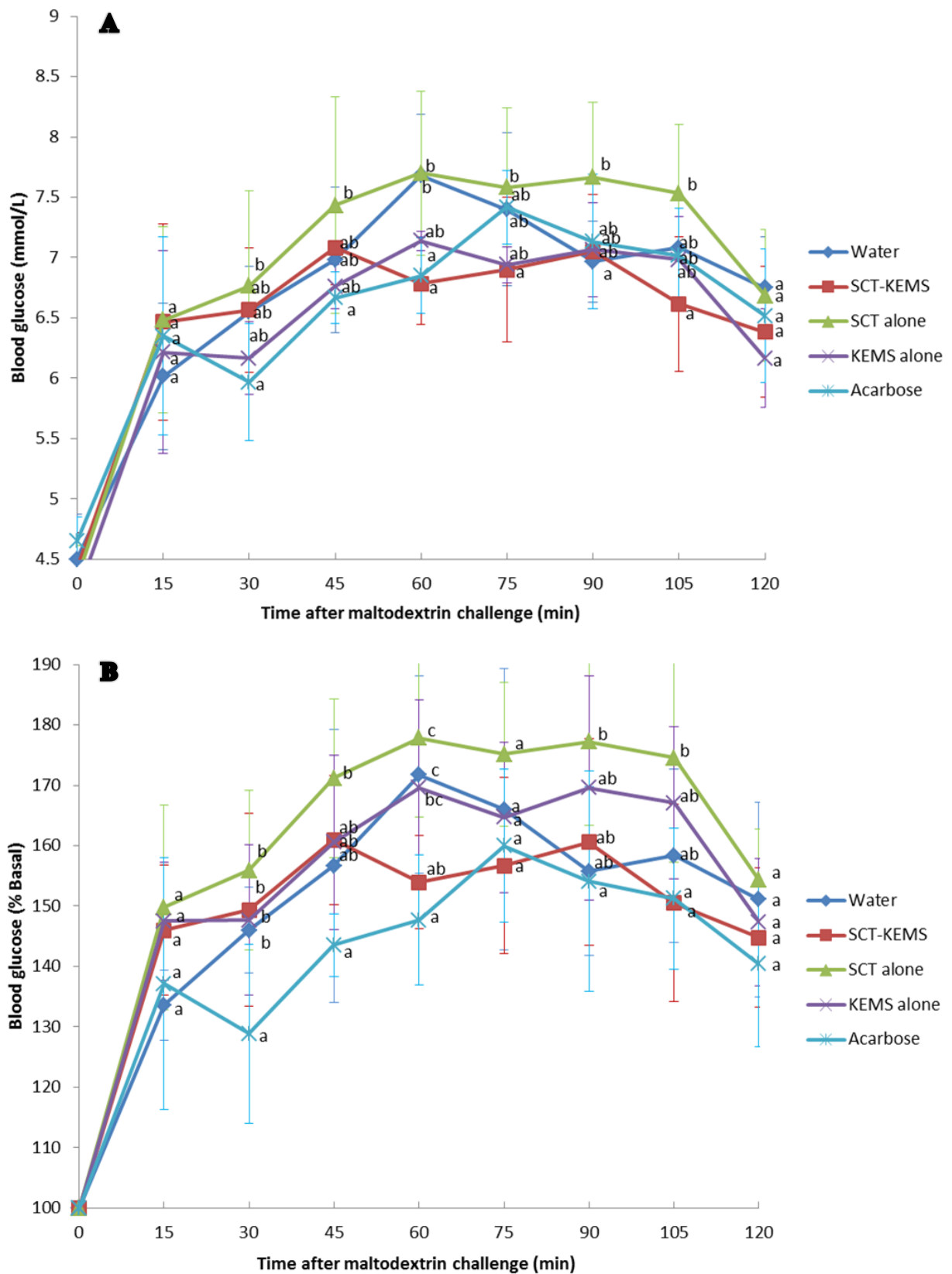
Figure 4.2.6: Blood glucose response curve after oral maltodextrin challenge, Experiment 3. A: Blood glucose response of rats treated with acarbose, SCT-KEMS, KEMS alone, SCT alone and the control, water. B: Data normalised to time 0. Results expressed as mean $\pm$ SD, n=6 rats. Values with different letters differ significantly at each time point ($p<0.05$).
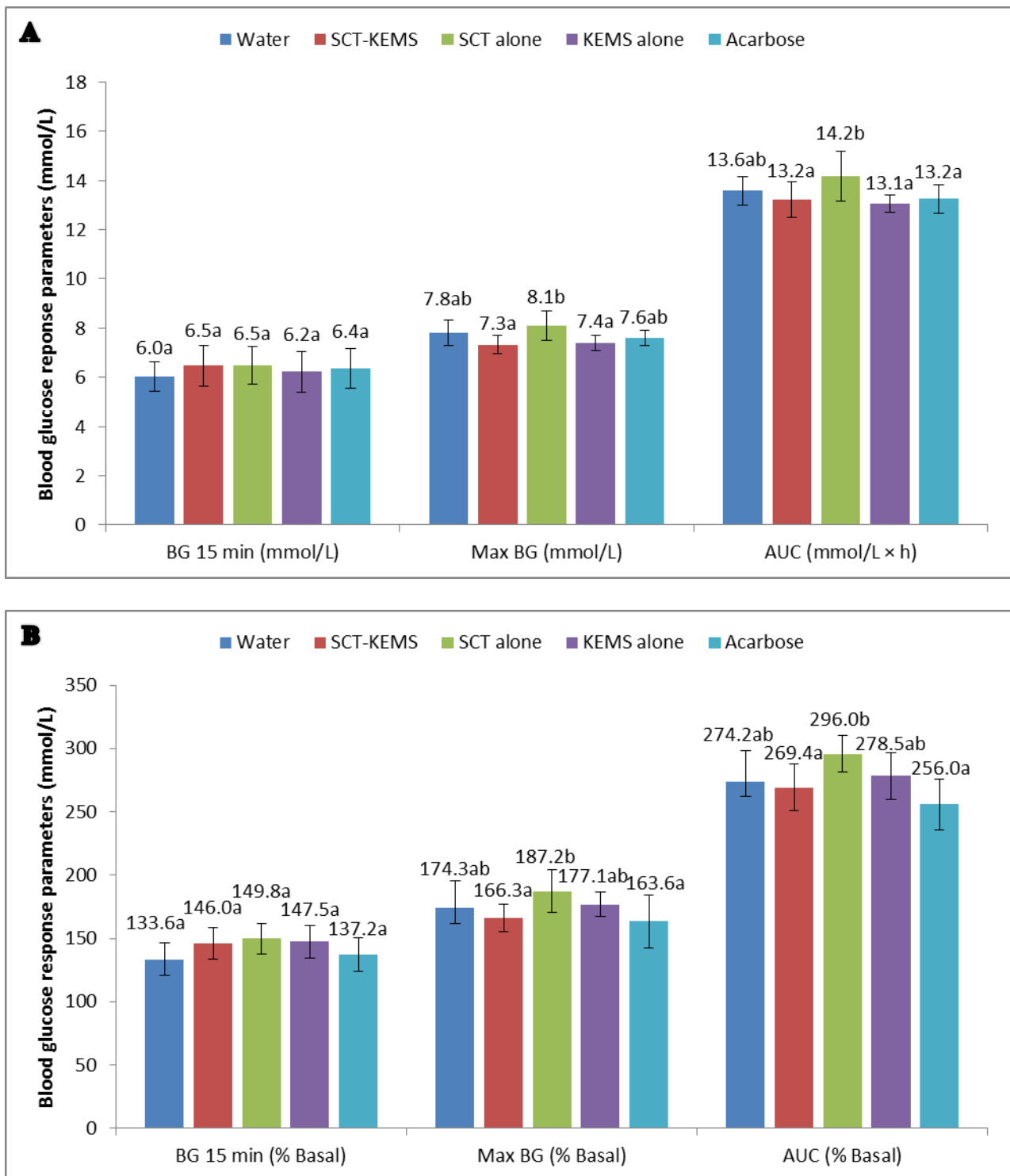
Figure 4.2.7: Blood glucose (BG) response after maltodextrin challenge, Experiment 3. A: The BG at 15 min, maximum BG over analysed time period and area under blood glucose curve (AUC) of rats treated with SCT-KEMS, SCT alone, KEMS alone, acarbose or the water control. B: Data normalised to time 0. Results expressed are mean ± SD, $n = 6$. Values with different letters differ significantly ($p<0.05$).

4.2.4.3 Serum insulin

As mentioned with respect to the blood glucose data, repetition of the OSTT probably caused the rats considerable stress. This is supported by the fact that the serum insulin data from Experiments 2 and 3 showed that all the rats had highly elevated serum insulin levels regardless of the treatment administered. As suggested, in response to the stress of the first OSTT the rats probably upregulated corticosterone secretion. The upregulation could in turn stimulate insulin secretion to levels observed with corticosteroid-induced hyperinsulinaemia (van Raalte, Ouwens and Diamant, 2009). Therefore, insulin measurements of Experiments 2 and 3 were not repeated and only the serum insulin results of Experiment 1 will be presented and discussed.
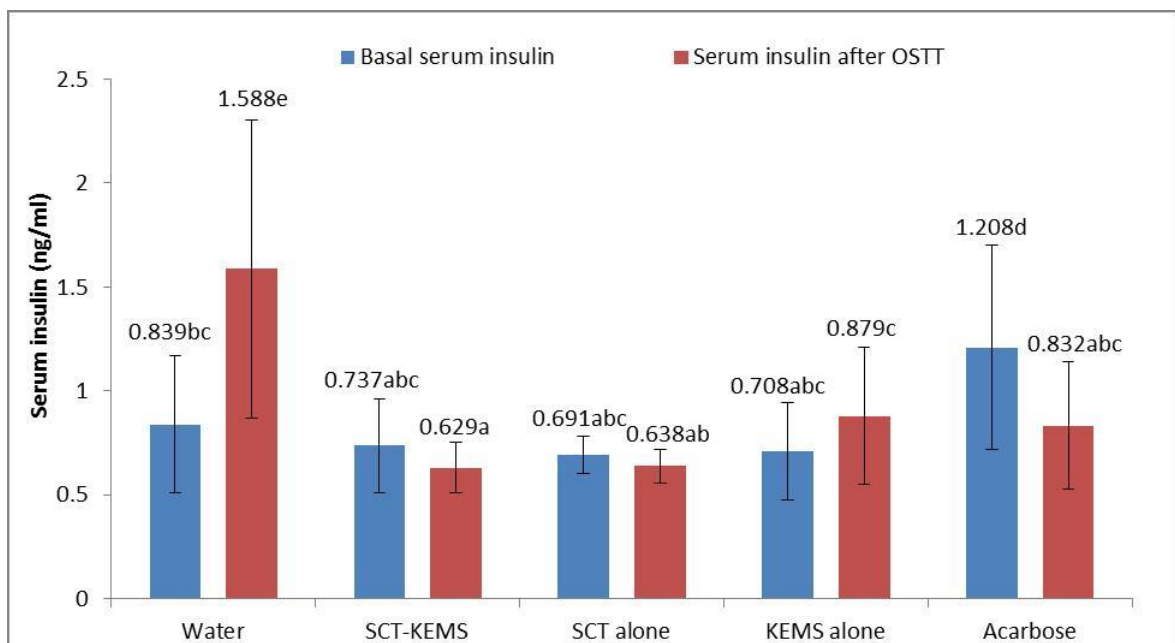
Figure 4.2.8: Insulin before (basal) and after maltodextrin challenge, Experiment 1:Serum insulin of rats treated with SCT-KEMS, SCT alone, KEMS alone and acarbose. Results expressed as mean ± SD, n=6 (acarbose after OSTT, n=5). Values with different letters differ significantly ($p < 0.05$).

In Experiment 1, basal insulin levels were not different between the control and SCT-KEMS, SCT alone and KEMS alone treatments (Figure 4.2.8). The acarbose group, however, had significantly higher ($p < 0.05$) basal insulin levels. The SCT-KEMS, SCT alone and acarbose treatments prevented elevation of insulin levels after the OSTT. By contrast, the water control showed significant increase ($p < 0.05$) (89%) in serum insulin

78

after the OSTT. All the treatments had a reduced insulin response compared to the water control. In fact, SCT-KEMS and SCT alone decreased (p<0.05) the insulin level after the OSTT by 60%, while acarbose showed a 48% reduction compared to the water control. The KEMS alone treatment showed a significant (p<0.05) increase in serum insulin after the OSTT compared to the SCT-KEMS and SCT alone treatments.

Surprisingly, the SCT alone treatment also did not elevate serum insulin levels, despite having the highest blood glucose levels in all three experiments (see Figure 4.2.8). Nomura, Takahashi, Nagata, Tsutsumi, Kobayashi, Akiba, Yokogawa, Moritani, and Miyamoto (2008) found that some flavonoids actually inhibit glucose uptake in adipose cells by inhibiting insulin-stimulated phosphorylation of the insulin receptor and translocation of the glucose transport molecule 4 (GLUT-4). Whereas a study by Pinent et al. (2004) found that condensed tannins from grape seed extracts have insulin-like effects in insulin sensitive cell-lines.

Kim and Park (2012) and Park, Lee, Chung and Park (2012) studied the anti-diabetic effects of sorghum phenolic extracts rich in tannins on streptozotocin-diabetic rats and high-fat fed mice, respectively. Kim and Park (2012) found that the anti-diabetic effect of their sorghum extract was not due to enhanced blood glucose removal, but rather inhibition of hepatic gluconeogenesis. Park et al. (2012) attributed the anti-diabetic effects of their sorghum extracts to increased insulin sensitivity via regulation of the peroxisome proliferator-activated receptor gamma (PPAR-γ) from adipose tissue. The inhibition of glucose uptake by flavonoids, insulin-like effects of grape seed condensed tannins and increased insulin sensitivity observed with sorghum phenolic extracts may be possible reasons why SCT alone did not stimulate insulin secretion but maintained high blood glucose levels.

The blood glucose data showed that KEMS alone also had anti-hyperglycaemic potential (Experiments 1 and 2). In contrast, the serum insulin results showed that the KEMS alone treatment was the only treatment to show increased insulin levels after the OSTT. Higuchi, Hira, Yamada and Hara (2013) showed that hydrolysates of zein (the maize prolamin protein, which shares extensive homology with kafirin) improved glucose tolerance in healthy rats by stimulating glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) secretion. GLP-1 and GIP are incretin hormones known

79

to increase insulin secretion (Ross et al., 2004) and therefore their observation might be an explanation for the raised insulin level and the anti-hyperglycaemic effects of the KEMS alone treatment.

Insulin maintains glucose homeostasis by stimulating glucose utilization by skeletal muscle and adipose tissue (Muniyappa, Lee, Chen and Quon, 2008). In healthy subjects, insulin secretion is stimulated in proportion to the blood glucose level (Wilcox, 2005). Insulin secretion was probably not stimulated after the SCT-KEMS, KEMS alone and acarbose treatments as the blood glucose level was not high enough to trigger an insulin response. Consequently, the insulin data indicate that the observed decreased blood glucose level after SCT-KEMS treatment was due to the inhibition of digestive amylases and not insulin-stimulated glucose clearance.

4.2.5 Conclusions

This study indicates that SCT encapsulated in KEMS can decrease blood glucose levels similarly to acarbose. SCT-KEMS can also prevent elevation of serum insulin in healthy rats after an OSTT. Kafirin microparticles are effective SCT encapsulating agents as they appear to mask the bitterness and astringency of SCT and deliver the SCT to the small intestine. Therefore, encapsulation of SCT in kafirin microparticles has potential as a nutraceutical for the management of hyperglycaemia associated with metabolic syndrome and T2D.

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CHAPTER 5: GENERAL DISCUSSION

The general discussion is divided into three main parts. The first part critically evaluates the important methods as applied in this study. The second section gives possible explanations and mechanisms for the results found in this study. The third section proposes ways in which the morphology and encapsulation efficiency can further be improved and suggests additional *ex-vivo* and *in vivo* studies that can be applied to SCT-KEMS and KEMS for their application as nutraceuticals for T2D.

5.1 Methodologies

In the Vanillin-HCl assay, the flavonoid A-ring at the C-6 position (Figure 2.5) reacts with vanillin and forms a red chromophore and thus the assay detects any monomeric or polymeric flavanols using catechin as a standard (Price et al., 1978). However, catechin is known to overestimate the amount of condensed tannins due to their complex and variable structures (Schofield et al., 2001). In this study the condensed tannin content of the dried extract was 312 mg/100 mg catechin equivalents, clearly a vast overestimation. It has been suggested that purified tannins should be used to give more accurate values (Price et al., 1978). Additionally, the Vanillin-HCl method proved very problematic when used to quantify the amount of SCT encapsulated in the aqueous ethanol prepared SCT-KEMS. The condensed tannin content obtained for these SCT-KEMS was approx. 3 mg/100 mg. With 11% binding of SCT to aqueous ethanol KEMS, a SCT content of about 34 mg/100 mg was expected. Therefore 3mg/100 mg was clearly too low to be accurate. To try and enhance the release of condensed tannins from aqueous ethanol prepared KEMS and quantify them using the Vanillin-HCl method, the samples were sonicated. However even after sonication only 4 mg/100 mg catechin equivalents were detected in the aqueous ethanol prepared SCT-KEMS. It is known that complexation between kafirin and condensed tannin leads to insoluble complexes that are extremely difficult to extract (Emmambux and Taylor, 2003). As majority of the SCT bound to the outside of the acetic acid prepared SCT-KEMS (Figure 4.1.2 D), the Vanillin-HCl assay was capable of quantifying the SCT bound to the acetic acid prepared KEMS. However, as the majority of the SCT were encapsulated inside the aqueous ethanol prepared SCT-KEMS they were not adequately quantified using this assay.

The acetic acid method of preparing KEMS was initially the preferred method for encapsulating the SCT. The larger internal surface area of KEMS made by phase separation from glacial acetic acid was thought to be advantageous as the larger surface area was assumed to result in higher SCT binding (Taylor et al., 2009a). However, TEM showed that KEMS prepared by the acetic acid method did not effectively encapsulate the SCT. The SCT were either left behind in the dispersed phase, or bound to the outside of the KEMS. Kafirin can be extracted from sorghum flour using 60% aqueous ethanol at elevated temperature (Sastry and Virupaksha, 1969). Also, condensed tannins can be extracted with 50% aqueous ethanol (Downey and Hanlin, 2010). Therefore the aqueous ethanol method was selected to prepare KEMS. TEM showed that most of the SCT were effectively encapsulated inside the aqueous ethanol prepared KEMS, although these KEMS aggregated into clumps rather than spherical microparticles.

The aqueous ethanol method has been previously found to produce spherical protein microparticles (Liu, Sun, Wang and Zhang and Wang, 2005; Taylor, 2008). Hence, the clumped morphology was unexpected. The morphology of the SCT-KEMS did not differ from the KEMS alone, indicating that the addition of the SCT was not responsible for the clumping of the KEMS. The method used to prepare the aqueous ethanol KEMS in this instance was slightly different and probably resulted in the different morphology. The rapid (3.6 ml/min) and manual addition of the distilled water could have resulted in the clumped morphology. Fast precipitation of the protein used to prepare microparticles can lead to larger and irregular shaped particles (Yeo and Park, 2004).

TEM was used to visualise the internal structure of KEMS. Osmium tetroxide staining of KEMS allowed binding of SCT to be clearly visualised. However, lead counterstaining led to dark black blotches on some TEM images (Figure 4.1.2), which were somewhat similar to the dark areas indicative of SCT binding. Fortunately the black blotches were clearly distinguishable and were also observed with TEM of KEMS alone. Thus, lead staining was omitted. However, the resolution and contrast of the TEM images were greatly reduced. Finally, halving the time required for lead staining led to improved images.

The *in vitro* protein digestibility of the acetic acid prepared KEMS (Table 4.1.2) was lower than that found by Taylor et al. (2009a) and Anyango, Duneas, Taylor, and Taylor (2012) using the same protocol. A possible explanation is that the chemical composition of the

86

kafirin used in this study was different and might have contained a higher percentage of γ-kafirin. It has been found that γ-kafirin was less digestible than either total or residual kafirin (after γ-kafirin removal) and the lower digestibility was attributed to the fact that γ-kafirin exists primarily in the form of disulphide cross-linked oligomers (Taylor et al., 2007). The high temperature (70°C) used to dissolve the kafirin during preparation of the aqueous ethanol KEMS could also have had an effect on the protein digestibility. High temperatures increase the amount of disulphide crosslinking in the kafirin (Belton et al., 2006) and thereby decrease the protein digestibility. Additionally, the considerably lower surface area of the aqueous ethanol KEMS compared to the acetic acid prepared KEMS probably limited digestibility of the aqueous ethanol KEMS. A smaller surface area would lead to decreased enzyme accessibility and Duodu et al. (2003) found that enzyme accessibility to the kafirin protein plays an important role in determining digestibility.

Alpha-amylase inhibition was assayed based on the methods by Tadera et al. (2006) and the Ceralpha method of McCleary and Sheehan (1987). The procedure involves using a substrate that is a defined maltosaccharide with an α-linked nitrophenyl group at the reducing end of the chain and a chemical blocking group at the non-reducing end. The substrate is completely resistant to attack by β-amylase, glucoamylase and α-glucosidase, making the assay highly specific for α-amylase. However, it was quite challenging to assess amylase inhibition by the KEMS. In the assay for α-amylase, tri-sodium phosphate (pH 11) was used to raise the pH and thereby stop the reaction and sodium carbonate (pH 11) to stop the α-glucosidase reaction. Upon addition of tri-sodium phosphate and sodium carbonate to the SCT-KEMS bright red sediment formed, which was presumably SCT released from the SCT-KEMS. To prevent this problem the inhibition reactions were stopped using ice. However, it was found that tri-sodium phosphate and sodium carbonate was required for adequate colour development. Therefore KEMS were removed from the reaction mixtures just before addition of the stopping reagents. The reaction mixtures without KEMS were then used for absorbance readings to determine the amylase inhibition of KEMS.

The number of animals in the treatment groups (n=6) of the *in vivo* study was probably suboptimal to obtain unequivocal differences between the treatments as variability between and within groups were quite large. Smaller sample sizes (n=6-8) have been used in oral glucose tolerance tests (reviewed by Pinent et al., 2012) while sample sizes of 10 (Gad, El-

87

Sawalhi, Ismail and El-Tanbouly, 2006) and up to 20 (Wolf et al., 2002) have been used for OSTT in healthy rats. The rationale behind the sample size used in this study was mainly based on the **"Three Rs"** (replace, reduce and refine) principles that ensure ethical use of animals in testing (Hooijmans, Leenaars and Ritskes-Hoitinga, 2010). The "reduce" principle was employed to the sample size by determining the minimum number of animals required to obtain significant results. Therefore the number of rats used in this study was based on similar studies that found significant differences in treatment when using n=6 (Subramanian, Asmawi and Sadikun, 2008; Ali et al., 2013). However, in view of the vulnerability in the data, a sample size of about 10 animals per treatment group could have resulted in clearer results in the present study.

To avoid delays, readily available rats were sourced from breeders. Unfortunately, these rats were already 15 weeks old when the study commenced. Additionally, the rats used in this study were rather large (260-350 g). Similar studies used male rats weighing between 150 and 250 g and aged around 8 weeks (Al-Awwadi, Azay, Poucheret, Cassanas, Krosniak, Auger, Gasc, Rouanet, Cros and Teissedre, 2004; Tiwari, Swapna, Ayesha, Zehra, Agawane and Madhusudana, 2011). The higher weight and older age might have influenced the animals' mechanisms of glucose metabolism and therefore caused the large variability in the results. To avoid age-related metabolic disturbances, it is suggested that rats used in these types of studies should be classified as adult, but not old. It is also suggested that rats between 150 and 200 g and a sample size of about 10 animals per treatment group could give better results.

Repetition of the OSTT was clearly a flaw in the *in vivo* study. During Experiments 2 and 3 it was observed that the rats were visibly more stressed compared to the first OSTT. Stress in rats leads to upregulation of corticosterone, a steroid hormone known to influence carbohydrate metabolism (Balcombe, Barnard and Sandusky, 2004; Ayala et al., 2010). Additionally, the upregulation could stimulate insulin secretion to levels observed with corticosteroid-induced hyperinsulinaemia (van Raalte, Ouwens and Diamant, 2009).). Therefore repetition of the OSTT probably evoked stress responses during Experiments 2 and 3 that overrode the treatment effects. Therefore, the OSTT should not be repeated using the same animals.

The originally intended method of treatment administration was by gavage (orogastric tube feeding). However, due to the larger size of the rats and consequently greater treatment volume required, it was not possible to get the KEMS through an 18-gauge gavage tube. The treatments were therefore orally administered via syringe. Although this was not the preferred method, it worked well and had the advantage of indicating which of the treatments the rats preferred to consume. The ease with which rats took in the SCT-KEMS compared to the SCT alone clearly demonstrated the ability of KEMS to mask the astringent and bitter taste of SCT (Kobue-Lekalake et al., 2007). Additionally, as food is consumed by mouth the oral administration of the treatments was advantageous in comparison to gavage.

The insulin values within the treatment groups were very inconsistent as the individual rats in each treatment group had different insulin levels after the OSTT. Such large variability in insulin levels can be attributed to each rat's individual stress response (Ayala et al., 2010). Another limitation in this study was that insulin was only measured before (basal) and 120 min after the OSTT. The main reason for the limitation in the amount of measurements was to reduce stress and pain. Studies that measured the serum insulin levels at the same time intervals as the blood glucose levels found that serum insulin peaked in proportion to the glucose spike (Itoh et al., 2004; Higuchi, Hira, Yamada and Hara, 2013). Therefore it is possible that in this present work the insulin peak was missed. Nonetheless, as the SCT-KEMS and acarbose treatments did not cause a spike in glucose level, it can be assumed that insulin levels were not elevated. In a normal/healthy situation, insulin will rise proportionally to blood glucose level (Wilcox, 2005) and as blood glucose return to normal, insulin levels would as well. However, if hyperglycaemia is induced, there would be higher final volumes of insulin in the blood. Therefore only two blood samples were required for insulin measurement.

5.2 Research Findings

The IC_{50} values obtained for SCT (Figure 4.1.1) indicate that SCT are an effective inhibitor of starch digesting enzymes, in particular α-glucosidase and could therefore be an effective anti-hyperglycaemic agent. Virtual screening of α-glucosidase inhibitors showed that the two structures illustrated in Figure 5.1 A were the strongest binders of the active site of α-glucosidase among the 20 known inhibitors examined (Park, Ko and Park, 2008). These

structures seem to show some structural homology with phenolic compounds that are components of sorghum polyphenols (Figure 5.1 B) (Awika and Rooney, 2004). These similarities appear to provide some explanation for the very low α-glucosidase IC_{50} found in this study (Figure 4.1.1). Conversely, the structure of acarbose indicates no apparent structural homology (Figure 5.3 C) with the chemical structures of the virtually determined α-glucosidase inhibitors. Providing a possible reason for the much higher α-glucosidase IC_{50} found with acarbose compared to SCT.
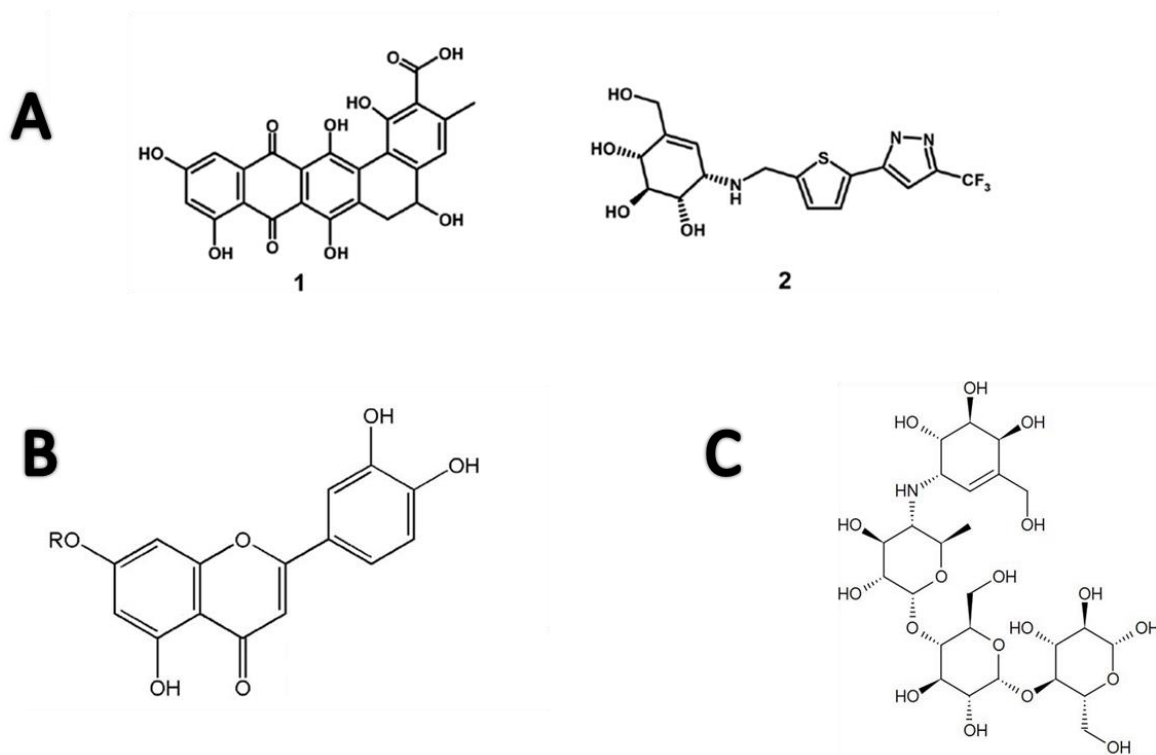
Figure 5.1: Chemical structures of α-glucosidase inhibitors. A: Top two scoring chemical structures obtained from homology modelling and virtual screening of α-glucosidase inhibitors with AutoDock software (adapted from Park, et al., 2008). B: Basic structure of flavonoid monomer found in sorghum phenolic extracts (adapted from Awika and Rooney, 2004). C: Two-dimensional structure of acarbose (Evans and Rushakoff, 2010).

The time it took to prepare the acetic acid prepared KEMS by coacervation was about 120 minutes (1.4 ml/min). According to Yeo and Park (2004) delayed precipitation of the polymer used as the encapsulation agent causes slow solidification of microparticles, thereby resulting in lower encapsulation efficiency. Their observation forms the basis of a potential mechanism to explain the lower encapsulation efficiency of the acetic acid prepared SCT-KEMS (Figure 5.2) compared to the aqueous ethanol prepared SCT-KEMS.

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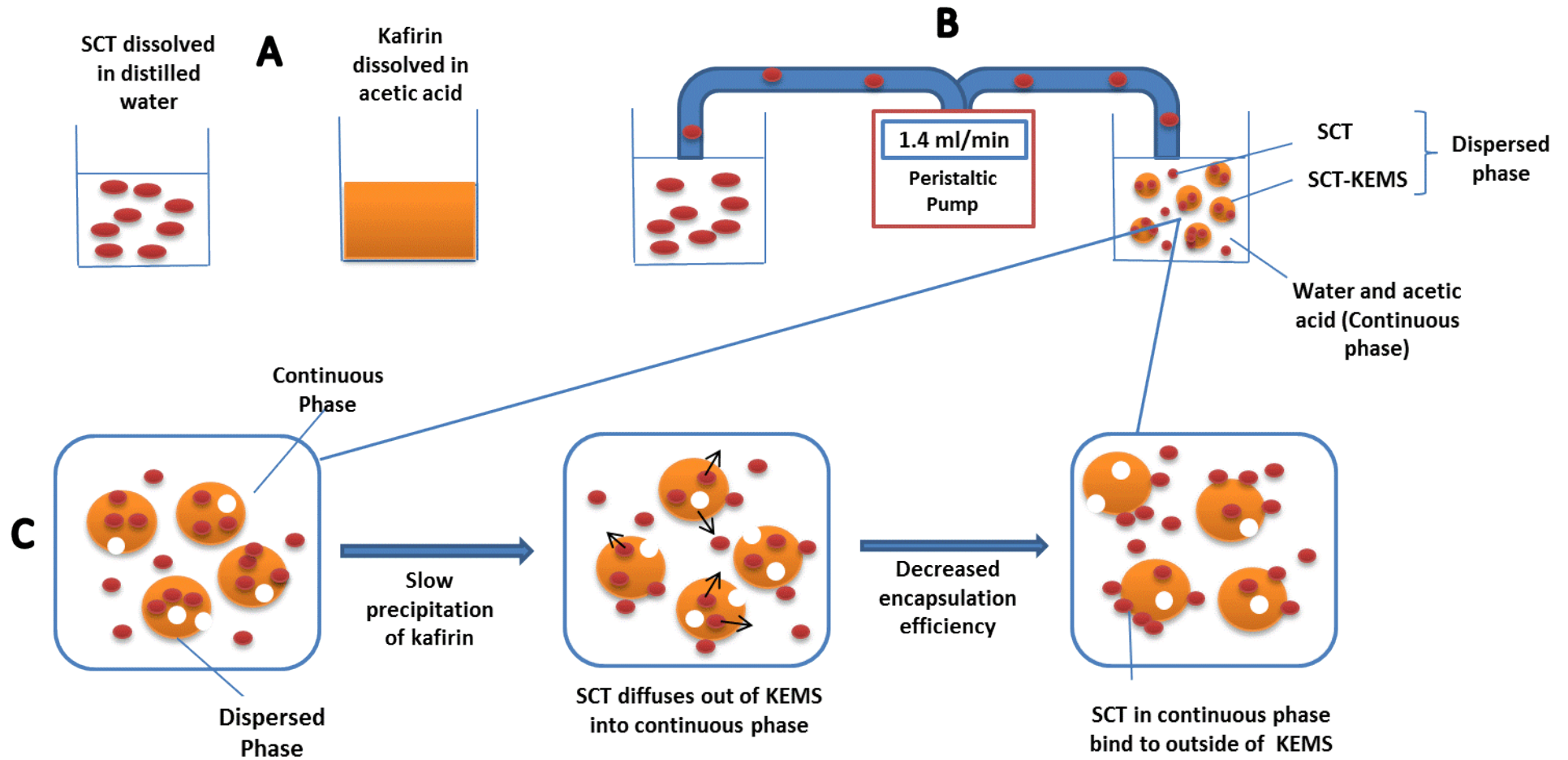
Figure 5.2: Model to explain the lower encapsulation efficiency of acetic acid prepared SCT-KEMS. A: Solutions of SCT in water and kafirin in glacial acetic acid. B: The addition of SCT to the kafirin via a peristaltic pump at 1.4 ml/min and formation of KEMS. C: Enlarged view of conditions leading to lower encapsulation efficiency of acetic acid prepared SCT-KEMS.

91

The lower encapsulation efficiency of the acetic acid prepared SCT-KEMS is presumably related to the SCT being dissolved in water and the kafirin in acetic acid separately (Figure 5.2 A) prior to kafirin precipitation at a rate of 1.4 ml/min (Figure 5.2 B). Therefore it is proposed that the lower encapsulation efficiency might have been caused by encapsulated SCT that had diffused into the continuous phase and either remained in the continuous phase or bound to the surface of already formed microparticles (Figure 5.2 C). The outside of kafirin microparticles have been proposed to be rich in γ-kafirin which would stabilize the partially formed microparticles by disulphide bonds (Taylor, 2008). Further, Taylor, et al., 2007 have shown that γ-kafirin bound more SCT than α- or β-kafirin due to its higher proline content. Therefore some of the SCT left in the continuous phase bound to the outside of the already formed acetic acid prepared microparticles.

Conversely, the higher encapsulation efficiency of the aqueous ethanol prepared KEMS was probably a result of mixing the SCT and kafirin in aqueous ethanol separately and then adding them together prior to coacervation (Figure 5.3 A). Additionally, Yeo and Park (2004) stated that fast precipitation of the polymer used for encapsulation leads to rapid solidification and higher encapsulation efficiency. Therefore the faster precipitation rate of 3.6 ml/min for the aqueous ethanol prepared SCT-KEMS contributed to the higher encapsulation efficiency (Figure 5.3 B). The faster precipitation rate for the aqueous ethanol prepared KEMS probably led to rapid solidification of the kafirin microparticle and limited diffusion of SCT into the continuous phase (Figure 5.3 C).

Amylase inhibition by the KEMS alone was unexpected. The kafirin used to prepare the KEMS did not exhibit any inhibition against α-amylase nor α-glucosidase (data not presented). It is known that change in pH, ionic strength and nature of the ions present affect protein conformation. Belton et al. (2006) reviewed that heating kafirin resulted in changes in conformation that may be sufficient to allow protein-protein interaction. Therefore a conformational change in the protein could have occurred during KEMS preparation and led to adsorption of the enzymes to the KEMS surface. Such adsorption could influence the inhibition assay and result in the observed "inhibition" by KEMS alone before and after simulated digestion. Additionally, certain food derived protein hydrolysates exhibit α-glucosidase inhibitory activity (Lacroix and Li-Chan, 2013; Yu, Yin, Zhao, Yu, Liu, Liu, and Chen, 2011). Therefore the inhibitory activity of KEMS alone after simulated digestion could also have been due to the hydrolysed kafirin.

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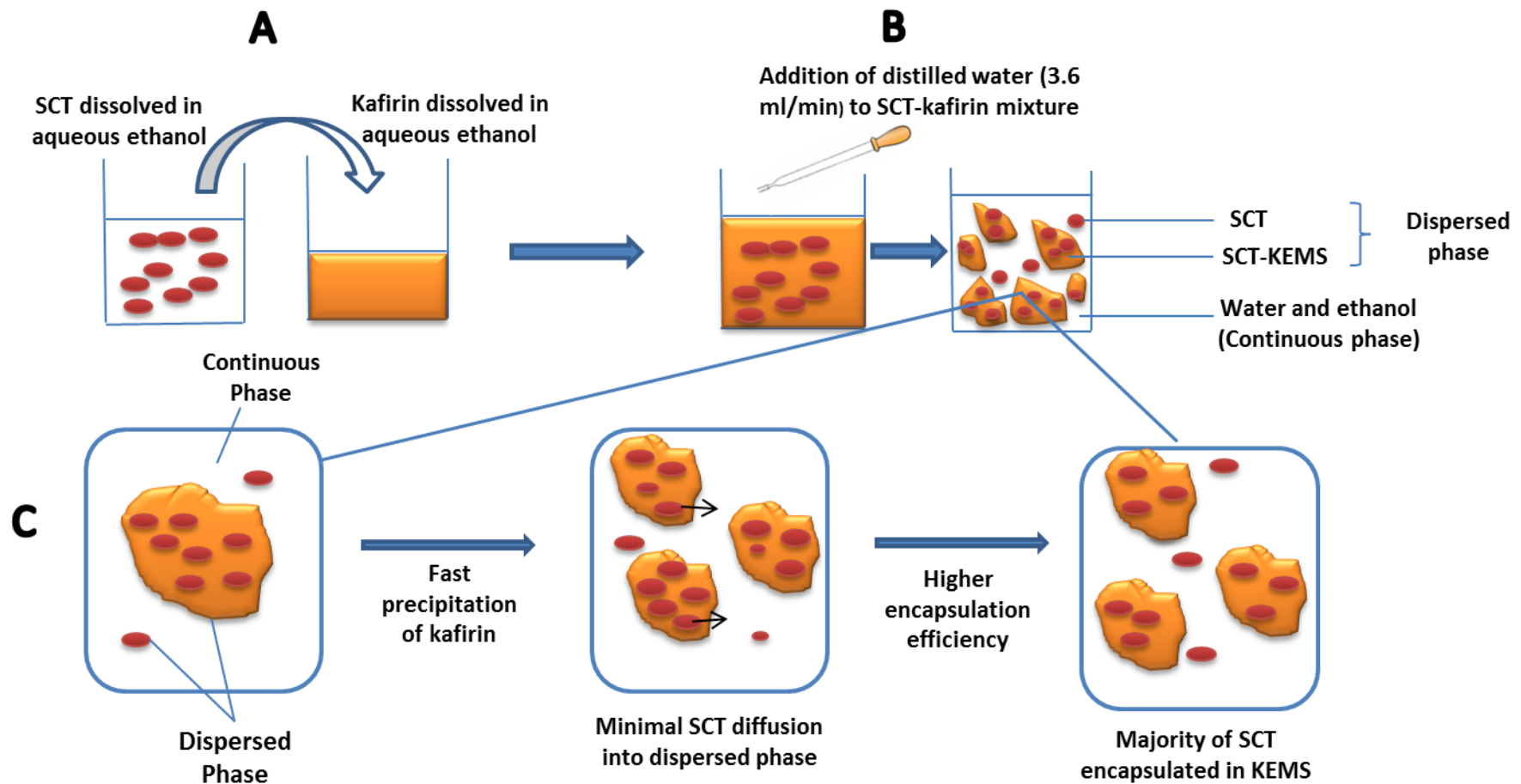
Figure 5.3: Model to explain the higher encapsulation efficiency of aqueous ethanol prepared SCT-KEMS. A: SCT and kafirin is separately dissolved in aqueous ethanol and then added together. B: The microparticles are formed upon addition of water via a pipette (3.6 ml/min). C: Enlarged view of conditions leading to higher encapsulation efficiency of SCT-KEMS**.**

93

In the in *vitro* component of this study, the α-amylase and α-glucosidase inhibitory effects of SCT-KEMS were clearly indicated, while SCT alone indicated almost no inhibition after simulated digestion. Furthermore, findings from the *in vivo* study provide evidence that the SCT alone (i.e. unencapsulated SCT) were not able to reduce blood glucose levels, while the SCT-KEMS did. The suggested mechanisms for the observed inhibition by the SCT-KEMS but not the SCT alone are illustrated in Figure 5.4. This model proposes that during the first stage of gastrointestinal digestion (in the oral cavity) some SCT alone bound with salivary proteins and α-amylases, while the SCT-KEMS passed through to the stomach without binding. During gastric digestion, the SCT alone that passed through the oral cavity would bind to pepsin and other proteins in the stomach, while the SCT-KEMS were hardly digested by pepsin. Transit through the stomach is relatively short compared to transit through the small intestine (Hur et al., 2011) and kafirin is resistant to pepsin digestion (Taylor et al., 2009a). Therefore release of SCT-KEMS by diffusion in the stomach would be limited. Upon reaching the small intestine, SCT alone would be incapable of inhibiting α-amylase and α-glucosidase due to binding with various enzymes and proteins of the gastrointestinal tract (Figure 5.4 A). In the small intestine, the SCT-KEMS would release the SCT by diffusion and pancreatic protein digestion. The released SCT would then be able to effectively inhibit α-amylase and α-glucosidase (Figure 5.4 B).

Only the acarbose treatment significantly reduced the glucose spike at 15 min compared to the control (Figure 4.2.3). As acarbose was not encapsulated and did not have to be released its effect on amylase activity was instant. However, as it is known that protein intake delays gastric emptying (Ma, Stevens, Cukier, Maddox, Wishart, Jones, Clifton, Horowits and Rayner, 2009), the release of SCT from KEMS in the small intestine probably resulted in them inhibiting the digestive amylases at a later stage compared to acarbose. The *in vitro* findings also indicated that SCT was a more potent inhibitor of α-glucosidase compared to acarbose (Figure 4.1.1 C). Alpha-amylase is responsible for the hydrolysis of starch into maltotriose and maltose which are then acted upon by α-glucosidase to release glucose (Kajaria, Ranjana, Tripathi, Tripathi and Tiwari, 2013). Therefore potent α-glucosidase inhibitory activity in the small intestine could be more important in controlling the release of glucose from maltodextrins than α-amylase inhibition. Hence, this may have been the cause of the apparent stronger blood glucose lowering effect of the released SCT compared to acarbose (Figures 4.2.2A and 4.2.3A).

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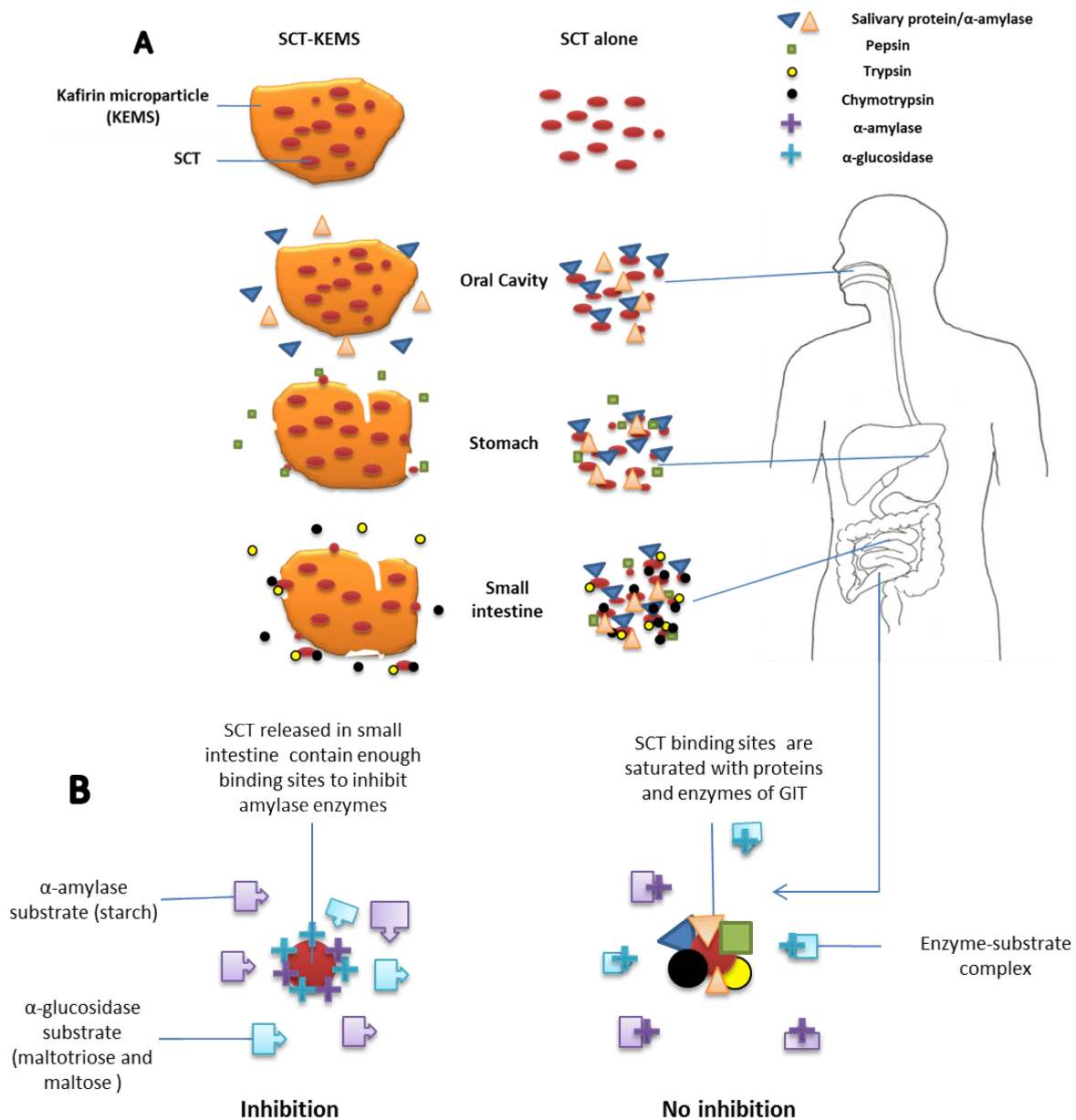
Figure 5.4: Proposed mechanisms for the gastrointestinal transit of SCT and SCT-KEMS and the influence of digestive enzymes and proteins on the ability of SCT alone and SCT-KEMS to inhibit digestive amylases in the small intestine. A: SCT-KEMS and SCT alone transit through the oral, gastric and small intestinal stages of the gastrointestinal tract (GIT). B: Enlarged view of SCT "released" in small intestine and able to inhibit intestinal amylases and of SCT alone unable to inhibit intestinal amylases due saturation of binding sites.

The glucose spike after KEMS alone treatment was lower compared to the water control and the SCT alone (Figure 4.2.2 A). Since KEMS comprise kafirin prolamin protein and the fact that protein ingestion delays gastric emptying (Ma et al., 2009) can be the reason for the observed lower spike. However, after 30 min there was a rapid increase in blood glucose level. It is presumed that glucose digestion could now commence fully as the majority of the maltodextrin would have reached the small intestine. In addition to slowing down gastric emptying, protein ingestion before a meal could stimulate incretin release and thereby decrease post-prandial glycaemia levels (Ma et al., 2009). As mentioned, incretins are gut hormones released in response to orally ingested nutrients that lead to a reduction in blood glucose due to enhanced insulin secretion (Ross et al., 2004).

Notably, KEMS alone was the only treatment that had increased serum insulin levels after the OSTT (Figure 4.2.8). Zein (maize prolamin protein) hydrolysate have showed improved glycaemic outcome when fed to a T2D rat model (Higuchi et al., 2013). These researchers proposed that the incretin stimulating effect of zein hydrolysates was responsible for improved glucose tolerance. The higher serum level after the OSTT suggests that kafirin in the KEMS could have led to an incretin stimulated insulin response (Ma et al., 2009; Higuchi et al., 2013). Furthermore, as *in vitro* digested KEMS alone inhibited α-amylase and α-glucosidase activity (Table 4.1.3) the blood glucose lowering effects of KEMS alone could have been a combination of insulin-simulated glucose removal and digestive amylase inhibition.

Fifteen minutes after maltodextrin administration there was a decrease in blood glucose level for the water control, SCT alone and KEMS alone treatments (Figure 4.2.2A). The decrease can be attributed to the initial burst of insulin that is released in the first 5−10 min after the β-cells are exposed to rapid increase in glucose (Caumo and Luzi, 2004). The water control had an elevated final blood insulin level, suggesting that glucose was absorbed into the blood stream constantly and resulted in the elevated level (Figure 4.2.8). The blood glucose level then gradually increased again after the decrease from the initial insulin burst. The second-phase insulin secretion occurs more gradually and is directly related to the degree and duration of the blood glucose stimulus (Caumo and Luzi, 2004). It has been found that hyperglycaemia-induced hyperinsulinaemia typical of T2D, could lead to deterioration and eventual "exhaustion" of insulin secreting β-cells (Ross et al., 2004). SCT-KEMS treatment did not cause a change in insulin levels as excessive insulin

96

secretion was avoided. This effect could be another important mechanism by which SCT-KEMS might attenuate T2D complications. Importantly, the stable insulin level seems to confirm that the blood glucose lowering effect of the SCT-KEMS treatment was due to amylase inhibition and not due to insulin-stimulated glucose removal.

5.3 Future research

The findings indicate that SCT-KEMS inhibit amylases of the small intestine both *in vitro* as well as *in vivo*. To optimize the applicability of SCT-KEMS as a nutraceutical, their encapsulation efficiency should be further enhanced and their morphology should be improved. As a way of increasing the encapsulation efficiency of KEMS, SCT could be encapsulated in KEMS made with higher concentrations of the γ-kafirin sub-class of kafirin proteins. As stated, γ-kafirins has been found to bind more SCT than α- and β-kafirin due to their higher proline content (Taylor et al., 2007). Higher encapsulation efficiency should help ensure delivery of more SCT to the small intestine and thereby decrease the amount of KEMS required for a bioactive effect. Morphological enhancement of SCT-KEMS could involve creating particles that are smaller, spherically shaped and evenly sized. Nanoparticles have several benefits compared to micron-sized particles as they provide a greater surface area and have the potential to increase solubility due to a combination of large interfacial adsorption of the core compound, enhanced bioavailability and improved controlled release, which enables better precision delivery of encapsulated materials (reviewed by Fang and Bhandari, 2010). Nano-sized SCT-KEMS could be obtained by carefully controlling the mixing and precipitation rate of the kafirin suspension to avoid agglomeration.

It is known that biochemical activities of flavonoids and their metabolites depend on their chemical structure and the relative orientation of various moieties in the molecule (Yao, Jiang, Shi, Toma, Barbera, Datta, Singanusong and Chen, 2004; Xiao, Ni, Kai and Chen, 2013). Some researchers found that various fractions of the same plant phenolic extracts have different effects on hyperglycaemia (Khan, Islam, Hossain, Asadujjaman Wahed, Rahman, Anisuzzaman, Shaheen and Ahmed, 2010; Ali et al., 2013). Studies have also found that proanthocyanidin polymers show much stronger inhibitory activity against α-amylase than oligomers (reviewed by Xiao et al., 2013), whereas proanthocyanidin oligomers show greater inhibitory activity than polymers against α-glucosidase (Lee, Cho,

Tanaka and Yokozawa, 2007). The SCT extract used in this study was not characterised in terms of its phenolic composition. Future studies should include a detailed characterisation of the size and types of condensed tannins present in a SCT extract and prepare SCT-KEMS using the size and types of condensed tannins that will result in the highest possible digestive amylase inhibition.

In this study healthy rats were used to demonstrate the potential of SCT-KEMS as an anti-hyperglycaemic nutraceutical. Future studies could involve using rodent models with altered glucose metabolisms (Ross et al., 2004). Rodent models in which T2D is induced with the consumption of high-fat and/or high-sugar diets can be advantageous to determine the mechanisms and pathophysiologic progress of T2D and the effect of potential treatments such as the SCT-KEMS on diabetes prevention and/or management. The Rhesus monkey which develops age and diet-related obesity and insulin resistance and the Gottingen pig (Srinivasan and Ramarao, 2007) can be used to demonstrate the anti-diabetic effects of SCT-KEMS in larger animals. Furthermore, the complexity of human physiology makes human studies with normoglycaemic and Type 2 diabetic patients vital for confirming the anti-hyperglycaemic and other health–benefiting effects of SCT-KEMS.

Increasing endogenous incretins, especially glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), has great potential to improve glucose tolerance and pancreatic β-cell function (Hira et al., 2009; Higuchi et al., 2013). Orally available incretin releasers, including dietary proteins or peptides, are considered promising agents for preventing and treating diabetes and obesity (Higuchi et al., 2013). In this study, the KEMS alone treatment demonstrated insulin stimulating effects. However, due to the limited insulin data, demonstration of this effect was very preliminary and therefore KEMS alone should be further assessed to elucidate their insulin stimulating effects. Assessment can include *ex-vivo* (cell-line) into the effects of SCT-KEMS, SCT alone and KEMS alone on dipeptidyl peptidase-IV (DPP4) inhibition (González-Abuín, Martínez-Micaelo, Blay, Pujadas, Garcia-Vallvé, Pinent, and Ardévol, 2012) and GLP-1 and GIP secretion/expression in appropriate muscle and adipose cell lines (Higuchi et al., 2013; Montagut et al., 2010).

CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

The *in vitro* study established that aqueous ethanol extracts of SCT from the bran of a high tannin sorghum cultivar have the potential to strongly inhibit digestive amylases. SCT have a superior α-glucosidase inhibitory activity compared to acarbose, the standard inhibitor. However, SCT require an encapsulated delivery system to preserve their inhibitory activity against digestive amylases.

KEMS prepared by the aqueous ethanol method has a superior SCT encapsulation efficiency to KEMS prepared by the acetic acid method. The aqueous ethanol prepared SCT-KEMS can reduce digestion by protease enzymes during simulated digestion and deliver SCT to the active site where they can inhibit digestive amylase.

The *in vivo* study showed that KEMS are effective SCT encapsulating agents as they appear to mask the astringency and bitterness of SCT and deliver the SCT to the small intestine of rats. Oral administrations of SCT-KEMS can prevent a glucose spike caused by digestion of rapidly available starch and decrease the AUC and maximum blood glucose levels after an oral starch tolerance test on healthy male Spague-Dawley rats. Additionally, SCT-KEMS can prevent elevation of serum insulin levels.

SCT-KEMS prepared using materials local to sub-Saharan African seem to have real potential as an anti-diabetic and anti-metabolic syndrome nutraceutical. SCT-KEMS can be particularly beneficial for residents of sub-Saharan Africa that do not have access to adequate healthcare and are suffering from hyperglycaemia associated with metabolic syndrome and T2D.

It is recommended that the encapsulation efficiency, size and morphology of SCT-KEMS be further improved to enhance their applicability as a nutraceutical. It is also recommended that diet-induced diabetic animal models and human studies are used to enhance understanding of the anti-diabetic effects of SCT-KEMS. Additionally, *ex-vivo* studies with appropriate cell lines will aid in elucidating other potential mechanism by which SCT-KEMS and KEMS alone exhibit anti-diabetic potential and should be investigated.

CHAPTER 7: REFERENCES

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Appendix A: List of publications and presentations based on this research

Journal Article

Links, M. R., Taylor, J., Kruger, M. C., Taylor, J. R. N. (2015). Sorghum condensed tannins encapsulated in kafirin microparticles as a nutraceutical for inhibition of amylases during digestion to attenuate hyperglycaemia. Journal of Functional Foods 12, 55-63.

Conference proceeding

Links, M.R. Van der Merwe, C. F., Taylor, J., Duodu, K. G., and Taylor, J. R. N. 2013. Condensed tannin encapsulation in kafirin (sorghum prolamin protein) microparticles. Conference proceedings of the 51st Annual Conference of the Microscopy Society of Southern Africa. Pretoria, South Africa.

Poster presentations

Links, M. R. Taylor, J., Kruger, M. C., Taylor, J. R. N. 2014. Encapsulation of sorghum condensed tannins in kafirin microparticles to inhibit digestive amylases. The 25th Congress of the Nutrition Society of South Africa. Johannesburg, South Africa and SAAFOST Northern branch Student Day. Pretoria, South Africa.

Taylor, J. R. N., Duodu, K. G., Taylor, J., Du Plessis, I., and Links, M. R.2014. Potential of Sorghum and other cereal grain phenolics to prevent and alleviate metabolic syndrome and type 2 diabetes. ICC International Symposium on Bioactive Compounds in Cereal Grains and Foods. Vienna, Austria.