The effects of *in vitro* digestion on polyphenolic and organic acid profile and antioxidant capacity of raw and cooked *Momordica charantia*.

Amy Van Elslande

School of Dietetics and Human Nutrition
McGill University
Winter 2012

A thesis submitted to McGill University
in partial fulfillment of the requirements of the degree of
Master of Science

Dedicated to my parents, who have always supported me unconditionally.

ABSTRACT

Ample studies relate the putative health promoting properties of the medicinal food *Momordica charantia* via an assessment of polyphenols, total phenolics (TP) and antioxidant capacity in the raw (R) form. However, few studies have investigated cooked *M. charantia* in this regard despite the fact that *M. charantia* is always eaten cooked. In the current study, quantification of polyphenols and organic acids as well as selected antioxidant indices were analyzed in four treatment groups including raw (R), cooked (C); R + digested (D) and C + D with a sample size of 10. A significant main effect of digestion was observed for all phenolics (except for catechin [CAT] and chlorogenic acid [CGA]), organic acids (ascorbic, fumaric, and malic acids), TP, ferric ion reducing antioxidant potential (FRAP) and 2,2'-azino-bis[3-ethylbenzothiazoline-6-sulphonic] acid (ABTS) antioxidant capacity assays. TP concentration was greatest in the R treatment which had significantly ($P < 0.05$) higher concentrations than C, R + D and C + D samples of *M. charantia*. HPLC analysis revealed protocatechuic acid (PA) as the phenolic compound in the greatest concentration in the C samples with a significant increase ($P < 0.05$) over the R sample. After digestion, there were significantly lower concentrations of protocatechuic acid in the C + D groups. Gallic acid (GA) was the phenolic compound observed in the highest concentration in the R + D and C + D treatments. There were significantly ($P < 0.05$) greater ascorbic acid (AA) concentrations by 24% in the C + D treatment relative to the C samples as well as significantly greater AA concentrations in the R + D treatment relative to the R sample. Fumaric acid (FA) was significantly ($P < 0.05$) greater in both digested treatments versus the R or C samples. GA was also strongly significantly ($P < 0.05$) positively correlated with both AA ($r = 0.88$) and FA ($r = 0.69$). FC and FRAP showed a significant ($P < 0.05$) positive correlation ($r = 0.56$). The present study shows that there are significant alterations in phenolic and organic acid concentrations and antioxidant capacity after cooking and digestion, indicating that such indices should be measured after cooking and digestive enzymatic processes to better understand the putative health properties associated with consumption of *M. charantia*.

RÉSUMÉ

De nombreuses études rapportent les propriétés médicinales de *Momordica charantia* à l'état cru via une évaluation des polyphénols, des composés phénoliques totaux (PT) et de la capacité antioxydante. Cependant, *M. charantia* est toujours consommée cuite et peu d'études ont jusqu'ici étudié ses propriétés médicinales lorsque cuite. Dans cette étude, la quantification des polyphénols et des acides organiques ainsi que des indices antioxydants ont été analysés dans quatre groupes de traitement : cru (CR), cuit (C), (CR) + digéré (D) et (C) + (D) (n = 10). Un effet principal significatif de la digestion a été observé pour tous les composés phénoliques (sauf pour la catéchine [CAT] et l'acide chlorogénique [CGA]), des acides organiques (acides ascorbique, fumarique et malique), les PT ainsi que via le pouvoir de réduction des ions ferriques (PRIF) et l'activité antioxydante par 2, 2'-azino-bis [3-éthylbenzothiazoline-6-sulfonique] (ABTS). Le groupe CR a révélé une concentration en PT significativement supérieure ($P < 0,05$) aux échantillons C, CR + D et C + D. L'analyse par HPLC a montré que l'acide protocatéchuique (AP) était le composé phénolique en plus grande concentration dans les échantillons C. Une augmentation significative en AP ($P < 0,05$) fut constatée dans l'échantillon C comparativement à l'échantillon CR. Après la digestion, des concentrations significativement plus faibles en acide protocatéchuique ont été mesurées dans le groupe C + D. L'acide gallique (AG) fut le composé phénolique en plus grande concentration dans les échantillons CR + D et C + D. Une concentration en acide ascorbique (AA) significativement supérieure ($P < 0,05$) de 24% fut observée pour les groupes de traitement C + D par rapport aux échantillons C. Une concentration en acide ascorbique (AA) supérieure fut également observée pour les groupes de traitement CR + D par rapport à l'échantillon CR. L'acide fumarique (AF) fut significativement ($P < 0,05$) en plus grande quantité dans les deux traitements digérés en comparaison aux échantillons CR ou C. AG fut également significativement ($P < 0,05$) corrélé positivement à la fois avec AA ($r = 0,88$) et AF ($r = 0,69$). PT et le FRAP ont montré une corrélation positive ($r = 0,56$) significative ($P <0,05$). La présente étude démontre des altérations significatives dans les concentrations d'acides phénoliques et organiques et pour la capacité antioxydante après la cuisson et la digestion. Cela indique que ces indices devraient être mesurés après la cuisson et le traitement à la digestion par des procédés enzymatiques afin de mieux comprendre les propriétés de santé attribuées à la consommation de *M. charantia* .

ACKNOWLEDGEMENTS

Thank you to my supervisors, Dr. Stan Kubow and Dr. Danielle Donnelly and my committee member Dr. Tim Johns for their support, editing and suggestions. Kebba Sabally, Atef Nassar and Behnam Azadi deserve a big thank you for their expertise for helping me to develop my laboratory protocols. A huge thank – you to Aydin Sarang for helping with my digestibility protocol.

CONTRIBUTION OF AUTHORS

Manuscript: The effects of *in vitro* digestion on polyphenolic and organic acid profile and antioxidant capacity of raw and cooked *Momordica charantia*.

Amy Van Elslande: (Candidate): Performed assays for *in vitro* digestion, ABTS and FRAP. Analyzed samples using high performance liquid chromatography, organized and analyzed data. The candidate wrote the above mentioned manuscript with the help of co-authors.

Stan Kubow (Supervisor, Associate Professor, School of Dietetics and Human Nutrition, McGill University): Guided and advised candidate on all aspects of the study including literature review, methodology and final editing.

Danielle Donnelly (Co-supervisor, Associate Professor, Department of Plant Science, McGill University): Guided candidate on thesis direction and helped edit the final manuscript.

Kebba Sabally (Postdoctoral Fellow): Ordered materials for all assays, helped with protocol development for HPLC. Guided lab work for FRAP assay.

Atef Nassar (Postdoctoral Fellow): Guided candidate on preparation of ABTS assay and statistical analysis.

Behnam Azadi (Research Assistant): Guided candidate on digestibility assay.

V.J. Lam (Undergraduate research student): Conducted Folin-Ciocalteu assay for total phenolics.

ABBREVIATIONS

AA	Ascorbic acid
ABTS	2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
AGEs	Advanced glycation end products
AO	Ascorbate oxidase
AR	Aldose reductase
BES	Barbados Eye Study
CAE	Chlorogenic acid equivalents
CAT	Catechin
CGA	Chlorogenic acid
C	Cooked
D	Digested
DDW	Double distilled water
DHAA	Dehydroascorbic acid
DPPH	1,1-diphenyl-2-picrylhydrazil
DW	Dry weight
EC50	Half maximal effective concentration
EtOH	Ethanol
FA	Fumaric acid
FC	Folin-Ciocalteu
FDW	Freeze dried weight
FRAP	Ferric ion reducing antioxidant potential
FW	Fresh weight
GA	Gallic acid
GAE	Gallic acid equivalents
GHb	Glycosylated hemoglobin
GI	Glycemic index
GSH	Glutathione
Hb	Hemoglobin

H2O	Water
HPLC	High performance liquid chromatography
IC50	Half maximal inhibitory concentration
LPS	Lipopolysaccharide
MA	Malic acid
NO	Nitric oxide
NOS	Nitric oxide synthase
o-CA	*o*-coumaric acid
ORAC	Oxygen radical absorbance capacity
PA	Protocatechuic acid
PPAR	Peroxisome proliferator-activated receptor
R	Raw
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SCFA	Short chain fatty acids
SEM	Standard error of the mean
SOD	Superoxide dismutase
SWE	Subcritical water extraction
TAA	Total ascorbic acid
T2D	non-insulin dependent type 2 diabetes mellitus
t-CiA	*t*-cinnamic acid
TCM	Traditional Chinese Medicine
TE	Trolox equivalents
TEAC	Trolox equivalent antioxidant capacity
TP	Total phenolics
VA	Vanillic acid

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

I. INTRODUCTION

1.1 Statement of the Problem

Barbados, an island nation in the Caribbean West Indies, is experiencing a diabetes epidemic. Barbados has a small (293,000 people) (WHO 2006), predominantly African-heritage population affected by a high cost of food due to decreasing local agriculture, increasing food imports (Long 1982), tourism, urbanization and a western lifestyle (Rotimi et al. 1995; Cunningham-Murie et al. 2008). A distinct trend toward overweight and obesity in the population has occurred over the last two decades (Nemesure et al. 2007). High rates of amputation are also reported, a consequence of untreated diabetes leading to diabetic neuropathy (Hennis and Fraser 2004; Hennis et al. 2004; Hambleton et al. 2009). The high mortality rate of non insulin dependent, type 2 diabetes mellitus (T2D) in the Caribbean may be due to delayed diagnosis, poor control of diabetic symptoms, lack of information or adherence to beneficial lifestyle changes and cultural factors (Foster 1993). Inaccessibility to health care is also a contributor, as poorer households are less likely to utilize health services (Racine et al. 2009).

Current estimates suggest that by 2030, there will be 366 million cases of diabetes across age groups, world-wide (Wild et al. 2004), 90% of which are attributable to T2D (Zimmet et al. 2001). The projection for the year 2025 is 48% prevalence of T2D for developing countries, specifically a 150% increase in prevalence between 1995 (15 million cases) to 2025 (39 million cases) for adults >20 years of age in Latin America and the Caribbean region (King et al. 1998). The estimated indirect cost of diabetes to the healthcare system is over 800 million US dollars in the English speaking Caribbean, which includes Barbados (Barcelo et al. 2003).

There is an associated genetic risk of developing diabetes which is more than double if one parent is diabetic and almost quadruple if both parents have diabetes (Knowler et al. 1981). A Barbadian cohort involved in the Barbados Eye Study (BES) consisted of over 4000 individuals, 40-84 years-old, predominantly black, male and female (~40/60%) participants (Hennis et al. 2002). The

prevalence of diabetes (primarily T2D) was variable depending on ethnicity, with a 17.5 % predominance in blacks and 16.7% occurring in the >30 year age group (Hennis et al. 2002). Comparatively, the prevalence decreased to 12.5% in those of mixed descent (black/other) and plummeted to 3.8% in white participants in the same age category (Hennis et al. 2002). According to the 9 year follow up to the BES study, the incidence of diabetes was 15.6% for those under 50 years (Nemesure et al. 2008). The ethnic dominance of the black population (~ 90%) (Sharma et al. 2007) is reflected here as the proportion of black participants is about 93% in this simple, random sample (Hennis et al. 2002). Of the study participants, 85% had a BMI over 25 (Hennis et al. 2002). This is in sharp contradiction to other predominantly black nations, such as Nigeria or Jamaica, where the prevalence of T2D in adult males was only 2% (BMI 21.7) and 5% (BMI 23.4) respectively which correlated with a more physically demanding lifestyle and lower caloric consumption (Cooper et al. 1997). A more recent longitudinal report found Nigerian men are gaining 0.31 kg/year compared to Jamaican men at 1.37 kg/year with the greatest gains in those with normal BMI (18.5- 24.9) at baseline (Durazo-Arvizu et al. 2008).

Barbados has experienced an accelerated socioeconomic transformation since being colonized and this has affected current dietary patterns (Hennis and Fraser 2004). As the movement from rural to urban areas continues, the ancient traditional knowledge and pattern of medicinal plant usage changes, however oral transfer of bush knowledge from healers to individuals is common (Longuefosse and Nossin 1996; Quinlan and Quinlan 2007). Compiling ethnobotanical data for the purpose of providing novel, drug precursors can devalue traditional medicine while simultaneously increasing the cost of treatment (Pedersen and Baruffati 1985; Cox and Balick 1994; Oubre et al. 1997; Bondurant and Sox 2005). Also, standardization of plant- based medicines can be problematic as efficacy is dependent on dose (Vuksan and Sievenpiper 2005). Metformin is a diabetic medication that has been in use since the 1950's and is derived from French lilac, *Galega officinalis* (Vuksan and Sievenpiper 2005). This latter example

demonstrates the multifaceted benefit of combining traditional medicinal knowledge with clinical, empirical study (Oubre et al. 1997).

As secondary metabolites of almost all higher plants, polyphenols form the bulk of antioxidant compounds in the human diet (Bravo 1998; Kahkonen et al. 1999; Singleton et al. 1999). Polyphenols prevent oxidative damage physiologically by scavenging or preventing the formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) and neutralizing molecules that may oxidize (Benzie and Strain 1996). Polyphenols donate hydrogen from their phenolic hydroxyl group(s), thus neutralizing free radicals (Parr and Bolwell 2000). As such, polyphenolic compounds as antioxidants are invaluable at preventing or ameliorating the symptoms of chronic disease, including T2D, which characteristically amplify the production of free radicals.

1.2 Study Rationale

Health care systems are overburdened in developing countries such as Barbados (Cunningham-Murie et al. 2008). A local, accessible pharmacopeia could be utilized as a reference for potential drug interactions, as well as providing a resource for those wishing to combat chronic diseases naturally, thus lowering the overall cost to the medical system (Pedersen and Baruffati 1985).

Likely indigenous to Asia, *Momordica charantia* is a member of the Cucurbitaceae family (Walters and Deckerwalters 1988). All parts of the plant are utilized, with the fruits and leaves regularly consumed as vegetables as well as for their purported amelioration of diabetic symptoms (Bakare et al. 2010). *M. charantia* is grown and regularly consumed in Asia (Virdi et al. 2003), Africa, Latin America (Lans 2003) and the Caribbean (Delgoda et al. 2004; Chandra et al. 2008) including Barbados (Carrington 2007; Fonseka et al. 2007) and some species are found growing wild (Wu and Ng 2008).

M. charantia is a rich source of polyphenols (Horax et al. 2005; Kubola and Siriamornpun 2008; Horax et al. 2010) as well as macro and micronutrients (Bakare et al. 2010; Horax et al. 2010), including ascorbic acid (Meerabai et al. 2007; Wang et al. 2007; Myojin et al. 2008) and has been extensively studied for

its health effects as a medicinal food (Virdi et al. 2003) with hypoglycemic properties (Srivastava et al. 1993; Tongia et al. 2004; Fonseka et al. 2007), insulin potentiating ability (Roffey et al. 2007), and a capability to ameliorate aberrant fatty acid metabolism (Chao and Huang 2003; Chen et al. 2003; Shih et al. 2008).

M. charantia is always eaten cooked; however, its *in vivo* and *in vitro* nutritional properties are frequently assessed after extraction from raw material (Srivastava et al. 1993; Horax et al. 2005; Horax et al. 2010). It has been analyzed in various *in vitro* studies that used heat in the extraction process (Ansari et al. 2005; Budrat and Shotipruk 2008; Kubola and Siriamornpun 2008) or extraction after cooking (Ng et al. 2011). To our knowledge, the effects of cooking and *in vitro* digestion have not been examined simultaneously in *M. charantia* to assess the phenolic and organic acid profile and its antioxidant capacity.

1.3 Hypothesis

Cooking and *in vitro* enzymatic digestion improve the polyphenolic and organic acid profiles and the antioxidant capacity of *M. charantia.*

1.4 Objectives

Although studies have assessed the phenolic profile, ascorbic acid content and antioxidant capabilities of cooked *M. charantia* (Ng et al. 2011) these effects have not been studied after both cooking and *in vitro* digestion. To our knowledge, fumaric acid and malic acid have not been quantified in *M. charantia*. The objectives of this study were to determine the phenolic and organic acid profiles of *M. charantia* as well as its antioxidant capacity after cooking and *in vitro* digestibility treatments.

Table 1.1 Methodology for *M. charantia* extraction and quantification and key references.

Compound	Method	Key References
Total polyphenols	Folin Ciocalteau	Singleton et al. (1988)
Cooking protocol	Volumetric flask, 3 min, 100˚C	Saura-Calixto et al. (2000)
Digestibility assay	*In vitro* gastric and pancreatic digestion	Sarang et al. (2011) Tagliazucchi et al. (2010)
Gallic acid, protocatechuic acid, catechin, chlorogenic acid, vanillic acid, *o*-coumaric acid, *t*-cinnamic acid, ascorbic acid, fumaric acid and malic acid	High pressure liquid chromatography (HPLC)	Shakya and Navarre (2006) (polyphenols and ascorbic acid) Schwartz et al. (1962) (fumaric and malic acids)
FRAP	Ferric ion reducing antioxidant potential	Benzie and Strain (1996)
ABTS (hydrophilic)	2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic) acid	Re et al. (1999)

II. REVIEW OF LITERATURE

2.1 Introduction

2.1.1 *Momordica charantia*

Thought to be indigenous to the Asian continent, *Momordica charantia* is a vinous, herbaceous dicot with green lobed foliage and many small, yellow flowers (Walters and Deckerwalters 1988). It is commonly reported and widely researched as a medicinal plant with antidiabetic properties (Srivastava et al. 1993; Marles and Farnsworth 1995; Fonseka et al. 2007; Roffey et al. 2007; Horax et al. 2010). Fruits are warty or wrinkled and oblong, 2 to 7 cm in length, turning from green to orange/red with maturation (Walters and Deckerwalters 1988). Common names include kerala, bitter gourd (India) (Virdi et al. 2003), karawila (Sri Lanka) (Fonseka et al. 2007) cerasee, miracle vine and bitter melon (Caribbean) (Carrington 2007). It is used extensively in Traditional Chinese Medicine (TCM) and Ayurveda as well as being a culinary medicinal (Virdi et al. 2003; Shih et al. 2009). *M. charantia* has also been documented in Jamaica for its hypoglycemic (Delgoda et al. 2004; Chandra et al. 2008) and antihypertensive properties (Delgoda et al. 2004) as well as in Barbados and Sri Lanka as a general antidiabetic (Carrington 2007; Fonseka et al. 2007).

The primary mineral content of *M. charantia* fruit, in mg/g, are phosphorous (5.8 ± 0.3), potassium (42.7 ± 2.1), magnesium (3.0 ± 0.1), sulphur (1.1 ± 0.1) and calcium (2.7 ± 0.3) (Horax et al. 2010). Mature *M. charantia* fruit was high in trace elements such as vanadium, zinc, copper, molybdenum and chromium (Kosanovic et al. 2009) as well as iron and manganese (Horax et al. 2010). *M. charantia* (fruit skin, flesh) was able to restore plasma magnesium concentrations in streptozotocin diabetic rats (Chandra et al. 2008).

Moisture content of fresh, mature *M. charantia* fruit was approximately 92% of its fresh weight (Horax et al. 2010). Starch content has been measured as approximately 4.9% of the dry weight with roughly 14% contribution from soluble dietary fibre and 30% from insoluble dietary fibre (Horax et al. 2010). The crude fibre is about 14% of the dry weight (Bakare et al. 2010). Lipid content has been

reported to range from 6% (Bakare et al. 2010) to 10% dry weight (Horax et al. 2010).

Protein content of *M. charantia* (cv. Thinneyville White) has been measured to be as low as 9% of the dry weight with 17 of the amino acids represented (Horax et al. 2010), however, Bakare et al. (2010) measured crude protein at almost 28% (cv. not reported). Both the former and latter authors cite a standard method for protein extraction (American Association of Cereal Chemists) (Bakare et al. 2010; Horax et al. 2010). Many factors contribute to the range of nutritional components in *M. charantia* including soil composition and fertilization practices (Meerabai et al. 2007), cultivar (Horax et al. 2005; Wu and Ng 2008), harvesting and storage conditions (Meerabai et al. 2007; Wang et al. 2007; Myojin et al. 2008), plant maturity and drying method (Zhang et al. 2009) as well as extraction technique (Horax et al. 2005). Interestingly, L-arginine content was found to be 45.6 ± 2.0 (mg/g) with a nitrate content of 4.2 ± 0.5 mg/g (Horax et al. 2010). In comparison, hazelnuts, which are considered a good plant source of L-arginine, have about 20.3 mg/g (Koksal et al. 2006).

2.2. Diabetes Etiology

2.2.1 Insulin

Diabetes is a condition that impairs cellular ability to combat oxidative stress due the interaction of increased ROS and depressed antioxidant status (Chung et al. 2003). T2D, obesity and hypertension share the simultaneous decrease of cellular glucose uptake and glycogenesis which lead to chronic hyperglycemia and hyperinsulinemia (Ferrannini et al. 1987).

As plasma glucose rises, insulin is secreted from the β-cell of the pancreas (Ashcroft et al. 1984). The main role of insulin is to regulate blood glucose by stimulating cells (muscle and adipose) for glucose uptake as well as depressing hepatic glucose synthesis (Bloch-Damti and Bashan 2005). Insulin also prevents the liberation of free fatty acids from adipocytes, augments the esterification of fatty acids in muscle and adipocytes and promotes protein anabolism (Randle et al. 1963) as well as regulating vasodilation (Laakso et al. 1990; Baron 1994).

Adipocytes *in vitro* were more effective at absorbing glucose when insulin levels were low when a water soluble extract of *M. charantia* was added indicating an insulin potentiating effect at suboptimal levels of insulin (Roffey et al. 2007).

Skeletal muscle is the primary tissue involved in insulin-mediated glucose uptake (Laakso et al. 1990; Baron et al. 1991). The GLUT4 transporter is involved in intracellular uptake of glucose in skeletal muscle (Tan et al. 2008). Insulin mediated glucose uptake is decreased in T2D due to impaired skeletal muscle permeability and attenuation of insulin mediated blood flow (Laakso et al. 1992). The cucurbitane glycosides (triterpenoids, momordicosides) extracted from *M. charantia*, were found to increase GLUT4 translocation to the cell surface, *in vitro*, in skeletal myocytes as well as in adipocytes (Tan et al. 2008). Also, *M. charantia* extract increased the number of GLUT4 transporters in skeletal muscle as well as PPARγ in adipose tissue ameliorating high glucose and insulin levels in rats (Shih et al. 2009).

Insulin resistance is defined as a debilitated response to insulin in target tissues such as muscle and adipose coupled with increased hepatic gluconeogenesis (Kim et al. 2006; van den Oever et al. 2010) and is characterized by elevated baseline 24 hour plasma glucose levels, hyperinsulinemia and adiposity (McLaughlin et al. 2002). Hyperinsulinemia, a condition where the glucose utilizing cells of the body (skeletal and adipose) are not responsive to insulin, is correlated with obesity (Meigs et al. 1997) and atherosclerosis (Stamler et al. 1960; Cruz et al. 1961) and can slow gastric movement postprandially, negatively interfering with carbohydrate metabolism and cellular absorption of glucose (Eliasson et al. 1995). *M. charantia* was able to depress the uptake of glucose from the intestinal tract by increasing enterocyte utilization of glucose in an *in vitro* model utilizing everted rat intestines (Mahomoodally et al. 2007). Three causes of insulin resistance have been postulated: defective pancreatic β-cell secretion; plasma insulin antagonists; and failure of target tissues to respond to insulin (Olefsky and Kolterman 1981). Pancreatic tissue showed significantly less damage by lipid peroxidation in T2D mice given *M. charantia* juice (Sitasawad et al. 2000). *In vitro*, mouse islet cells and rat insulinoma cells treated with *M. charantia*

extract were protected from lipid peroxidation and apoptosis (Sitasawad et al. 2000).

2.2.2 Hyperglycemia

Hyperglycemia is chiefly responsible for the initiation of insulin resistance due to the increase in oxidative stress caused by glucose toxicity which impairs endothelial function and attenuates systemic perfusion (Akbari et al. 1998; Renaudin et al. 1998; Beckman et al. 2001; Kim et al. 2006). *M. charantia* juice given to T2D mice was shown to improve blood glucose levels to normoglycemia within 5 days of treatment (Sitasawad et al. 2000). Juice or ethanolic extract of *M. charantia* fruits decreased serum glucose and blood lipids in normal and alloxan diabetic rats (El Batran et al. 2006). Antihyperglycemic effects of *M. charantia* aqueous extract showed similar glucose lowering effects to glibenclamide (Virdi et al. 2003; Harinantenaina et al. 2006), while also preventing kidney damage (Virdi et al. 2003). The glucose lowering effect may be attributable to the ability of *M. charantia* to inhibit enzymatic gluconeogenesis as well as increasing glucose utilization (Shibib et al. 1993). Extracts of *M. charantia* inhibited α-amylase and α-glucosidase enzyme activity, thereby, interrupting glucose digestion and absorption from the proximal intestine (McCue et al. 2005; Fonseka et al. 2007). After *in vitro* digestion with amylase and glucosidase, *M. charantia* extract (solvent not mentioned) incubated with cooked white rice reduced the conversion of starch to glucose by up to 63% (Fonseka et al. 2007).

Clinical trials involving human diabetic subjects given an aqueous extract of *M. charantia* showed a decrease of 54% in blood glucose levels after 3 weeks of treatment (Srivastava et al. 1993) and those given white rice plus cooked *M. charantia* had up to a 31% reduction in glycemic index over those subjects given white rice alone (Fonseka et al. 2007). The control of blood glucose has been postulated to be one of the best measures to prevent the secondary symptoms of diabetes (Peterson et al. 1977).

2.2.3 Polyol pathway and advanced glycation end products

Aldose reductase (AR) is the primary and rate limiting enzyme involved in the polyol pathway (Srivastava et al. 2003). The activation of aldose reductase results in excess intracellular conversion of glucose to sorbitol which accumulates intracellularly and disrupts cellular function due to osmotic changes (Chandra et al. 2002) ultimately converting to fructose (Chung et al. 2003). Advanced glycation end products (AGEs) are formed when glucose forms a non-enzymatic attachment to the amino group of protein known as non-enzymatic glycosylation and accumulates irreversibly on cell membrane, plasma and structural proteins (Brownlee et al. 1984). Glycated or glycosolated hemoglobin (GHb) is a measure of chronic blood glucose exposure (Kilpatrick 2000; Hennis et al. 2002). GHb is specifically related to recent mean plasma glucose levels but not glycemic fluctuations (Kilpatrick 2000). Approximately 4-6% of circulating plasma hemoglobin (Hb) in healthy adults is $HbA_{1c,}$ a glycosylated form (Fitzgibbons et al. 1976). Participants in the BES were shown to have GHb over 10% (Hennis et al. 2002). In mice, complementary oral feeding with *M. charantia* extract lowered HbA_{1c} levels by up to 70% over those mice fed a high fat diet alone (Shih et al. 2008).

2.2.4 Overweight, Obesity and Hyperlipidemia

In obesity (BMI>30kg/m^2), an individuals' ability to respond to insulin is hampered with a decrease of insulin mediated glucose uptake between 40% (whole body) to 60% (leg muscle) (Laakso et al. 1990; Baron et al. 1991). Chronic high circulating plasma free fatty acids have an inhibitory effect on glucose utilization by decreasing muscle glycogen synthesis and inhibiting glucose metabolism (Roden et al. 1996), contributing to insulin insensitivity and IGT (Randle et al. 1963). In rats fed a high fat diet plus freeze-dried *M. charantia* juice, free fatty acid concentration in plasma increased, indicative of fat mobilization and increased fat metabolism (Chen et al. 2003). The same diet also improved insulin sensitivity and decreased serum insulin and leptin levels leading to reduced visceral adiposity (Chen et al. 2003).

In rats, *M. charantia* was demonstrated to decrease leptin expression in white adipose tissue (Shih et al. 2008; Shih et al. 2009). *M. charantia*, whole fruit (skin, flesh, seeds) ethanolic extract stimulated peroxisome proliferator activated-receptor (PPAR) α (expressed in liver, cardiocytes, myocytes; controls lipolysis) and PPARγ (expressed in white and brown adipose tissue; modulates the cellular insulin response, adipocyte differentiation and lipogenesis) in an *in vitro* mouse hepatoma model (Chao and Huang 2003). The PPAR family are ligand activated transcription factors that regulate energy metabolism and inflammation (Chao and Huang 2003). A noticeable effect of *M. charantia* (*n*-butanol extract) orally administered was the decrease in the abdominal fat accumulation and concurrently reduced blood lipids and increased lipoprotein lipase activity in rats fed a high fat diet (Shih et al. 2008). The authors proposed that the effects of *M. charantia* in rats was partially due to its simultaneous action on PPARα and PPARγ (Shih et al. 2008). Momordicosides isolated from *M. charantia* also contributed to enhanced fatty acid oxidation *in vitro* (Tan et al. 2008). As mentioned, adverse hepatic histological changes occur in people and animals with T2D (Dowman et al. 2010) but *M. charantia* has shown preventative effects (Teoh et al. 2009). In streptozotocin induced diabetic rats typical features of a diabetic liver such as inflammation, accumulation of fatty deposits within hepatocytes and collagen deposits in the portal vasculature were ameliorated with the administration of an *M. charantia* saline solution (Teoh et al. 2009).

The above empirical evidence supports the ability of *M. charantia* extracts to counter the adverse effects of aberrant metabolism that occur during T2D. Potentiation of insulin (Roffey et al. 2007), improvements in glucose uptake and glycemic control via GLUT4 (Tan et al. 2008; Shih et al. 2009) and PPARs (Shih et al. 2008) as well as improvements in lipid parameters and reductions in adiposity (Tan et al. 2008; Teoh et al. 2009) are some of the mechanisms by which *M. charantia* exerts its beneficial effects. These effects may be attributed to the ability of *M. charantia* to scavenge free radicals due to its nutritional and phenolic profile (Budrat and Shotipruk 2008; Myojin et al. 2008; Horax et al. 2010).

2.3 Free radicals and free radical scavenging ability of *M. charantia*

Free radicals generated during normal biological metabolic processes are unstable, extremely reactive molecules having at least one unpaired electron and include reactive oxygen and reactive nitrogen species (ROS, RNS) (Slater 1984; Halliwell 1994; Opara 2002). Free radicals have repeatedly been implicated in cellular and tissue damage affecting carbohydrates, proteins, lipids and nuclear cells (Slater 1984). The damaging effects of a free radical depend upon its concentration and its particular attraction to physiologically significant molecules (Slater 1984). Free radicals are a natural product of aerobic metabolism but their accelerated production and the ensuing persistent oxidative stress of chronic diseases such as T2D leads to cellular damage (Bashan et al. 2009). The most reactive oxidizers will exert their effects close to their point of synthesis such as reactions that occur within a plasma membrane which can produce irreversible, pathological, secondary consequences that emanate throughout the cell (Slater 1984). These damaging effects include lipid peroxidation, DNA modification, AGE accumulation and protein denaturation and this accrual of damage is collectively referred to as oxidative stress (Bashan et al. 2009). Studies have found that *M. charantia* ethanolic extract scavenging of the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical to be 12.6 ± 0.5 (mg extract/mg DPPH, EC_{50}) (Horax et al. 2010). Heat treatment (boiling for 30min under reflux in methanol) of *M. charantia* (peeled, seeds removed, variety not mentioned) found DPPH capacity was 5 times greater than in a typical room temperature methanolic extract (Ansari et al. 2005) (See Table 8.). Aqueous extract of *M. charantia* was measured for its ability to scavenge DPPH radical and was not found to be effective (McCue et al. 2005).

2.3.1 Nitric oxide (NO) and superoxide ($O_2^{\cdot-}$)

Nitric oxide is a ubiquitous molecule implicated in almost all cellular activities (Moncada and Higgs 2006; Luiking 2009). Endogenous NO, synthesized from L-arginine, is produced by the endothelial cell by endothelial nitric oxide synthase or eNOS (Furchgott et al. 1984; Stuehr 1999) and by adipocytes through inducible NOS (iNOS) and eNOS (Ribiere et al. 1996). nNOS is responsible for

production of neuronal NO, but is expressed in all insulin responsive tissue (except adipose) and mtNOS produces mitochondrial NO (Brodsky et al. 2002). NO formation in the endothelium is substrate dependent (L-arginine and BH_4) and eNOS dependent (Moncada and Higgs 2006). Endothelial dysfunction, exacerbated by oxidative stress, is characterized by eNOS deficiency which can be due to substrate deficiency (L-arginine, BH4), increased ADMA (asymmetric dimethylarginine) or the accelerated decomposition of endothelium generated NO to its reactive metabolites (Kurowska 2002) such as peroxynitrite. When L-arginine or BH_4 are deficient, the uncoupling of eNOS leads to $O_2^{\cdot-}$ production (Xia et al. 1996; Luiking 2009). This uncoupling redirects reductase-heme electron flow to molecular O_2 instead of L-arginine with resultant $O_2^{\cdot-}$ production (Sullivan and Pollock 2006). In diabetic non-obese rats the hyperglycemic state was postulated to simultaneously couple and uncouple NOS, therefore increasing $O_2^{\cdot-}$, NO and peroxynitrite simultaneously (Fujii et al. 2010). The NO scavenging ability of *M. charantia* was roughly 76% *in vitro* when an ethanolic extract of 1000µg/ml was added to sodium nitroprusside (a NO donor) in phosphate buffered saline (Jagetia and Baliga 2004). An aqueous extract of *M. charantia* was also found to suppress lipopolysaccharide (LPS) induced production of prostaglandin E_2 (PGE_2) in RAW 264.7 macrophages (Huang and Wu 2002). A methanolic extract of *M. charantia* was shown to stimulate murine peritoneal macrophages by increasing the secretion of NO (Juvekar et al. 2009).

The superoxide radical ($O_2^{\cdot-}$), produced by the reduction of molecular oxygen, interacts with NO and can cause damage by a variety of mechanisms (Kurowska 2002; Fridovich 2008) such as contributing to atherogenesis and vascular plaques (Sullivan and Pollock 2006) and promoting AGEs intracellularly (Nishikawa et al. 2000). Hyperglycemia up regulates the production of $O_2^{\cdot-}$ during the electron transport phase of mitochondrial glycolysis (Nishikawa et al. 2000). $O_2^{\cdot-}$, which is very reactive (Fridovich 2008) extracts hydrogen atoms indiscriminately from physiologically important molecules and contributes to the oxidation of sugars (Asahi et al. 2000). In a diabetic, inflammatory state the endothelium produce more $O_2^{\cdot-}$ and H_2O_2, which subsequently increase hydroxyl

radical (·OH) production, therefore, the vasculature is more prone to oxidative damage (Pieper et al. 1997). Partial eNOS uncoupling may accelerate the simultaneous release of $O_2^{\cdot-}$ and NO leading to excess peroxynitrite generation (Cai and Harrison 2000). An aqueous extract of wild *M. charantia* (*M. charantia* Linn. var. *abbreviata* Ser.) was more potent than an ethanolic extract at scavenging DPPH but both extracts were effective inhibitors of $O_2^{\cdot-}$ and lipid peroxidation and their activity exceeded that of vitamin E (Wu and Ng 2008).

Superoxide dismutase (SOD) is responsible for reducing $O_2^{\cdot-}$ leading to the formation of H_2O_2 which subsequently reacts with glutathione peroxidase and catalase acting in concert with SOD as antioxidants (Kim and Rhee 2004). In alloxan induced diabetic rat cardiac tissue aqueous extracts of *M. charantia* were shown to increase glutathione (GSH) and SOD, prevent lipid peroxidation (Tripathi and Chandra 2009). *M. charantia* was also able to repress the enzymatic and non-enzymatic expression of $O_2^{\cdot-}$ in plasma and red blood cells which was as effective as glibenclamide at restoring SOD levels (Chandra et al. 2008).

2.4 Antioxidants, polyphenols and organic acids

It is agreed that consumption of phytochemical and antioxidant rich foods such as fruits, vegetables, whole grains, legumes and fish promote optimal health (Thompson et al. 1999; Fung et al. 2001) and is correlated with decreased oxidative stress thus lowering the incidence of chronic disease (Green et al. 1982; Lii et al. 2009). The opposite is true of a Western style diet including red and processed meat, high fat dairy products and sugar rich foods (Fung et al. 2001). The plasma antioxidant profile of subjects who consumed an experimental diet that doubled their fruit and vegetable intake to 10 servings per day showed significant improvement in antioxidant status based on the plasma oxygen radical absorbance capacity (ORAC) assay values (Cao et al. 1998). Human subjects fed a high fat meal were observed to have a decreased response to L-arginine, impaired endothelial antiplatelet activity (reduction in NO), increased coagulation and fibrinolysis (Esposito et al. 2003). The addition of antioxidant vegetables

(tomatoes, peppers, both 100g; and carrots, 200g) to the high fat meal attenuated these negative physiological responses (Esposito et al. 2003).

2.4.1 Polyphenols

As a component of all plant materials, polyphenols may form the bulk of antioxidant compounds in the human diet (Bravo 1998; Kahkonen et al. 1999; Singleton et al. 1999). Phenolics are thus named because they are derived from the amino acid phenylalanine as secondary metabolites synthesized through the shikimate pathway in plants (Arts and Hollman 2005). In measures of total antioxidant capacity such as trolox equivalent antioxidant capacity (TEAC), many polyphenolic flavonoids, including catechin, have been shown to be more effective than ascorbate (Rice-Evans et al. 1995). Polyphenols prevent oxidative damage physiologically by scavenging or preventing the formation of ROS and RNS and neutralizing other molecules that may oxidize (Benzie and Strain 1996). Polyphenols donate hydrogen from their phenolic hydroxyl group, neutralizing free radicals through their redox potential (Parr and Bolwell 2000). Their defining chemical structure includes at least one aromatic ring in conjunction with one or more hydroxyl groups (Arts and Hollman 2005) and they are categorized mainly as flavonoids and phenolic acids (Virgili and Marino 2008). In general, polyphenols are soluble in aqueous solutions (Haslam and Cai 1994).

Phenolic acids are subdivided into derivatives of benzoic acid or cinnamic acid with the latter being the most abundant in foods (Manach et al. 2004). Gallic, vanillic and protocatechuic acid are included in the hydroxybenzoic acid family while chlorogenic acid (a quinic acid conjugate of caffeic acid) is a hydroxycinnamic acid (Jaganath and Crozier 2010). Phenolics such as chlorogenic acid and ferulic acid, were shown to improve glucose uptake in cultured L6 rat myotubes, and the effects showed synergy with common hypoglycemic medications metformin and THZ (2,4-thiazolodinedione) (Prabhakar and Doble 2009).

Phenolic acids found in *M. charantia* (80% EtOH) extract were catechin, gentisic acid, epicatechin, gallic acid, chlorogenic acid, vanillic acid,

protocatechuic acid, *trans*-cinnamic acid and *o*-coumaric acid (Horax et al. 2005; Horax et al. 2010). Budrat and Shotipruk (2008) report catechin > gentisic > gallic > chlorogenic as the prominent phenolics extracted using a subcritical water extraction technique at 200˚C. *P*-coumaric acid, tannic acid, ferulic acid, benzoic acid and caffeic acid have also been identified (Kubola and Siriamornpun 2008) (Table 4.8). Most agree that catechin is found in the largest quantities (Horax et al. 2005; Budrat and Shotipruk 2008; Horax et al. 2010) with up to 86% of phenolic content (Budrat and Shotipruk 2008). However, Kubola et al. (2008) suggest that gallic acid is the most concentrated, in all parts of the plant (seeds, flesh, leaf). Ethanolic (80%) extraction of oven dried *M. charantia* peel and flesh was proposed to yield the maximum polyphenolics compared with other preparation methods (Horax et al. 2010). In agreement with the latter finding, Wu and Ng (2007) found that *M. charantia* aqueous extract was higher in total flavonoids and an ethanolic extract contained more phenolics. However, in studies where heating above 100˚C was involved in the extraction process, total phenolic (Folin-Ciocalteu) concentrations were much greater (Budrat and Shotipruk 2008; Kubola and Siriamornpun 2008; Ng et al. 2011) (Table 4.8).

Gallic acid is a phenolic widely dispersed in food and a major contributor to the phenolic content of black tea (Hodgson et al. 2000). Gallic acid, also known as gallate or 3,4,5-trihydroxybenzoate, forms the base unit of the hydrolysable tannins (Jaganath and Crozier 2010). Gallic acid is most frequently found in its simple esterified forms, gallotannins, ellagitannins, and condensed tannins (proanthocyanidins) formed of (+)-catechin or (-)-epicatechin units (Haslam and Cai 1994). Esterification of flavan-3-ols produces catechin gallates which are hydroxylated to form gallocatechins (Jaganath and Crozier 2010) such as epigallocatechin-gallate commonly found in fresh green tea leaves (Miura et al. 2001). GA was found to be a potent scavenger of the ferric ion in the FRAP assay (Pulido et al. 2000) as well as in the TEAC assay, where it was followed by vanillic acid (VA), protocatechuic acid (PA) and *o*-coumaric acid (*o*-CA) (RiceEvans et al. 1996). GA contains several active hydroxyl groups and has been

demonstrated to be a powerful scavenger of the DPPH radical followed by PA, VA and *o*-couA (Karamac et al. 2005).

Protocatechuic acid has demonstrated insulinomimetic effects in human omental adipocytes *in vitro* by stimulating GLUT4 translocation to the cell surface as well as increasing adiponection expression and nuclear PPARγ levels (Masella et al. 2010). Cyanidin-3-β-D-glucoside, a component of black rice (commonly consumed in Asian cuisine), is primarily metabolized to PA in rats and inhibits inflammatory cytokines IL-4 and TNF-α (Han et al. 2009). Similar inhibition of IL-1β, TNF-α, prostaglandin E2, COX-2 pathway, NO and iNOS was demonstrated by protocatechuic acid applied to lipopolysaccharide stimulated RAW 264.7 cells (Min et al. 2010).

In vitro NO production was decreased by 30% with the addition of vanillic acid, which also effectively scavenged O_2^-, $ONOO^-$ and its metabolite •OH (Kang et al. 2006).

2.4.2 Flavonoids

As a group, the flavonoids are structurally similar, consisting of 2 aromatic rings bound by 3 carbons which form an "oxygenated heterocyclic ring" (Manach et al. 2004). The many configurations of the central C ring divide the flavonoids into different structural classes (Jaganath and Crozier 2010). Flavonoids are further divided into the anthocyanins and anthoxanthins, the latter including the flavan-3-ols (Han et al. 2007). Flavonoids represent about two thirds of the total dietary polyphenols in the human diet (Han et al. 2007). Flavonoids such as quercetin demonstrate an ability to inhibit glycogen phosphorylase, a regulator of glycogen catabolism (Jakobs et al. 2006).

In the Zutphen elderly study, polyphenolic flavonoids found in commonly consumed foods such as apples (quercetin) and tea (catechins) were correlated with a reduced risk of coronary events in men (Hertog et al. 1993). By donating a hydrogen atom to the peroxy radical, flavonoids prevent the autooxidation of fatty acids and strengthen the vasculature by limiting the permeability of capillaries (Torel et al. 1986). The flavonols quercetin and myricetin show significant

inhibition of NO production induced by lipopolysaccharide and interferon in RAW 267.4 macrophages (Wang and Mazza 2002).

The flavanols or flavan-3-ols, a class of flavonoids, consist of monomeric (catechins) and polymeric (proanthocyanidins) structures (Manach et al. 2004). The flavan-3-ols are perhaps the most commonly consumed flavonoids in the standard North American diet (Jaganath and Crozier 2010). Polymers composed of catechin or epicatechin units are the most abundant proanthocyanidins (condensed tannins) found in plant tissue (Jaganath and Crozier 2010) . Catechin contains 5 hydroxy groups (Daisy et al. 2010) which may explain its potent antioxidant properties, a higher degree of hydroxylation being related to free radical scavenging (Rice-Evans et al. 1995). Green tea extract with a high component of epigallocatechin-gallate (a catechin polymer) maintained 90% of its potency even after 24 hours of storage indicating minimal oxidation (Miura et al. 2001). In ApoE deficient mice, green tea extract prevented the accumulation of aortic plaque and smooth muscle cell proliferation (Miura et al. 2001). Catechins from green tea showed a protective effect in microwave exposed rats by increasing SOD and glutathione peroxidase and reducing lipid peroxidation (Kim and Rhee 2004). Catechin isolated from the medicinal plant *Cassia fistula* demonstrated an ability to restore several parameters of glucose metabolism as well as stimulating GLUT4 mRNA expression suggesting an insulinomimetic effect (Daisy et al. 2010).

2.4.3 Ascorbic acid, fumaric acid and malic acid

L-ascorbic acid (AA), an enediol (Singleton et al. 1999), shares a structural relationship with the C6 sugars, such as glucose, and is a reductone (containing a carbonyl group adjacent to the enediol group) which makes it a powerful antioxidant (Davey et al. 2000). AA is able to scavenge ROS including superoxide and the more damaging hydroxyl radical as well as regenerating α-tocopherol *in vivo* (Padh 1990). In healthy humans, experimentally induced hyperglycemia markedly decreased brachial endothelial dilation, which was restored after AA administration due to an increase in NO availability and depressed synthesis of $O_2^{\cdot-}$ (Beckman et al. 2001). AA was also able to restore NO stimulated vascular dilation

in diabetic patients (Ting et al. 1996). *In vivo*, ascorbic acid exists as an L-ascorbate anion containing one valence electron and is highly concentrated in neural and immunological tissues (Davey et al. 2000).

Total ascorbic acid concentration includes both L-ascorbic acid and dehydroascorbic acid (Baardseth et al. 2010). The copper containing enzyme ascorbate oxidase (AO) is responsible for the oxidation of AA to dehydroascorbic acid (DHAA) (Sekiya et al. 1990). AO is abundant in members of the Cucurbitaceae family including cucumber and other squashes (Nakamura et al. 1968; Davey et al. 2000). AO activity in cucurbit cell culture (pumpkin callus) has been demonstrated to increase with the addition of copper sulfate ($CuSO_4$) and was attributed to an increase in transcription (Esaka et al. 1988).

Oxidation of AA to DHAA is a reversible process and is the primary mechanism for its antioxidant potential (Novakova et al. 2008). The oxidation of AA to DHAA is accelerated by temperature fluctuations, basic pH, exposure to light and oxygen, the presence of metals as well as enzymatic action (Novakova et al. 2008; Baardseth et al. 2010). The degradation of AA to DHAA is most prominent when a vegetable is in the raw state and at temperatures of 30˚C - 60˚C (Munyaka et al. 2010). *M. charantia* AA content is stable at 5 - 10˚C (Tatsumi et al. 2006) but once cut and stored for 5 days at 5˚C, AA content was shown to decrease by about 78% (Wang et al. 2007). Cooking temperatures below 60˚C do not hinder the oxidation of AA to DHAA, but the AA content of crushed broccoli was maintained when samples were cooked for 10 min at 80˚C due to the denaturation of AO (Munyaka et al. 2010). Other authors suggest that extended cooking time (15 min), especially boiling, dramatically decreased AA content in *Brassica rapa*, but these samples had been previously frozen (Francisco et al. 2010). Freezing at -40˚C has been shown to decrease AA content in both raw and blanched *M. charantia* samples (Myojin et al. 2008).

Due to their structural similarity, glucose and DHAA compete for transport into the cell (Schorah 1992). Therefore, the hyperglycemia of T2D impairs the cellular uptake of DHAA, increasing plasma DHAA concentrations and immobilizing the intra-cellular conversion of DHAA to AA (Schorah 1992). The *in*

vivo ratio of AA/DHAA is an indicator of antioxidant status and the ability of an aerobic organism to combat oxidative stress caused by ROS (Novakova et al. 2008). Both AA and DHAA are stable when held at a pH of 2 (Wechtersbach and Cigic 2007). While it is good practice to include measures of total ascorbic acid (L-ascorbate + DHAA) many researchers do not. Both AA and DHAA can interfere with the Folin-Ciocalteu (FC) reagent (Singleton et al. 1999). Therefore, separate analysis and quantification of both ascorbic acid forms is indicated as total ascorbic acid (TAA) has the ability to interfere with total phenolic (TP) results if present in large quantity (Singleton et al. 1999).

Fumaric acid (2-Butenedioic acid) is a "short chain-carboxylic acid containing a double bond" (Dibner and Buttin 2002). FA in the proximal GI tract has been shown to improve digestibility of proteins and amino acids (Mroz et al. 1997) by reducing fermentative lactic acid bacteria within the lumen (Blank et al. 2001). It is, therefore, commonly included in the feed of livestock to increase gastrointestinal acidity and improve feed to gain ratios and growth (Dibner and Buttin 2002).

2.4.5 Digestion, bioavailability and bioaccessibility of polyphenols and organic acids including their putative effects on gut health

The bioavailability of a food depends upon the effects of digestive enzymes in the gastrointestinal tract, the small intestine (duodenum and jejunum) being the primary site of enzymatic nutrient absorption (Serafini et al. 1998; Saura-Calixto et al. 2007). There is a minor contribution to bioavailability from colonic absorption (Saura-Calixto et al. 2007). The structure, including molecular weight, glycosylation and esterification of a polyphenol affects its intestinal absorption (Scalbert et al. 2002). This case has been observed for (+)-catechin and (-)-epicatechin which are stable during oral (synthetic saliva) and gastric digestion and have an absorption rate between 40 - 80% (respectively) in the presence of Caco-2 cells (Laurent et al. 2007). During metabolism the antioxidant activity of the ingested compound may be diminished as it is transformed to its metabolites and the parent compound may not reach the blood stream (Olthof et al. 2003).

Extractable or free phenolics have low molecular weights, are soluble in aqueous and organic solvents and are associated with soluble dietary fibre (Sayago-Ayerdi et al. 2007). Frequently, the data reported in the literature measures phenolic content from the supernatant or soluble fraction of a plant extract, without regard for any bound phenolics remaining in the residual pellet (Saura-Calixto et al. 2007).

Bioaccessibility is another term that is used to describe the process of colonic metabolism which can improve the transport of certain nutrients across the intestinal epithelial barrier rendering them bioavailable (Saura-Calixto et al. 2007). Non-extractable phenolics or the bound component corresponds with insoluble fibre (Sayago-Ayerdi et al. 2007) and is included in the indigestible fraction of a plant material (Saura-Calixto et al. 2000). The non-extractable polyphenols are generally found as esters, polymers or glycosides, which are not absorbed until extensive metabolism (Tagliazucchi et al. 2010). These bound or non-extractable phenolics are thought to belong to the proanthocyanidins also known as condensed tannins (catechin and epicatechin polymers) and other compounds contained in the fibrous parts of the plant (Saura-Calixto et al. 2010). As an example, *M. charantia* is rich in total dietary fibre composing roughly 46% of its dry weight (Horax et al. 2010) as well as the proanthocyanidin precursors catechin and epicatechin (Horax et al. 2010). The indigestible fraction of food, including insoluble fibre, proteins, resistant starch and some polyphenols such as lignin, are resistant to enzymatic and mechanical digestion and nourish the colonic microflora (Saura-Calixto et al. 2000).

Both extractable (Bravo et al. 1994) as well as non-extractable polyphenols, may be important factors in the bioactivity of polyphenolic compounds contained in whole foods (Saura-Calixto et al. 2000). Simulated mastication and chemical extraction methods yielded similar amounts of bioaccessible total polyphenols, including catechin (Tagliazucchi et al. 2010). In this respect, the mechanical processing of a food during *in vitro* laboratory testing cannot replace the action and activity of the oral cavity, due to a lack of bacterial interaction with the food bolus (Sato et al. 2008). Also, the bioaccessible total phenolics that are extracted during

mechanical extraction processes are not equal to the available phenolic activity after exposure to the full spectrum of oral, gastric and intestinal digestive enzymes *in vivo* (Saura-Calixto et al. 2000; Saura-Calixto et al. 2007). While the extractable polyphenolic fraction of a food is released during mastication, it is during the gastric phase of digestion that the non-extractable polyphenols become bioavailable, a process that continues during pancreatic and colonic digestion (Saura-Calixto et al. 2000; Tagliazucchi et al. 2010). Epigallocatechin gallate, catechin, resveratrol and quercetin increased the rate of pepsin activity when incubated with denatured hemoglobin *in vitro* suggesting improved protein digestion with the ingestion of polyphenols (Tagliazucchi et al. 2005). Green tea extract has also been shown to attenuate plasma cholesterol and triglyceride levels (Miura et al. 2001). Certain polyphenols, including gallic acid, were shown to repress the activity of α-amylase as well as trypsin during *in vitro* studies (Rohn et al. 2002). The repression of α-amylase and α-glucosidase has also been reported with addition of *M. charantia* extracts inhibiting carbohydrate metabolism *in vitro* (Fonseka et al. 2007). In rats, urinary excretion rates of some phenolics have been shown to increase after sterilization of the intestinal tract, indicating the importance of proximal and colonic gut microflora in the metabolism of phenolic compounds (Goodwin et al. 1994). The same authors also concluded that output of phenylacetic acid does not equal input during experimental conditions, which indicates that there may be additional endogenous phenolic metabolism in the upper intestinal tract, as urinary metabolites are generally absorbed in the proximal intestine (Goodwin et al. 1975).

Chlorogenic acid (Bermudez-Soto et al. 2007), caffeic acid and catechin display a high degree of stability during gastric digestion (Tagliazucchi et al. 2010). At the end of gastric and pancreatic digestion, over 62% of the total polyphenols were bioavailable (based on an enzyme peroxidase assay for total polyphenols), 56% of which were flavonoids (Tagliazucchi et al. 2010). Although the authors state that there was no difference between chemical and simulated mastication, it is difficult to differentiate the total amount of polyphenols extracted by these methods, but the values for chemical extraction appear to be less than that

for enzymatic digestion (Tagliazucchi et al. 2010). Catechin and epicatechin were found to be stable during *in vitro* digestion, however, the experimental gastric juice (0.24% hydrochloric acid-0.2% sodium chloride solution, pH 1.8) was devoid of pepsin and pancreatin, because of their capacity to bind proteins (Zhu et al. 2002). Eliminating digestive enzymes from *in vitro* studies certainly limits the relevance of the findings although others have found that catechin and epicatechin remain stable during the gastric phase of digestion (Tagliazucchi et al. 2010) and catechin is highly bioavailable (Bravo et al. 1994). *In vitro* studies using rat small intestine reveal that between 40-70% of catechin remaining in the lumen was protein bound (Carbonaro et al. 2001). *M. charantia* has a protein content of 8.8% (Horax et al. 2010) to 27.88% of its dry weight (Bakare et al. 2010). Catechin and epicatechin were observed to be stable at a pH between 2 and 7.4 (Zhu et al. 2002). Stability declined as the digestive milieu gained alkalinity, with (+)-catechin epimerizing to epicatechin and (-)-epicatechin epimerizing to catechin (Zhu et al. 2002). It has been found that (-)-epicatechin was far more effective than its isomer (+)-catechin at stimulating NO production in human endothelial cells *in vitro,* indicating the importance of this epimeration during digestion on the physiological effects of these flavan-3-ols (Ramirez-Sanchez et al. 2010).

In vitro studies using segments of rat small intestine, show that catechin can cross the epithelial barrier with approximately 10% detectable in the surrounding medium (Carbonaro et al. 2001). Analysis of fecal content in live rats fed a diet containing purified catechin for 3 weeks showed only 3% excretion of catechin, indicating both excellent bioavailability and bioaccessibility *in vivo* (Bravo et al. 1994). Approximately 40-60% of catechin subjected to colonic fermentation in a simulated rat colon was recovered in the fecal content as assessed by HPLC as well as recovery of up to 95% of tannic acid (polymers of gallic acid units), which was also shown to increase fecal bulk, water and protein content (Bravo et al. 1994). Tannic acid appears to be absorbed by the epithelium, but not does not enter into the surrounding medium *in vitro* (Carbonaro et al. 2001). *In vitro,* catechin increased the production of butyric acid, a short chain fatty acid implicated as an important factor for the health of colonic microflora (Bravo et al. 1994).

About 50% of ingested chlorgenic acid is potentially bioavailable to humans, indicated by its recovery in the urine of human subjects (Olthof et al. 2003). Within the human colon, chlorogenic acid is converted to quinic and caffeic acids by the intestinal flora and is further metabolized within the liver and kidney to benzoic acid and excreted in the urine as hippuric acid (*N*-benzoylglycine) (Olthof et al. 2003). Hippuric acid, due to the loss of hydroxyl groups during metabolism, has negligible antioxidant properties (Olthof et al. 2003).

Organic acids perform an acidifying function in the gastrointestinal tract, affecting the microbiotic flora by reducing pH (Dibner and Buttin 2002). These effects are dependent on the specific organic acid and its rate of dissociation (Dibner and Buttin 2002). Ascorbic acid is a major component of healthy human gastric secretions and the concentration has been found to be greater than that in plasma (Schorah et al. 1991). Persons with gastric pathology were found to have increased gastric pH level, accompanying a decrease in secreted ascorbic acid concentrations potentially depressing the ability of the gastric epithelium to combat free radicals (Sobala et al. 1991). Other beneficial functions of organic acids in the GI tract include enhanced pancreatic enzyme secretion and function as well as improved regeneration of the mucosal layer (Dibner and Buttin 2002).

Lack of dietary organic acids in the stomach and proximal intestine contributes to the growth of pathogenic coliform or lactic acid bacteria, the latter being specifically repressed by fumaric acid (Knarreborg et al. 2002). Physiologically, the proximal intestine maintains a static pH balance and dietary organic acids are an important factor in maintaining a healthy microbial environment by acidifying the lumen and its contents (Dibner and Buttin 2002).

In weaned piglets, the addition of formic, fumaric and n-butyric acids to feed increased protein digestibility in the ileum, including amino acids (Mroz et al. 1997). Moreover, the addition of fumaric acid to feed ameliorates the concentration of fermentative microbiota, such as lactic acid, in the ileum of weaned piglets (Blank et al. 2001). A combination of organic acids (phosphoric, citric, fumaric and malic acids) was able to reduce cecal ammonia and acetic acid concentrations in swine (Piva et al. 2002).

Organic acids found in banana and sweet potato, such as malic, oxalic and citric acids were released slowly and differentially after oral, gastric and pancreatic digestion, with each stage of digestion contributing to their release from the solid matrix (Sabboh-Jourdan et al. 2011). Further, malic acid was absorbed into Caco-2 cells but was not transported across the basolateral layer (Sabboh-Jourdan et al. 2011). However, malic acid as an additive in the feed of lambs, was not effective at promoting feed intake or growth (Carro et al. 2006) but did increase propionate concentrations in sheep ruminant fluid (Jalc et al. 2002).

2.4.6 Effect of heat treatment, including cooking, on polyphenols and organic acids

Heat treatment improved TP status in *M. charantia* (Budrat and Shotipruk 2008; Kubola and Siriamornpun 2008; Ng et al. 2011). Freezing and cooking as part of the commercial canning process improved the bioavailability and bioaccessibility of chlorophyll and its metabolites in *Pisum sativum* (peas) (Gallardo-Guerrero et al. 2008). Analysis of polyphenols in unsoaked *Phaseolus vulgaris* L. beans (cv. Jalo Precoce), cooked to 100˚C, including cooking water, showed an increase in total flavonoids (quercetin, kaempferol; + 18.6mg/100g) and only a slight decrease in total phenolics (*p*-coumaric, ferulic and hydroxycinnamic acid derivatives; - 4.2 mg/g) (Ranilla et al. 2009). However, heating to 100˚C with inclusion of cooking water actually improved the ferulic acid profile by about 60% (Ranilla et al. 2009). In a study on the kinetic rate of triglyceride oxidation in the absence and presence of selected polyphenols, protocatechuic acid was equally effective at preventing lipid oxidation at 90˚C and at ambient temperatures (Marinova and Yanishlieva 2003). Heat treatment of ginseng was shown to increase its ability to scavenge $O2^{\cdot-}$, $ONOO^-$ and ·OH (Kang et al. 2006). *M. charantia* is eaten as a cooked vegetable, the processing of which can include soaking in salt or vinegar, soaking or preheating in water to remove bitterness prior to cooking (Wang et al. 2008). Heat treatment (in water, 5 min) between 50-75˚C increased proteolytic activity in *M. charantia* and was closely associated with photosynthetic enzyme and chlorophyll degradation (Wang et al. 2008).

The free radical scavenging ability (DPPH) of *M. charantia* extract was shown to increase from 5% inhibition to 80% inhibition after heat extraction (Ansari et al. 2005). Also, boiled *M. charantia* (fruit, cut, boiled in water) showed an increase in FRAP activity and TP concentrations (Ng et al. 2011). Proteolytic activity of *M. charantia in vitro* was enhanced by heat treatment from 50 – 75˚C (Wang et al. 2008).

2.4.7 Measures of total phenolics and antioxidant capacity: Folin-Ciocalteu, FRAP and ABTS (hydrophilic) assays

While it is more difficult to quantify a broad range of individual antioxidant compounds, including phenolics and vitamins, quantifying total phenolics is a simple and valid technique used by many researchers (Wachtel-Galor et al. 2008; Ng et al. 2011). There is a wide range of TP content reported in mature *M. charantia* (Table 4.8) which appears to be dependent on extraction method (Budrat and Shotipruk 2008; Kubola and Siriamornpun 2008; Horax et al. 2010) or heat treatment involving cooking (Ng et al. 2011).

In four different varieties of *M. charantia* TP concentrations ranged from 5.39 ± 0.06 – 7.75 ± 0.07 CAE/g (chlorogenic acid equivalents ± SE) in oven dried samples vs. 6.40 ± 0.06 - 8.9 ± 0.09 CAE/g in freeze dried samples (Horax et al. 2005). A more recent study found the highest TP values (up to 14.8 ± 0.3 GAE mg / 100g) in 80% ethanol extract (Horax et al. 2010). Wu and Ng (2008) found that TP in *M. charantia* ethanolic extract contained 68.8 mg/g vs. 51.6 mg/g in an aqueous extract. Heat treatment has been documented to improve TP status in *M. charantia* (Ansari et al. 2005; Budrat and Shotipruk 2008; Kubola and Siriamornpun 2008; Ng et al. 2011). *M. charantia* TP concentrations of 7.69 ± 33.9 mg GAE/ g were reported after microwaving in water, freeze drying and aqueous extraction (Ng et al. 2011). After a subcritical water extraction (SWE) technique at 200˚C, 52 mg GAE / g (DW) were observed (Budrat and Shotipruk 2008). TP concentrations increased to 324 ± 1.63 mg GAE/ g when mature fruits were boiled in distilled water (Kubola and Siriamornpun 2008).

Techniques that utilized heat in the extraction process including SWE which also utilizes high pressure, may have affected the bound polyphenols (such as proanthocyanidins) by degrading the fibre matrix and releasing more polyphenols for quantification (Ansari et al. 2005; Saura-Calixto et al. 2007; Budrat and Shotipruk 2008; Kubola and Siriamornpun 2008).

The ferric reducing ability of plasma or ferric reducing antioxidant power (FRAP) assay, assesses the redox potential of a food extract or pure compound (Benzie and Strain 1996; Benzie and Strain 1999). Cooking (boiling and microwaving) *M. charantia* prior to aqueous extraction increased the FRAP value by greater than 200% over the raw samples (Ng et al. 2011). FRAP values of pure standards measured as EC_1 have been observed in the following order gallic acid (180 µmol/L) > trolox (322 µmol/L) catechin (348 µmol/L) > ascorbic acid (392 µmol/L) at 4 min incubation (Pulido et al. 2000).

ABTS, which is oxidized to $ABTS^{\bullet+}$ (2,2'-azino-bis[3-ethylbenzothiazoline-6-sulphonic acid]) when reacted with potassium persulfate, is another frequently used assay which again utilizes a colorimetric method (Re et al. 1999). With addition of a dietary antioxidant, donation of hydrogen ions fuels a redox reaction producing a reduction in colour and decreasing absorbance which is negatively correlated with increasing antioxidant activity (Re et al. 1999). Kotikova et al. (2011) found that, in tomatoes, up to 83% of the total antioxidant capacity as measured by ABTS assay was due to the hydrophilic component, including AA and phenolic compounds. *M. charantia* subjected to SWE at 150˚C had the most potent IC_{50} (3.90 µg / ml) as compared with SWE at 130˚C, 180˚C and 200˚C (4.37; 5.42; 5.49 µg / ml), respectively (Budrat and Shotipruk 2008). The IC_{50} (or EC_{50}) value represents 50% or half of the maximal efficacy of an extract (Budrat and Shotipruk 2008).

III. LINKING STATEMENT

T2D as a preventable, chronic disease is a major world health concern especially when the trend away from traditional foods and an urban lifestyle are considered (Wild et al. 2004; Cunningham-Murie et al. 2008). The oxidative stress caused by chronic hyperglycemia can be alleviated by the ingestion of dietary polyphenols and organic acids which act as antioxidants due to their ability to scavenge harmful physiological ROS and RONS either directly by acting as reducing agents or by halting the propagation of more damaging reactive intermediates (Halliwell and Gutteridge 1995; Singleton et al. 1999; Beckman et al. 2001). *Momordica charantia* is an extensively documented, widely grown plant eaten as a cooked vegetable and utilized for its medicinal value, including anti-diabetic properties, in Asia, Africa, Latin America and the Caribbean (Lans 2003; Virdi et al. 2003; Delgoda et al. 2004; Fonseka et al. 2007). During gut metabolism the antioxidant activity of the ingested polyphenol or organic acid is altered as it is transformed to metabolites, in turn affecting the absorption of nutrients (Saura-Calixto et al. 2000; Olthof et al. 2003; Saura-Calixto et al. 2007; Saura-Calixto et al. 2010; Tagliazucchi et al. 2010) as well as gut health (Dibner and Buttin 2002). *In vitro* models of digestion which include gastric and pancreatic digestive enzymes are a good alternative to *in vivo* studies (Saura-Calixto et al. 2000; Tagliazucchi et al. 2010). Additionally, cooking, freezing (Gallardo-Guerrero et al. 2008; Ranilla et al. 2009; Ng et al. 2011) and extraction method affect the phenolic profile, frequently increasing TP and improving the antioxidant capacity of *M. charantia* (Ansari et al. 2005; Horax et al. 2005; Budrat and Shotipruk 2008; Kubola and Siriamornpun 2008).

In the current study, the effects of cooking and *in vitro* enzymatic digestion were assessed in ethanolic extract from raw (R), cooked (C), R + digested (D) and C + D *M. charantia* fruit. The major outcomes were a significant increase in GA, AA, FA and ABTS scavenging after digestion. Since *M. charantia* is always eaten as a cooked vegetable, assessing its polyphenolic and organic acid profile as well

as its antioxidant capacity after both cooking and digestion is an important contribution to understanding its purported medicinal effects.

IV. MANUSCRIPT

The effects of *in vitro* digestion on polyphenolic and organic acid profile and antioxidant capacity of raw and cooked *Momordica charantia*.

Amy Van Elslande[1], V. J. Lam[1], Kebba Sabally[1], Atef Nassar[2], Behnam Azadi[1] Danielle J. Donnelly[2] and Stan Kubow[1]

[1]School of Dietetics and Human Nutrition, [2]Department of Plant Science McGill University, Macdonald Campus, 21,111 Lakeshore Road, Ste-Anne-de-Bellevue, H9X 3V9, QC, Canada

4.1 Abstract

Ample studies relate the putative health promoting properties of the medicinal food *Momordica charantia* via an assessment of polyphenols, total phenolics (TP) and antioxidant capacity in the raw (R) form. However, few studies have investigated cooked *M. charantia* in this regard despite the fact that *M. charantia* is always eaten cooked. In the current study, quantification of polyphenols and organic acids as well as selected antioxidant indices were analyzed in four treatment groups including raw (R), cooked (C); R + digested (D) and C + D with a sample size of 10. A significant main effect of digestion was observed for all phenolics (except for catechin [CAT] and chlorogenic acid [CGA]), organic acids (ascorbic, fumaric, and malic acids) TP, ferric ion reducing antioxidant potential (FRAP) and 2,2'-azino-bis[3-ethylbenzothiazoline-6-sulphonic] acid (ABTS) antioxidant capacity assays. TP concentration was greatest in the R treatment which had significantly ($P < 0.05$) higher concentrations than C, R + D and C + D samples of *M. charantia*. HPLC analysis revealed protocatechuic acid (PA) as the phenolic compound in the greatest concentration in the C samples with a significant increase ($P < 0.05$) over the R sample. After digestion, there were significantly lower concentrations of protocatechuic acid in the C + D groups.

Gallic acid (GA) was the phenolic compound observed in the highest concentration in the R + D and C + D treatments. There were significantly ($P < 0.05$) greater ascorbic acid (AA) concentrations by 24% in the C + D treatment relative to the C samples as well as significantly greater concentrations in the R + D treatment relative to the R sample. Fumaric acid (FA) was significantly ($P < 0.05$) greater in both digested treatments versus the R or C samples. GA was also strongly significantly ($P < 0.05$) positively correlated with both AA ($r = 0.88$) and FA ($r = 0.69$). FC and FRAP showed a significant ($P < 0.05$) positive correlation ($r = 0.56$). The present study shows that there are significant alterations in phenolic and organic acid concentrations and antioxidant capacity after cooking and digestion, indicating that such indices should be measured after cooking and digestive enzymatic processes to better understand the putative health properties associated with consumption of *M. charantia*.

4.2 Introduction

Momordica charantia is a medicinal plant that is a rich source of antioxidant compounds including polyphenols (Horax et al. 2005; Kubola and Siriamornpun 2008; Horax et al. 2010) and ascorbic acid (Meerabai et al. 2007; Wang et al. 2007; Myojin et al. 2008) and a potential source of other organic acids which have been shown to play a role in gut and digestive health (Schorah et al. 1991; Sobala et al. 1991; Dibner and Buttin 2002; Knarreborg et al. 2002; Sabboh-Jourdan et al. 2011). *M. charantia* has been reported to have hypoglycemic properties (Srivastava et al. 1993; Tongia et al. 2004; Fonseka et al. 2007), insulin potentiating ability (Roffey et al. 2007), and a capability to ameliorate aberrant fatty acid metabolism (Chao and Huang 2003; Chen et al. 2003; Shih et al. 2008).

Cultivar has an effect on the nutritional content, including polyphenols, of *M. charantia* (Horax et al. 2005). Horax et al. (2005) quantified TP concentration in 4 cultivars of *M. charantia* using extracts of freeze dried or oven dried (60˚C) fruits and found a range of 5.39 ± 0.06 – 8.90 ± 0.09 mg CAE/ mg dry weight. Although *M. charantia* is always eaten cooked, its *in vivo* and *in vitro* nutritional properties have been frequently assessed after extraction from the raw material

(Srivastava et al. 1993; Horax et al. 2005; Horax et al. 2010). Ng et al. (2011) reported TP concentrations of 582.9 ± 47.9 mg GAE/100 g (DW) in raw *M. charantia* extract (H_2O). Wu and Ng (2007) reported TP concentrations of 51.6 mg GAE/g dry weight in an aqueous extract vs. 68.8 mg GAE/g dry weight in an ethanolic extract.

On the other hand, *in vitro* studies that used heat in the extraction process (Ansari et al. 2005; Budrat and Shotipruk 2008; Kubola and Siriamornpun 2008) or extraction after cooking (Ng et al. 2011) have shown significant alterations in polyphenolic profiles and antioxidant capacity measures of *M. charantia*. Horax et al. (2010) stated that an 80% ethanolic extraction (80˚C) from *M. charantia* fruit (flesh and peel, oven dried at 40˚C) yielded the highest phenolic concentration (14.8 ± 0.3 mg GAE/g of extract) compared to 0, 20, 40, 60 and 95% ethanol concentration. In two other methods utilizing heat extraction (100˚C vs. 200˚C) and water as the solvent, Kubola et al. (2008) and Budrat et al. (2009), reported TP concentrations of 324 ± 1.63 and 52.63 (mg GAE/g DW), respectively. In the only study to specifically report the effects of cooking (boiling in water for 5 min, aqueous extraction, freeze dried supernatant) on *M. charantia*, Ng et al. (2011) reported TP concentrations of 766.3 ± 14.9 mg GAE/100g. Ansari et al. (2005) found that there was an increase of 83% in 1,1-diphenyl-2-picrylhydrazil radical (DPPH) inhibition when a heat extraction process (boiled in 60% methanol under reflux) was utilized vs. a cold extraction (60% methanol) of *M. charantia*. The latter finding is similar to results obtained by Kubola et al. (2008) who reported DPPH inhibition of about 81%. Ng et al. (2011) reported a significant increase in FRAP values after boiling *M. charantia* (raw 2.2 ± 0.1 vs. boiled 8.2 ± 0.7 mmol FE/100 g) but boiling did not alter the TEAC value.

The small intestine, mainly the duodenum and jejunum, is the primary site of enzymatic digestion and nutrient absorption (Serafini et al. 1998; Saura-Calixto et al. 2007), with a minor contribution from colonic metabolism (Saura-Calixto et al. 2007). Mechanical processing (such as chewing or blenderizing) and digestion including gastric and pancreatic enzymes, release soluble polyphenols (Saura-Calixto et al. 2000; Tagliazucchi et al. 2010). Bound polyphenols may be partially

metabolized in the proximal intestine but the majority are released by colonic metabolism (Saura-Calixto et al. 2000; Tagliazucchi et al. 2010). Heat treatment, such as during cooking, improves the capacity of digestive enzymes to breakdown the insoluble fibre matrix of foods, including *M. charantia*, allowing a greater release of polyphenols (Ng et al. 2011). Budrat et al. (2009) also reported increases in concentration of specific polyphenols (CAT, gentisic acid, GA and CGA) when *M. charantia* fruits were extracted in water at temperatures between 130˚C and 200˚C. To our knowledge, the effect of cooking and *in vitro* digestion on the polyphenolic, organic acid and antioxidant capabilities of *M. charantia* has not been assessed previously

The present study assessed indices associated with antioxidant capacity including total phenolic compounds (TP), the individual polyphenolic acids gallic acid (GA), protocatechuic acid (PA), catechin (CAT), chlorogenic acid (CGA), vanillic acid (VA), *o*-coumaric (*o*-CA) acid and *trans*-cinnamic acid (*t*-CiA), the organic acids ascorbic acid (AA), malic acid (MA) and fumaric acid (FA) as well as the ferric ion reducing antioxidant potential (FRAP) and 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid; ABTS; hydrophilic) assays in *M. charantia* (R; C; R + D and C + D) subjected to cooking and *in vitro* enzymatic digestion.

4.3 Materials and methods

4.3.1 Chemicals

Chemicals, solvents and standards used for HPLC quantification of polyphenolic compounds and organic acids (HPLC grade) and spectrophotometric analysis of total phenolic compounds and antioxidant capacity were purchased from Sigma-Aldrich (Oakville, Ontario, Canada)**.** Standards included GA, PA, CAT, CGA, VA, *o*-CA and *t*-CiA, AA, MA and FA.

4.3.2 Preparation of standards

Phenolics and organic acids were prepared based on the method of Shakya and Navarre (2006). Stock standards were prepared in 100% methanol to 10 mg/ml. Working standards were prepared in 100% methanol to 100 µg/ml. A

sample size of 20 µl for the intact phenolics was injected for HPLC analysis. The results were expressed in µg/g.

4.3.3 Preparation of samples

4.3.3.1 Procurement and treatment of fresh *M. charantia* fruits

Sixty *M. charantia* (China green phenotype) fruits, bought from a local Asian market, were washed and dried before processing. Washed fresh fruits (skin and flesh, with seeds removed) were homogenized and subjected to 4 different treatments: Raw (R), cooked (C), raw digested (R + D) and cooked digested (C + D). Three fruits per replicate were used with 10 replicates prepared for the R and R + D treatments and another 10 replicates for the C and C + D treatments for a total of n=10. All replicates were measured in triplicate.

M. charantia replicates were processed separately in a laboratory blender until homogenized to simulate mastication according to the method of Tagliazucchi et al. (2010). As significant portions of hydrophilic nutritional compounds are lost in the cooking water (Wachtel-Galor et al. 2008; Baardseth et al. 2010; Ng et al. 2011), no water was added to any of the samples for the cooking procedure. Cooked samples were subsequently processed according to the cooking method described by Saura-Calixto et al. (2000), with the following modifications: cooking time was reduced to 3 min and no water was added to the sample. Homogenized samples were cooked on a hot plate in a graduated beaker at 100˚C for approximately 3 min to simulate stir frying temperatures (Chuah et al. 2008). Triplicate 2 g aliquots of the R and C homogenate were freeze-dried in 50 ml capped tubes in a freeze drying unit (Christ Gamma 1-15 LSC, MBI Lab Equipment) for approximately 4 days (96 hours). The samples were subsequently ground into a homogenous powder and stored in aliquots in 4 ml capped glass tubes in a -20˚C freezer for later ethanolic extraction (Roffey et al. 2007).

4.3.3.2 In vitro digestion of samples

In vitro digestion followed a modified version of the method developed by (Sarang et al. 2011) with the addition of bile salts to the pancreatic enzyme mixture

(Tagliazucchi et al. 2010). Homogenized fresh or cooked samples (2 g) were portioned into 120 ml capped plastic cups to which was added 20 ml of simulated gastric fluid (HCl/KCl buffer 0.2 M) was added. The pH was adjusted to 1.5 and samples were pre-incubated for 45 min at 37˚C in a water bath. An aliquot of 200µl of pepsin/HCl-KCl buffer (1mg pepsin in 1ml 0.001 M HCl-KCl buffer, pH 1.5) was added to the heated samples, which were then incubated at 37˚C for 1hr in a shaking water bath. Approximately 262µl of 4M NaOH was added to raise the pH to 7.8 and stop the pepsin reaction. Samples were again pre-incubated at 37˚C for 45 min. An aliquot of 200 µl of pancreatin in phosphate buffer (pH 7.8) and 50 mg / ml bile salts were added and incubated in a shaking water bath at 37˚C for 45 min. Thereafter, Na_2CO_3 was added to stop the enzymatic reaction. Volume was adjusted up to 50 ml with Tris-Maleate buffer (pH 6.9) and pH was adjusted to 6.9 with 0.1M HCl. Samples were again pre-incubated at 37˚C for 45 min. A 5ml α-amylase solution in Tris-Maleate buffer was added (2mg of 28 units/5 ml Tris-Maleate buffer) and samples were incubated at 37˚C in a shaking water bath for 1 hr. Tubes were shaken and submerged in an ice bath to inactivate the enzyme. At the end of pancreatic digestion, samples were acidified with 6ml 0.4 M sodium acetate buffer and adjusted with 1 M HCl to pH 2.0 to protect the polyphenol content (Tagliazucchi et al. 2010). Samples were then freeze-dried in a freeze drying unit (Christ Gamma 1-15 LSC, MBI Lab Equipment) for approximately 4 days (96 hr) and subsequently ground to a homogenous powder which was stored in aliquots in a -20˚C freezer for later ethanolic extraction (Roffey et al. 2007).

4.3.3.3 Analysis of total phenolic compounds

Based on a method of Singleton et al. (1999) the TP were quantified using the Folin-Ciocalteu (FC) reagent. Briefly, 100µl of *M. charantia* extract (resolubilized in methanol), along with 2 ml double distilled water (DDW) and 220µl of Folin- Ciocalteu reagent (FCR) was incubated at room temperature for 30 min. A 1 ml aliquot solution of aqueous sodium carbonate was added and left at room temperature for 1h. Absorbance was read at 765 nm on a Beckman DU 640

spectrophotometer (Beckman Instruments, Fullerton, CA). Results were reported as GAE mg/g (Singleton et al. 1999).

4.3.3.4 Analysis of polyphenolic compounds and organic acid compounds

According to the method of Shakya and Navarre (2006), 100 mg freeze dried *M. charantia*, powdered samples were placed in 4 ml glass vials and 1.8 ml 80% ethanol was added. The tubes were vortexed for 20 min and centrifuged at 4˚C for 15 min at 3000x. The supernatant was transferred into clean 4 ml glass vial and 1.2 ml of extraction buffer (80% ethanol) was added to the residual pellet. The tubes were again vortexed for 20 min and centrifuged at 4˚C for 15 min at 3000x g. The supernatant was removed and added to the previous one. The supernatants were dried using a rotovapour vacuum dryer (Thermo Savant, SC210A Speedvac Plus, Waltham, MA), flushed with nitrogen gas and stored in a -20˚C freezer until analysis. Just prior to analysis, previously vacuum dried samples were rehydrated in 1.0 ml methanol, vortexed until solubilised and filtered through 0.2 µm Whatman nylon filters into HPLC vials. An HPLC system, (Varian 9050 UV detector, Varian 9012 Solvent Delivery System, Mississauga, Ontario, Canada) equipped with a tertiary pump, refrigerated auto-sampler and single variable wavelength detector was used for sample analysis. Phenolic materials were separated based on a modified method of Shakya and Navarre (2006) using a reverse phase HPLC Gemini-NX Phenomenex (5 µm, 100 mm×4.6 mm) column and a 4.6 mm×2.0 mm guard column (Shakya and Navarre 2006). The mobile phase was composed of solvent buffer A (10 mM formic acid, pH 3.5, with NH_4OH) and buffer B (100% methanol with 5 mM ammonium formate). The solvent gradient was as follows: 0-1 min 100% buffer A, 1-5 min 0-30 % buffer B, 5-8.5 min 30-70 % buffer B, 8.5- 20 min 70-100 % buffer B. UV detection was conducted at 280 nm. A flow rate of 1.0 ml/min was used and 20 µL of sample was injected.

Organic acids were separated based on a method by Schwartz et al. (1962). Briefly, 20µl samples were injected into the HPLC system (Varian 9050 UV detector, Varian 9012 Solvent Delivery System, Mississauga, Ontario, Canada).

Samples were eluted using a reverse phase HPLC Gemini-NX Phenomenex (5 µm, 100 mm×4.6 mm) column and a 4.6 mm×2.0 mm guard column. The samples and organic acid standards (fumaric and malic acids) were eluted for 12 min with 1.0 mL of aqueous 0.008 N H_2SO_4 under isocratic conditions at 210 nm. The R + D and C + D samples were diluted to 1:100 in methanol. The results are expressed in µg/g, DW (Schwartz et al. 1962).

4.3.3.5 Analysis of antioxidant capacity

While both the contribution of the hydrophilic and lipophilic portions of a sample contribute to its total antioxidant capacity (TAOC), the primary contribution of antioxidant power is contained in the hydrophilic fraction (Kotikova et al. 2011). In the current study, only the hydrophilic fraction was assessed. Lack of correlation between measures of antioxidant capacity reflects differential structural configurations and mechanisms of action on the ability of a specific polyphenol to scavenge diverse free radicals (Karamac et al. 2005) therefore it is common to include more than one measure of antioxidant capacity. In the current study, both the ferric ion reducing antioxidant power (FRAP) as well as ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) assay were used to quantify the hydrophilic fraction of *M. charantia* extract. The extraction process followed the method of Shakya and Navarre (2006) and is described above. The FRAP assay followed the procedure outlined by Benzie and Strain (1996) with ferrous sulphate as the standard. Solutions of 30 µl H_2O and 10µl sample were pipetted into a microtiter plate to which 200µl of FRAP solution was added, mixed for 10s and the absorbance at 595nm was taken after 8min in a plate reader (Beckman, DU 640, USA). The FRAP values of the samples in ferrous sulfate equivalent were calculated using the calibration curve generated and expressed as $FeSO_4$ equivalents µM/g, DW.

Using the method proposed by Re et al. (1999) ABTS (7mM) stock solution was prepared in DDW. Radical cation of ABTS ($ABTS^{\bullet+}$) was produced by reacting ABTS solution with potassium persulfate ($K_2S_2O_8$; 2.24mM) in the dark at room temperature for 12-16 hrs before use for complete oxidation of ABTS (Re et

al. 1999). After addition of $K_2S_2O_{8,}$ oxidation of ABTS starts immediately but absorbance is stable and maximal after > 6 h and for up to 2 days when stored in the dark at room temperature. The $ABTS^{\bullet+}$ solution was diluted with 95% ethanol to an absorbance of 0.70 (±0.02) at 734nm. 1.2ml of diluted $ABTS^{\bullet+}$ solution was added to 100µl of sample extracts in ethanol and absorbance was reported between 1 and 2 min. Antioxidant scavenging activity was expressed as µg/g TE, DW.

4.3.4 Statistical analysis

Using SAS (SAS Institute, Cary, NC) 9.2 differences between groups were analyzed by 2-Way ANOVA and Tukey's post-hoc test for multiple comparisons. When significant differences were observed among the main effects of C, D and C x D comparisons for phenolic acids, organic acids and antioxidant capacity were made by a priori linear contrasts. The specific contrasts included the following comparisons: 1) R vs. C, 2) R vs. R + D, 3) C vs. C + D, 4) R + D vs. C + D. A probability of $p < 0.05$ was accepted as the minimal level of significance. Pearson's correlation was used to determine associations between LSMeans of specific contrast groups.

4.4 Results

4.4.1 Total Phenolic Compounds

A significant main effect of digestion ($p < 0.0006$) on total phenolic content was observed (Table 4.1). The R sample showed the greatest TP concentrations (12185 ± 38.82 mg GAE/g) and was significantly greater than the C ($p < 0.0001$) and the R + D ($p < 0.02$) samples. The C + D treatment had significantly greater TP concentrations than the C samples ($p < 0.03$). There was a significant positive correlation between TP and *t*-CiA ($r = 0.33$; $p < 0.04$) as well as FRAP ($r = 0.56$; $p < 0.0002$).

4.4.2 Polyphenolic and organic acid profiles

Excluding CAT and CGA, a significant ($p > 0.05$) main effect of digestion was noted with respect to phenolic concentrations (µg/g FDW) (Table 4.1). There was a significant main effect of digestion on GA ($p < 0.0001$). R + D sample had

significantly greater GA concentrations than the R treatments ($p < 0.0009$) and C + D samples showed significantly greater concentrations of GA than the C treatment ($p < 0.0001$). There was a significant main effect of digestion on PA ($p < 0.0001$). C treatment showed significantly greater than the R ($p < 0.0043$) samples and the C + D ($p < 0.0001$) group. A significant main effect of digestion was observed in VA ($p < 0.004$). The R + D samples had significantly greater concentrations than the R ($p < 0.006$) and the C + D ($p < 0.0129$) samples. Significant main effects of digestion were observed for *o*-CA ($p < 0.0002$). The C sample had significantly greater concentrations than the C + D ($p < 0.0016$) samples. A significant main effect of digestion was observed for *t*-CiA ($p < 0.0008$) and significantly ($p < 0.0026$) decreased concentrations were noted in the R vs. the R + D treatment and R vs. the C ($p < 0.0288$) treatment.

Of the phenolics that responded with an increased concentration to the *in vitro* digestion treatment, GA and VA shared a strong, positive correlation ($r = 0.58$; $p < 0.0001$). PA and *o*-CA were also strongly, positively correlated ($r = 0.7$; $p < 0.0001$) and both decreased in concentration post-cooking and digestion. PA also shared a weak, positive correlation with *t*-CiA ($r = 0.40$; $p < 0.01$).

A significant main effect of digestion was observed for MA ($p < 0.0001$). A significant decrease in MA was observed in the C vs. the C + D samples ($p < 0.0001$) and the R vs. the R + D samples ($p < 0.0017$). A significant main effect of digestion was observed for FA ($p < 0.0001$). A significant increase in FA was observed in the C vs. the C + D ($p < 0.0001$) samples as well as between the R vs. the R + D ($p < 0.0001$) and the R + D vs. the C + D ($p < 0.01$) samples. A significant main effect of digestion was observed for AA ($p < 0.0001$). A significant increase in AA was noted between the R vs. the R + D ($p < 0.0001$) and the C vs. the C + D ($p < 0.0003$) groups.

MA showed a significant positive correlation with PA ($r = 0.47$; $p < 0.002$) and *o*-CA ($r = 0.59$; $p < 0.0001$). MA showed a strong, significant, positive correlation with ABTS ($r = 0.73$; $p < 0.0001$). MA showed a significant, negative correlation with GA ($r = 0.53$; $p < 0.0004$) and a strong, significant, negative correlation with FA ($r = 0.69$; $p < 0.0001$) and AA ($r = 0.61$; $p < 0.0004$). Both FA

and AA shared a strong, significant, positive correlation ($r = 0.79$; $p < 0.0001$) and both responded with an increase in concentration after digestibility treatments.

4.4.3 Antioxidant activity measured by FRAP and ABTS (hydrophilic) assays

A significant main effect of digestion on FRAP was observed ($p < 0.0001$) (Table 4.2). Significantly lower ($p < 0.0003$; $p < 0.0001$) FRAP activity was observed in the C and the R + D samples vs. the R samples. Significantly lower ($p < 0.03$) FRAP values were also observed in the C vs. the C + D samples. The FRAP assay showed a significant, positive correlation with TP ($r = 0.56$; $p < 0.0002$). FRAP shared a significant, positive correlation with *t*-CiA ($r = 0.41$; $p < 0.008$) as well as significant, weak, negative correlation with VA ($r = 0.31$; $p < 0.05$).

A significant main effect of digestion was observed ($p < 0.0001$) on ABTS. Significantly lower ABTS activity was observed in the R + D vs. the R ($p < 0.0001$) treatments and in the C + D as compared to the C ($p < 0.0001$) samples. ABTS shared significant strong, positive correlations with MA ($r = 0.73$; $p < 0.0001$) and significant moderate, positive correlations with PA ($r = 0.43$; $p < 0.005$), *o*-CA ($r = 0.54$; $p < 0.0003$) and *t*-CiA ($r = 0.52$; $p < 0.0006$). Higher concentrations of some phenolics and organic acids corresponded with an increase in activity in the ABTS assay that included significant strong negative correlations of ABTS with GA ($r = 0.74$; $p < 0.0001$), FA ($r = 0.94$; $p < 0.0001$) and AA ($r = 0.84$; $p < 0.0001$). There was also a significant negative correlation with VA ($r = 0.36$; $p < 0.02$).

4.5 Discussion

In the present research an effect of digestion on *M. charantia* in both the raw and the cooked form was observed in terms of antioxidant capacity measures, TP concentration, organic acids as well as individual polyphenolics (excluding CAT and CGA). In agreement with results of similar studies, the current study found that digestion may be a determinant in the release of nutritionally relevant

compounds from the food matrix (Saura-Calixto et al. 2000; Sayago-Ayerdi et al. 2007; Tagliazucchi et al. 2010).

In the present work, the greatest TP concentrations were associated with the raw samples of *M. charantia* (Table 4.1). Other authors have reported significant decreases of TP after the gastric phase of digestion, with an accompanying increase after pancreatic digestion in grapes (Tagliazucchi et al. 2010) and apples (Bouayed et al. 2011).

Lignin, a polyphenol and a primary component in plant cell walls, is associated with non-extractable polyphenols, (Saura-Calixto et al. 2000; Sayago-Ayerdi et al. 2007) which are relatively stable through pancreatic digestion and nourish the colonic microflora (Tagliazucchi et al. 2010). Total fibre (soluble and insoluble) content of fresh *M. charantia* is as much as 44 % (Horax et al. 2010) or roughly 13% of its dry weight (Bakare et al. 2010).

Many authors have utilized heat in the extraction process and TP ranged from 14.8 - 324 mg GAE/g (Kubola and Siriamornpun 2008; Horax et al. 2010) (Table 4.8). Heat extraction (subcritical water extraction) has been demonstrated to degrade lignin into phenolic compounds (Wahyudiono et al. 2008) and this process yielded 52.6 mg GAE/g of TP in *M. charantia* (Budrat and Shotipruk 2008). In the current study, although there was no significant main effect of cooking, there was a significant *a priori* contrast in the comparison of the raw vs. the cooked treatments showing a decrease in total phenolics in association with cooking alone (Table 4.1). The latter result is in contrast with previous findings showing that cooking may be responsible for a greater release of certain polyphenols and TP from the fibrous matrix from an assortment of foods typical in a Spanish diet (Saura-Calixto et al. 2000) and a *Hibiscus sabdariffa* L. decocted beverage (Sayago-Ayerdi et al. 2007) as well as *M. charantia* (Ng et al. 2011).

In the current work, pH may have played a role in greater stabilization of TP (Tagliazucchi et al. 2010) as there was a tendency for a smaller decrease in TP content between the R + D and R samples as compared to the R and C samples (Table 4.2). Also the C + D sample had significantly greater TP content than the C sample (Table 4.2). Ng et al. (2011) compared TP in raw and cooked (boiled, 5

min, in water) *M. charantia* extracts and showed a significant increase of 31% in TP concentrations in the boiled sample. In the current study cooking temperatures may have been too low (less than 80˚C), the heat may not have been evenly distributed throughout the samples or cooking time may have been of insufficient duration, which could explain the decreased as opposed to an increased TP concentration observed the C vs. the R samples.

In the present research, the measured TP concentrations in all samples were higher than those previously reported for *M. charantia* (Budrat and Shotipruk 2008; Kubola and Siriamornpun 2008; Myojin et al. 2008; Wu and Ng 2008; Horax et al. 2010; Ibrahim et al. 2010; Ng et al. 2011; Sahib et al. 2011) (Table 4.8). Variability in TP content of *M. charantia* reported within the literature can be ascribed to various extraction techniques and solvents, concentration, cultivar, shipping and storage time as well as duration and temperature of heat treatment (Horax et al. 2005; Budrat and Shotipruk 2008; Kubola and Siriamornpun 2008; Ibrahim et al. 2010; Ng et al. 2011). Also, the FC assay is not specifically sensitive to phenolics because other compounds such as AA and copper complexes are able to reduce it (Huang et al. 2005).

Ascorbic acid oxidase (AO) is a copper(II) containing enzyme (Sekiya et al. 1990) and may partly explain why TP concentrations in all treatments in the current study, especially in the R sample, are higher than those reported in the literature. There was extensive time between the *M. charantia* harvest and the various steps of processing (cutting, blenderizing, cooking, digestion, freezing and freeze drying) (Wang et al. 2007; Myojin et al. 2008; Munyaka et al. 2010) in the present work. Members of the Cucurbitaceae family (cucumber) are known to contain large quantities of AO in the peel tissue (Nakamura et al. 1968). AO may itself have specific reducing effects on FC reagent due to its copper content (Huang et al. 2005), also, it is active at ambient temperatures in fresh broccoli samples and is the main enzyme responsible for the oxidation of AA to DHAA (Munyaka et al. 2010).

AA or DHAA can both react with the FC reagent and their presence should be separately quantified and adjusted to reduce interference and increase the

accuracy of TP measurements because if they are present in large enough quantities they can contribute to an overestimation of TP content (Singleton et al. 1999). In the current study, this interference was not taken into consideration, however, the other *M.charantia* studies cited have not reported specifically on either AA (Budrat and Shotipruk 2008; Ng et al. 2011) or DHAA content (Myojin et al. 2008) in *M. charantia*, so there is little comparative evidence.

Protocatechuic acid (3,4-dihydroxybenzoic; PA), which was the phenolic seen in the highest concentrations in both the raw and cooked forms (Table 4.1), showed relatively lower concentrations when *M. charantia* was subjected to cooking and digestion. Purified PA has been found to be equally effective at preventing lipid peroxidation both at ambient temperature and at 90˚C (Marinova et al. 2002) suggesting heat alone may not negatively impact PA in its purified form. However, our results are suggestive that enzymatic digestion degrades PA when *M. charantia* has been subjected to cooking.

In contrast to PA, gallic acid (GA) was associated with a major increase in concentration following digestion of both the raw and cooked forms of *M. charantia*. The relatively high concentrations of GA in the C + D and R + D treatment groups is in accord with previous findings that GA is the phenolic found in the highest concentration in *M. charantia*, specifically when heat was utilized in the extraction process (Kubola and Siriamornpun 2008; Ibrahim et al. 2010). Tagliazucchi et al. (2010) found that after *in vitro* pancreatic digestion, a pure GA standard decreased in concentration by > 40%, however, its ABTS scavenging capacity increased by 18%. A similar increase in ABTS activity was noted in the current research; however, it was coupled with a significant increase in GA concentrations after pepsin and pancreatic *in vitro* digestion of *M. charantia.* The difference between the two studies may involve the solubility and subsequent *in vitro* enzymatic metabolism of a pure extract of GA vs. the synergistic interaction of polyphenols, organic acids and soluble fibre metabolism and their interaction with GA during *in vitro* pepsin and pancreatic digestion in *M. charantia*. The GA concentrations observed in the R (1.36 ± 0.03 µg /g) or C (17.97 ± 0.29 µg /g) treatments in the present study are lower than those seen in other studies utilizing

cold or heat extraction techniques, which ranged from 100 – 510 µg/g DW, respectively (Horax et al. 2005; Budrat and Shotipruk 2008). In the current study, the GA concentrations in the R + D (105.2 ± 3.15 µg/g) and C + D (158.3 ± 1.44 µg/g) samples were more in keeping with the latter studies. After supplementation of GA in the diets of sheep, only 25 – 50 % of GA was excreted in urine as resorcinol glucoronide based on ruminal or abomasal degradation, indicating that the majority of ingested GA was metabolized (Murdiati et al. 1992). Urinary metabolites of GA in humans include 4-*O*-methylgallic acid, 3-*O*-methylgallic acid, and 3,4-*O*-dimethylgallic acid corresponding to about 1.5 mg/day urinary excretion of total gallic acid ingested (Hodgson et al. 2000). Other authors reported only 4-*O*-methylgallic acid and natural GA in urine and plasma after a 50 mg oral dose in humans (Shahrzad and Bitsch 1998).

The small intestine, mainly the duodenum and jejunum, is the primary site of enzymatic nutrient absorption and metabolism within the proximal intestine and yields the bulk of plasma and urinary metabolites (Serafini et al. 1998). In the current study a significant increase of GA content was observed after *in vitro* gastric and pancreatic digestion, which suggests that GA can be further metabolized and may nourish the colonic microflora and provide an energy supply as observed when ruminant diets are supplemented with GA (Getachew et al. 2008). GA has also been shown to have α-amylase and trypsin inhibiting activities (Rohn et al. 2002).

In the present work digestion was associated with the greatest VA concentrations in the R + D samples of *M. charantia*. Other researchers have found that heat treatment of ginseng (98-100˚C) was shown to increase VA concentration, as well as individual phenolic concentrations, however, no further increases in VA were noted as temperatures reached 120˚C (Kang et al. 2006). In the present research, there was a significant decrease in VA concentrations between the R + D and C + D samples. VA was significantly, negatively correlated with FRAP (r = 0.31) and ABTS (r = 0.36). VA shared a significant, positive correlation with GA (r = 0.58) and AA (r = 0.50).

In the present study higher concentrations of AA were observed after digestion of cooked *M. charantia*, which is indicative of effects of acidification and digestive enzymatic activity on AA availability. The above findings are supported by the findings of Munyaka et al. (2010) who found that inactivation of AO was achieved by cooking crushed samples (broccoli) above 70˚C for 10 min to prevent the degradation of AA to DHAA. Analysis of AA content in *Phaseolus vulgaris* (green beans) found that AA increased by 34% after blanching and freezing, compared to raw samples, while TAA (total ascorbic acid; AA + DHAA) remained the same (Baardseth et al. 2010).

In the current study, samples proceeding through *in vitro* gastric digestion were acidified to pH 1.5 that may have contributed to the stabilization of AA concentration (Wechtersbach and Cigic 2007; Novakova et al. 2008) and is indicated by > 1800% increase in AA concentrations in the R + D vs. the R treatments and > 700 % increase in AA concentrations in C + D vs. the C treatments (Table 4.1). In the present study, AA content in the raw samples was substantially lower than previous measurements showing the content of AA in *M. charantia* up to 1.52 mg/ g (fresh weight) up to 4 days post harvest (Meerabai et al. 2007). Reports suggest that AA and DHAA content of whole *M. charantia* fruits are relatively stable at storage temperatures between 5-10˚C (Tatsumi et al. 2006) which suggests that if properly handled, nutritional quality in terms of AA content can be maintained for a number of days post harvest.

In the current study, UV filters were fitted over fluorescent light bulbs, limiting exposure to light - however, exposure to air is difficult to eliminate and accelerates the oxidative process, especially once the fruits are cut (Wang et al. 2007; Novakova et al. 2008). Wang et al. (2007) found that AA content of fresh *M. charantia* decreased from 94 – 21 mg/ 100g FW or roughly 78% after cutting and storing for 5 days at 10˚C whereas storage at 2˚C for up to 7 days produced no significant chilling injury.

Freezing *M. charantia* at -40˚C degrades AA content in both fresh (79.7 ± 14.6 - 70.3 ± 6.3 mg/100g) and blanched samples (54.6 ± 3.0 - 46.6 ± 6.0 mg/100g) from day 0 (fresh) to day 150 (Myojin et al. 2008). In the present study, digested

samples were adjusted to an end pH of 2 as it was suggested that an acidic pH would also protect polyphenolic content during frozen storage (Tagliazucchi et al. 2010). AA acid content in gastric secretions maintains stability after freezing with liquid nitrogen and frozen storage for up to 4 weeks (Schorah et al. 1991). DHAA, which was not quantified in the present research, can represent a large proportion of total AA content especially after storage and processing (Davey et al. 2000). DHAA contributes to the overestimation of TP concentrations, as does AA and AO (Singleton et al. 1999).

To our knowledge, this is the first study to quantify fumaric acid (FA) or malic acid (MA) in *M. charantia*. MA was not quantifiable in the R + D and C + D treatments as significant decreases in MA were observed after digestion treatment of the raw and cooked forms, which indicates concentrations of MA have no nutritional consequence after digestion of *M. charantia* samples in the current work. Conversely, FA was non-detectable in the R and C samples whereas significant increases in FA in raw and cooked *M. charantia* were observed after *in vitro* digestive treatments indicating that cooking *M. charantia* might improve the ability of digestive enzymes to breakdown the insoluble fibre matrix of foods (Tagliazucchi et al. 2010; Bouayed et al. 2011). The effects of organic acids on gut health have been well documented. In the case of FA, digestion may be the key determinant in its beneficial properties, which include enhanced protein digestibility (Mroz et al. 1997), acidification of the proximal lumen (Dibner and Buttin 2002) and decreased lactic acid production (Blank et al. 2001).

To our knowledge, no study has looked at the effects of digestion on antioxidant capacity in *M. charantia*. In the present study, FRAP values were the most concentrated in the raw *M. charantia* samples. However, an increase in FRAP antioxidant capacity was noted in the C + D treatment compared to the C sample, which had comparable values to the R sample (Table 4.2). This observation might indicate that FRAP values may be over-estimated when cooking or digestive enzyme action is not considered. Higher pH values are associated with increased FRAP values (Tagliazucchi et al. 2010). In the current study, initial pH measurements of raw *M. charantia* were over 11 and the digested

samples were acidified to pH 1.5. Tagliazucchi et al. (2010) reported increases in both FRAP and ABTS scavenging after gastric digestion of red grape, which was followed by an initial decrease at the baseline measurement of pancreatic digestion as the digestive milieu gained alkalinity. By the end of pancreatic digestion, ABTS values had significantly increased, whereas FRAP values did not (Tagliazucchi et al. 2010). The concomitant increase in alkalinity between the phases of gastric and pancreatic digestion is purported to affect the radical scavenging abilities of polyphenols due to changes in structure such as altered ionization of the hydroxyl groups (Tagliazucchi et al. 2010). Tagliazucchi et al. (2010) reported significant strong correlation between TP and FRAP values during gastric digestion (r = 0.993) and pancreatic digestion (r = 0.999) of grapes. In the current research, a similar positive correlation was noted between FRAP and TP. In contrast, increases in *M. charantia* TP concentrations and antioxidant capacity were not well correlated based on DPPH radical scavenging assay (Horax et al. 2010). Previous studies have noted that tests of antioxidant capacity can have low correlations with some plant products due to the varying quantities of phenolics present and their diverse capabilities at radical scavenging (Karamac et al. 2005) as well as interferences from non-phenolic compounds (Singleton et al. 1999).

In agreement with the decrease in FRAP antioxidant activity after digestion of raw samples seen in the present work, Bouayed et al. (2011) observed an overall reduction in FRAP values after gastric and pancreatic digestion of four apples varieties (raw). In the current work, the decrease of FRAP antioxidant capacity and TP content in the C samples followed by an increase in the C + D samples could be indicative of a greater release of bound polyphenols seen when cooking (Ng et al. 2011) is followed by digestion (Saura-Calixto et al. 2010). After gastric digestion, Bouayed et al. (2011), observed that the ABTS scavenging ability of apples was reduced, but increased after the pancreatic phase of digestion, which compares with the present finding of increased ABTS activity after both pepsin and pancreatin enzyme digestion. The capacity of individually assessed, pure standards of GA, caffeic acid, catechin, quercetin and resveratrol to

scavenge the ABTS radical were increased after pancreatic digestion, compared to a baseline measurement prior to gastric digestion (Tagliazucchi et al. 2010). Further, *in vitro* colonic digestion released more total phenolics in maize and the bound phenolics that were extracted were 8 times more concentrated than the free phenolics (Kljak et al. 2009). The action of digestive enzymes and intestinal microbes on plant fibre contributes to the physiological metabolism of polyphenols and their metabolites in the proximal intestine and the colon (Saura-Calixto et al. 2000; Saura-Calixto et al. 2010). For instance, Serra et al. (2012) report protocatechuic acid as the main colonic metabolite after *in vitro* quercetin digestion, however, metabolites of pure protocatechuic acid were phenylacetic acid and *p*-hydroxybenzoic acid. Pure GA is metabolized to phenylacetic acid and *p*-hydroxy benzoic acid after *in vitro* colonic digestion (Serra et al. 2012). In humans, 4-*O*-methylgallic acid, 3-*O*-methylgallic acid, and 3,4-*O*-dimethylgallic acid were the main urinary metabolites after tea ingestion (Hodgson et al. 2000). Bound phenolics, such as catechin gallate esters, need further metabolism in the colon and may protect the epithelial cells from oxidative damage while contributing to the SCFA concentrations (Saura-Calixto et al. 2007).

It is possible that the relatively strong ABTS antioxidant capacity measures noted in the current study might be related to the lyophilization and storage of samples at -40˚C until use. Myojin et al. (2008) attributed increased losses of TP and radical scavenging to the continuing enzymatic degradation of phenolic compounds at higher freezing (-18˚C) temperatures in the raw sample which was prevented by blanching *M. charantia* (Myojin et al. 2008). Freezing *M. charantia* at -40˚C improved the radical scavenging capacity of DPPH over a 5-month period with only minor decreases in TP content (Myojin et al. 2008). In the current research, freeze dried *M. charantia* was extracted after 4 weeks of freezing at -40˚C. The extracts were stored for up to 3 months at -20˚C. Freeze drying *M. charantia* may contribute to the breakdown of the fibre matrix in plant foods, especially in combination with blanching, which would explain the improvements in TP concentrations and radical scavenging. All samples were freeze dried prior to extraction in the current study; however, heating *M. charantia* alone was not

associated with altered antioxidant capacity measures or total phenolics. Previous studies show contrasting findings regarding the effects of heat treatment on antioxidant capacity measures of *M. charantia.* Ng et al. (2011) found that boiling *M. charantia* more than doubled the FRAP antioxidant values although they reported no significant differences between raw and boiled samples for DPPH or ABTS assays. Budrat et al. (2009) found that the antioxidant activity of *M. charantia* as measured by ABTS decreased in the heated extract (200˚C, SWE). In the current study, it was observed that FRAP values decreased after heat treatment -; however, ABTS values increased, which is contrary to the latter reports. In the present study, the adverse effect of heat treatment on FRAP values might be related to the relatively low heat (100˚C) applied for a short duration of 3 min used during stir fry cooking in comparison to previous studies that used higher temperatures or longer cooking times. In the current study we attempted to simulate cooking conditions that would traditionally be used in the preparation of *M. charantia* for human consumption, which is typically stir-frying, for 3-4 min (Virdi et al. 2003; Fonseka et al. 2007; Ng et al. 2011). In the present work, FRAP activity decreased after cooking whereas heat extraction has previously been shown to increase the free radical scavenging ability of *M. charantia* >80%, based on DPPH (Ansari et al. 2005). Ng et al. (2011) found that boiling increased the FRAP value > 200% compared to the raw extract of *M. charantia* but reported no significant differences between raw and boiled samples for DPPH or TEAC (ABTS) assays. The relatively low cooking temperatures (100˚C), uneven heat distribution and shorter cooking time in the present study could account for the differences in FRAP antioxidant capacity observed between studies.

In the current research TP concentrations initially decreased between the R and C samples, but again increased when the cooked *M. charantia* samples underwent pepsin-pancreatin digestion. This finding provides further support for the concept of a release of polyphenolic compounds from the fibre matrix after enzymatic digestion (Tagliazucchi et al. 2010; Bouayed et al. 2011). The current research is similar to the findings of Perez-Vicente et al. (2002) who reported no change of TP content (in pomegranate juice) after *in vitro* gastric digestion but

showed increases after pancreatic digestion. Several studies have indicated that the non-extractable polyphenolic content in foods comprises the bulk of dietary polyphenols and many of these are intact when they reach the colon (Arranz et al. 2010).

AA concentrations increased significantly after *in vitro* digestion of the cooked samples of *M. charantia*, which is in apparent contradiction to previous work showing a greater than 80% loss of AA after gastric and pancreatic digestion of pomegranate juice (Perez-Vicente et al. 2002). Myojin et al. (2008) found that both raw and blanched (boiling for 4 min) *M. charantia* suffered significant losses of AA during frozen storage at -40˚C. In the current study, it is possible that the effect of heating *M. charantia* at 100˚C together with the digestion process that acidified the sample may have inactivated AO and thus contributed to AA stabilization in the digested samples (Wechtersbach and Cigic 2007; Novakova et al. 2008; Munyaka et al. 2010). There is evidence that absorption and bioavailability of certain polyphenols (such as catechins) are enhanced by the presence of AA (Peters et al. 2010), which underline the synergistic effects of nutritional compounds towards increased bioavailability and bioactivity. Further research is needed to determine whether such synergistic benefits occur between the relatively high concentrations of AA and polyphenols present in *M. charantia*.

It should, then, be recommended that *M. charantia* be eaten as close to harvest as possible (Meerabai et al. 2007), cooked, without water, for approximately 10 min at a temperature ranging from 80 – 100˚C (Munyaka et al. 2010; Ng et al. 2011) to preserve AA content and encourage maximal polyphenol release (Saura-Calixto et al. 2000).

In summary, cooking and digestion of *M. charantia* was unique to the present study and is a more physiologically relevant mechanism by which to estimate the metabolic action of certain foods *in vivo*. FRAP antioxidant capacity improved after *in vitro* digestion after an initial decline after cooking alone. ABTS radical scavenging improved after *in vitro* digestion in both raw and cooked samples. Digestion was a major contributor to the release or stabilization of certain polyphenols and organic acids. Although raw *M. charantia* contained the greatest

concentration of TP, the significant increase of TP between the C and C + D sample would indicate that *in vitro* or *in vivo* studies should utilize the cooked fruits to more accurately assess phenolic content relevant to typical human consumption. Enzymatic digestion also contributed to higher GA, AA and FA concentration, which are important contributors to gut health in conjunction with their individual or synergistic roles as antioxidants.

Table 4.1 Mean organic acid and phenolic acid concentration based on high performance liquid chromatography including significant main effects of 2-Way ANOVA.

	Treatment[1]				Significance of main effects of ANOVA[3]		
Organic Acid	R	C	R + D	C + D	C	D	C X D
	FDW[2]						
Ascorbic Acid	2.25 ± 0.07	6.33 ± 0.09	43.73 ± 0.9	55.1 ± 0.17	NS	< 0.0001	NS
Malic Acid	40.77 ± 0.7	58.75 ± 1.27	0 ± 0.0	0 ± 0.0	NS	< 0.0001	NS
Fumaric Acid	0 ± 0.0	0 ± 0.0	786500 ± 4922	1008056 ± 8010	NS	< 0.0001	NS
Phenolic Acid							
Gallic Acid	1.36 ± 0.03	17.97 ± 0.29	105.2 ± 3.15	158.3 ± 1.44	NS	< 0.0001	NS
Protocatechuic Acid	18.13 ± 0.58	43.24 ± 0.66	16.97 ± 0.4	6.81 ± 0.09	NS	< 0.0001	NS
Catechin	0.11 ± 0.004	0.15 ± 0.002	0.15 ± 0.003	0.08 ± 0.001	NS	NS	NS
Chlorogenic Acid	0.04 ± 0.002	0.03 ± 0.002	0.003 ± 0.002	0.1 ± 0.004	NS	NS	NS
Vanillic Acid	0.18 ± 0.006	0.62 ± 0.02	11.2 ± 0.39	1.16 ± 0.02	NS	0.004	NS
o-coumaric Acid	0.12 ± 0.003	0.21 ± 0.004	0.02 ± 0.001	0.05 ± 0.002	NS	0.0002	NS
t-cinnamic Acid	0.25 ± 0.007	0.09 ± 0.001	0.04 ± 0.002	0 ± 0.0	NS	0.0008	NS

[1] Samples were analyzed in the raw (R) state, after cooking (C), after digestibility treatment (R + D) and after cooking and digestibility treatments (C + D).
[2]All values are least squares means ± SEM with n = 10 for all variables. Phenolics expressed as µg/ g based on FDW. Organic acids expressed as µg/g based on freeze dried weight (FDW).
[3]Effects were considered significant if $p < 0.05$ as determined by two-way ANOVA. NS = not significant.

Table 4.2 Mean TP, FRAP and ABTS (hydrophilic) concentrations based on high performance liquid chromatography including significant main effects of two-way ANOVA.

	Treatment[1]				Significance of main effects of ANOVA[3]		
	FDW[2]						
Assay	R	C	R + D	C + D	C	D	C x D
TP*	12185 ± 38.82	8671.5 ± 52.09	10185.8 ± 51.08	10608.5 ± 40.94	NS	0.0006	NS
FRAP $^{\alpha}$	30.52 ± 0.39	14.87 ± 0.24	11.94 ± 0.07	24.72 ± 0.14	NS	< 0.0001	NS
ABTS^{+}	0.69 ± 0.0009	0.61 ± 0.001	0 ± 0.0	0 ± 0.0	NS	< 0.0001	NS

[1] Samples were analyzed in the raw (R) state, after cooking (C), after digestibility treatment (R + D) and after cooking and digestibility treatments (C + D).
[2]All values are least squares means ± SEM with n = 40 for all variables based on FDW. *Total phenolics values expressed as mg GAE /g. $^{\alpha}$ FRAP values are expressed as µM $FeSO_4$ equivalent/g. $^{+}$ABTS values are expressed as µg TE /g.
[3]Effects were considered significant if $p < 0.05$ as determined by two-way ANOVA.
NS = not significant.

Table 4.3 Effects of cooking and digestibility treatments on phenolics, organic acids, TP, FRAP and ABTS (hydrophilic) assays.

	Significance of pre-planned contrasts			
	Treatments[1]			
Organic Acid	**R x C**	**R x R + D**	**C x C + D**	**R + D x C + D**
Ascorbic Acid	NS	< 0.0001	< 0.0001	NS
Malic Acid	NS	0.002	< 0.0001	NS
Fumaric Acid	NS	< 0.0001	< 0.0001	0.01
Phenolic Acid				
Gallic Acid	NS	0.0009	< 0.0001	NS
Protocatechuic Acid	0.004	NS	< 0.0001	NS
Catechin	NS	NS	NS	NS
Chlorogenic Acid	NS	NS	NS	NS
Vanillic Acid	NS	0.006	NS	0.013
o-coumaric Acid	NS	NS	0.002	NS
t-cinnamic Acid	0.03	0.003	NS	NS
Assay				
TP	< 0.0001	0.02	0.03	NS
FRAP	0.0003	< 0.0001	0.03	0.003
ABTS	< 0.0001	< 0.0001	< 0.0001	NS

[1] Samples were analyzed in the raw (R) state, after cooking (C), after digestibility treatment (R + D) and after cooking and digestibility treatments (C + D).

Table 4.4 Correlation coefficients between selected phenolics.

	Phenolic Acid				
Phenolic Acid	**Gallic Acid**	**Protocatechuic Acid**	**Vanillic Acid**	***o*-coumaric Acid**	***t*-cinnamic Acid**
Gallic Acid	-	-	0.58 Significant	-0.39 Significant	-0.40 Significant
Protocatechuic Acid	-0.28 Non-significant	-	-0.01 Non-significant	0.70 Significant	0.40 Non-Significant
Vanillic Acid	-	-	-	-0.29 Non-significant	-0.12 Non-significant
***o*-coumaric Acid**	-	-	-	-	0.43 Significant

Pearson's correlation was tested between organic acids and phenolics based on quantification using high performance liquid chromatography. Significance level is set a $p < 0.05$. Results were expressed as correlation coefficients and whether there was a significant association or not.

Table 4.5 Correlation coefficients between selected phenolics and organic acids.

	Phenolic Acid				
Organic Acid	**Gallic Acid**	**Protocatechuic Acid**	**Vanillic Acid**	***o*-coumaric Acid**	***t*-cinnamic Acid**
Ascorbic Acid	0.88 Significant	-0.26 Non-significant	0.50 Significant	-0.46 Significant	-0.38 Significant
Malic Acid	-0.53 Significant	0.47 Significant	-0.25 Non-significant	0.59 Significant	0.24 Non-significant
Fumaric Acid	0.70 Significant	-0.47 Significant	0.25 Non-significant	-0.55 Significant	-0.49 Significant

Pearson's correlation was tested between organic acids and phenolics based on quantification using high performance liquid chromatography. Significance level is set a $p < 0.05$. Results were expressed as correlation coefficients and whether there was a significant association or not.

Table 4.6 Correlation coefficients between selected phenolics, TP, FRAP & ABTS (hydrophilic) assays.

	Phenolic Acid				
Assay	**Gallic Acid**	**Protocatechuic Acid**	**Vanillic Acid**	***o*-coumaric Acid**	***t*-cinnamic Acid**
TP	0.13 Non-significant	-0.21 Non-significant	-0.20 Non-Significant	0.15 Non-significant	0.33 Significant
FRAP	-0.12 Non-Significant	-0.06 Non-significant	-0.31 Significant	0.11 Non-significant	0.41 Significant
ABTS	-0.74 Significant	0.43 Significant	-0.36 Significant	0.54 Significant	0.52 Significant

Pearson's correlation was tested between phenolic acids and TP, FRAP and ABTS based on quantification using high performance liquid chromatography. Significance level is set a $p < 0.05$. Results were expressed as correlation coefficients and whether there was a significant association or not.

Table 4.7 Correlation coefficients between organic acids, TP, FRAP & ABTS (hydrophilic) assays.

	Organic Acid		
Assay	**Ascorbic Acid**	**Fumaric Acid**	**Malic Acid**
TP	-0.15	0.30	-0.24
	Non-significant	Non-significant	Non-significant
FRAP	-0.14	-0.12	0.29
	Non-Significant	Non-significant	Non-significant
ABTS	-0.84	-0.94	0.73
	Significant	Significant	Significant

Pearson's correlation was tested between organic acids and TP, FRAP and ABTS based on quantification using high performance liquid chromatography. Significance level is set a $p < 0.05$. Results were expressed as correlation coefficients and whether there was a significant association or not.

Table 4.8 Summary of polyphenolic and ascorbic acid concentrations and antioxidant capacity of *M. charantia* reported in the literature and relevant to current study.

Compound quantified	**Literature**		
	Amount reported	**Part used**	**References**
Total Phenolics	5.39 ± 0.06 – 7.75 ± 0.07 mg CAE/mg	flesh, with peel	Horax et al. (2005)*
	51.6 – 68.8 mg GAE/g DW	flesh, with peel	Wu and Ng (2007) $^{\Delta}$
	324 ± 1.63 mg GAE/g DW	flesh, with peel	Kubola et al. (2008)
	226.9 ± 7.7 (unblanched) – 263.3 ± 9.5 (blanched) µM GAE/100g FW	flesh, with peel	Myojin et al. (2008)˜
	52.63 mg GAE/g (SWE) vs. 6mg GAE/g (MeOH) DW	flesh, with peel	Budrat et al. (2009) º
	3.51 ± 1.1 g GAE/100g DW	flesh, with peel	Sahib et al. (2009)
	14.8 ± 0.3 mg GAE/g DW	flesh, with peel	Horax et al. (2010)
Gallic Acid	10.23 ± 0.21- 20.66 ± 0.29 mg/100g DW	flesh, with peel	Horax et al. (2005)
	95.6 ± 0.97 mg/L DW	flesh, with peel	Kubola et al. (2008)
	0.51 (SWE) vs. 0.02 (MeOH) mg/g DW	flesh, with peel	Budrat et al. (2009)
	48.8 ± 1.4 mg/100g DW	flesh, with peel	Horax et al. (2010)
	26.7 ± 0.73 mg/100g DW	flesh, with peel	Ibrahim et al. (2010)
Protocatechuic Acid	2.07 ± 0.18 – 6.14 ± 0.23 mg/100g DW	flesh, with peel	Horax et al. (2005)
	11.8 ± 2.8 mg/100g DW	flesh, with peel	Horax et al. (2010)

Table 4.8 Continued

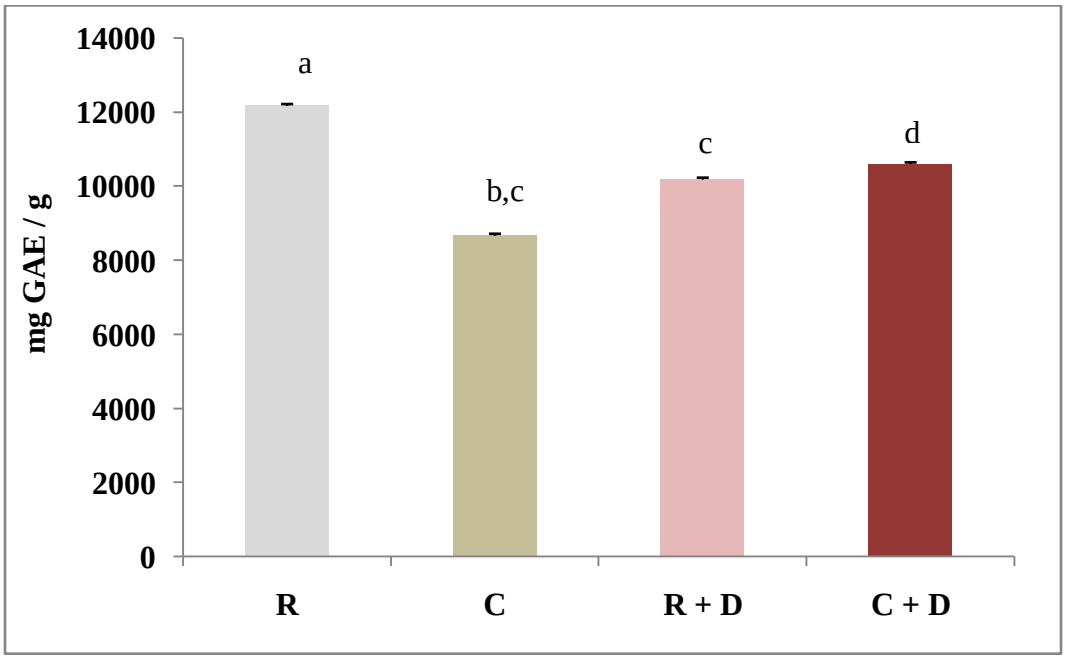
Compound quantified	Literature		
	Amount reported	**Part used**	**References**
Catechin	68.16 – 82.45 mg /100g DW	flesh, with peel	Horax et al. (2005)
	3.95 ± 0.52 mg/L DW	flesh, with peel	Kubola et al. (2008)
	47.34 mg/g DW	flesh, with peel	Budrat et al. (2009)
	154.5 ± 7.2 mg/100g DW	flesh, with peel	Horax et al. (2010)
Chlorogenic acid	6.42 ± 0.10 – 14.15 ± 0.25 mg/100g DW	flesh, with peel	Horax et al. (2005)
	0.12 mg/g DW	flesh, with peel	Budrat et al. (2009)
	66.4 ± 5.4 mg/100g DW	flesh, with peel	Horax et al. (2010)
Vanillic acid	1.84 ± 0.1 – 2.42 ± 0.09 mg/100g DW	flesh, with peel	Horax et al. (2005)
	9.3 ± 2.1 mg/100g DW	flesh, with peel	Horax et al. (2010)
***o*-coumaric acid**	1.96 ± 0.12 – 2.43 ± 0.02 mg/100g DW	flesh, with peel	Horax et al. (2005)
	26.8 ± 5.9 mg/ 100g DW	flesh, with peel	Horax et al. (2010)
	9.93 ± 1.2 mg/100g DW	flesh, with peel	Ibrahim et al. (2010)
***t*-cinnamic acid**	1.31 ± 0.04 mg/100g DW	flesh, with peel	Horax et al. (2005)
	8.5 ± 1.7 mg/100g DW	flesh, with peel	Horax et al. (2010)
Ascorbic acid	83 mg/100g FW	flesh, with peel	Tatsumi et al. (2006)
	152 mg/100g FW	flesh, with peel	Meerabai et al. (2007)
	94 mg/100g FW	flesh, with peel	Wang et al. (2007)
	54.6 ± 3.0 – 79.7 ± 14.6 mg/100g FW	flesh, with peel	Myojin et al. (2008)
	66000 ± 141.42 ppm	flesh, with peel	Bakare et al. (2010)

Table 4.8 Continued

Compound quantified	Literature		
	Amount reported	**Part used**	**References**
Dehydroascorbic acid	16mg/100g FW	flesh, with peel	Tatsumi et al. (2006)
FRAP	43.8 ± 0.008 µmol FeSO4/g	flesh, with peel	Kubola et al. (2008)
	2.2 ± 0.1 (raw) – 8.2 ± 0.7 (boiled) mmol FE/100g	flesh with peel	Ng et al. (2011)
ABTS	IC 50 5.49 ± 0.54 (SWE) vs. 17.34 ± 2.45 (EtOH) µg/ml	flesh with peel	Budrat et al. (2009)
	3.3 ± 0.9 (raw) – 3.5 ± 0.8 (boiled) mmol TE/100g	flesh with peel	Ng et al. (2011)
	IC 50 98.1 ± 2.4 - 204.5 ± 1.2	leaves, flesh (isolated compounds)	Lin et al. (2011)
DPPH	5 -88 % inhibition	flesh, with peel	Ansari et al. (2005) †
	60% inhibition	flesh, with peel	Wang et al. (2007)
	IC50 129.94 - 156.78 µg /ml)	flesh, with peel	Wu and Ng (2007)
	81.4 ± 0.31 % inhibition	flesh, with peel	Kubola et al. (2008)
	431.2 – 536.6 µmol TE/100g FW	flesh, with peel	Myojin et al. (2008)
	EC50 12.6 ± 0.5 mg extract/mg DPPH	flesh, with peel	Horax et al. (2010)
	82.8% ± 0.57 inhibition	flesh, with peel	Ibrahim et al. (2010)
	3.4 ± 2.0 – 5.3 ± 0.8 % inhibition	flesh, with peel	Ng et al. (2011)

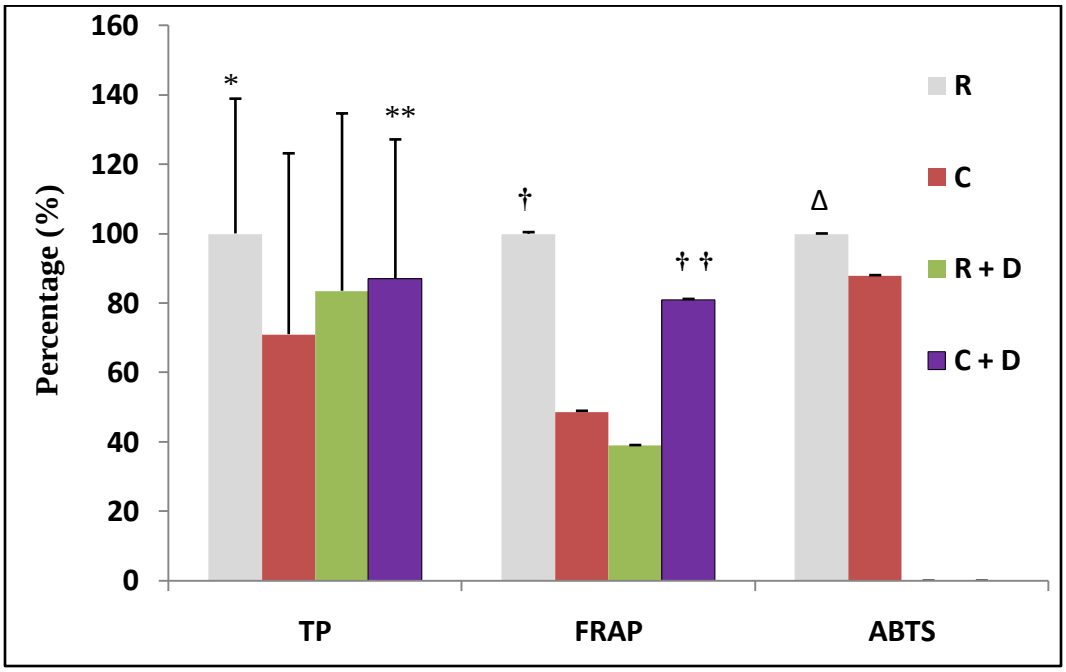
* TP reported in CAE (chlorogenic acid equivalents). Values are means ±SD. Phenolic amounts reported are for oven dried samples in the 4 cvs. *o*-coumaric acid in cvs. IG and IW non- detectable. *t*-cinnamic acid trace amounts only in IG, IW and CW cvs.
$^{\Delta}$TP and DPPH reported for both aqueous and EtOH extracts of wild *M. charantia* Linn. var. *abbreviata* Ser.
†Based on traditional solvent extraction or heat extraction under reflux.
˜ Blanched compared with unblanched *M. charantia.*
º Values reported for SWE extracts at 200˚C and EtOH extraction.
** Values reported for raw and boiled samples only. TEAC value reported as ABTS.

Figure 4.1 Total phenolic concentration in *Momordica charantia*



Differences were tested between 4 treatments (raw [R]; cooked [C]; R + digested [D]; and C + D) based on mg GAE/g DW. Data are represented as lsmeans ± SEM. lsmeans having the same letters are not statistically different from each other.

Figure 4.2 Percentage (%) of recovery of total phenolics, FRAP and ABTS after cooking and *in vitro* digestion of *Momordica charantia.*



Data obtained after EtOH (80%) extraction of raw (R) samples is considered 100% of TP and antioxidant activity contained in *M. charantia* samples. Data shown represent lsmeans ± SEM. * R ($p < 0.0001$, $p < 0.02$) significantly different than cooked (C) and R + digested (D) (2-way ANOVA, Tukey's post hoc test). ** C + D ($p < 0.03$) significantly different than C (2-way ANOVA, Tukey's post hoc test). † R ($p < 0.0003$, $p < 0.0001$) significantly different from C and R + D (2-way ANOVA, Tukey's post hoc test). †† C + D ($p < 0.03$, $p < 0.003$) significantly different from C and R + D (2-way ANOVA, Tukey's post hoc test). $^{\Delta}$ R ($p < 0.0001$, $p < 0.0001$) significantly different from C and R + D (2-way ANOVA, Tukey's post hoc test). C + D ($p < 0.0001$) significantly different from C (2-way ANOVA, Tukey's post hoc test).

V. SUMMARY AND CONCLUDING REMARKS

As diabetes rates climb across the continents (Wild et al. 2004), it is important to retain the knowledge of traditional healing systems to ameliorate the high cost of pharmaceutical treatments, not only on healthcare systems, but on the individual (Pedersen and Baruffati 1985; Cunningham-Murie et al. 2008). The health and nutritional benefits of *Momordica charantia*, especially as an antidiabetic medicinal food have been empirically documented (Srivastava et al. 1993; Virdi et al. 2003; Fonseka et al. 2007; Roffey et al. 2007). Its use in TCM and Ayervedic medical systems is a good indication of its purported effectiveness (Virdi et al. 2003; Shih et al. 2009). However, not much is currently known about the effects of cooking or digestion on *M. charantia* fruit, even though it is most commonly eaten cooked. Studies which have examined *M. charantia* after heat extraction techniques have reported increases in TP and individual phenolics, including GA as well as ABTS scavenging capacity (Budrat and Shotipruk 2008). FRAP values were increased after boiling *M. charantia* fruit (Ng et al. 2011). Other researchers report decreases in TP and AA concentrations as well as decreased DPPH scavenging capabilities after blanching *M. charantia* fruit (100˚C, 4 min) (Myojin et al. 2008) or no change in ABTS scavenging when *M. charantia* fruit was boiled (100˚C, 5 min) (Ng et al. 2011). Studies which have looked at human consumption of *M. charantia* (dried powder) have examined its glycemic index (Karthika 2006). Other authors have examined the effects of *M. charantia* extracts on specific processes of *in vitro* digestion such as α-amylase and α-glucosidase inhibition, which inhibit carbohydrate metabolism in the proximal intestine (Fonseka et al. 2007).

Significant differences were observed for polyphenolic and organic acid content as well as antioxidant capacity in the current study. Cooking did not have a significant main effect on polyphenols, organic acids or antioxidant capacity – however it cannot be ruled out as a contributor to the breakdown of insoluble plant fibre making them more available to interact with digestive enzymes and colonic microbes (Saura-Calixto et al. 2010). In the current study increased GA, AA and

FA concentrations observed after *M. charantia* was cooked and digested would indicate that these compounds benefit from heat treatment in order to be available for enzymatic digestion, metabolism and absorption. In the current study, cooking temperatures (100˚C, 3 min) were designed to reflect the conditions under which *M. charantia* is usually prepared.

Budrat et al. (2009) found higher TP concentration at 200˚C extraction of *M. charantia* vs. lower temperatures. Also, Ng. et al. (2011) found that boiling increased the TP concentrations in *M. charantia*. In the current study, although TP concentrations were highest in the R samples they again increased after cooking and enzymatic digestion were applied to *M. charantia.* Physiologically, this also makes sense as dietary components access the digestive tract through the mouth, and many foods are cooked.

Ng et al. (2011) found that FRAP values increased in *M. charantia* after boiling (100˚C, 5 min) which may have improved the release of phenolic compounds from the fibre matrix (Saura-Calixto et al. 2010). The FRAP assay, assesses the redox potential of a food extract or pure compound (Benzie and Strain 1996; Benzie and Strain 1999). In the current research, the raw *M. charantia* extract was the most effective FRAP scavenger but digestion improved FRAP values over cooking alone. In the present study, lower cooking temperatures as compared to Ng et al. (2011) may not have been sufficient to release the maximum antioxidant phenolic compounds. In the current study, compounds that responded significantly to cooking and/or digestion (GA, AA, FA) were strongly, significantly, negatively correlated with decreased absorbance shown with the ABTS assay. In the ABTS assay, a dietary antioxidant donates a hydrogen ion and fuels a redox reaction producing a reduction in colour and decreasing absorbance is negatively correlated with increasing antioxidant activity (Re et al. 1999). Therefore, a significant negative correlation as in the case of GA, AA and FA, with ABTS, may indicate strong antioxidant activity of these compounds.

Digestion was a determining and significant main effect leading to changes in all parameters examined (except for CAT and CGA) in the current study. *In vitro* digestion increased GA, VA, AA, and FA concentrations as well as ABTS

absorbance, but decreased TP, PA, *o*-CA, *t*-CiA, MA and FRAP concentrations compared to the raw samples. Therefore, our original hypothesis is partially supported whereby certain antioxidant capacity measures, most phenolics and all organic acids (based on HPLC quantification) were affected significantly by digestion treatment.

The statement that the parent compound does not reach the bloodstream to exert physiological effects does not undermine the benefits of dietary phenolic compounds or organic acids (Olthof et al. 2003). As an example, dietary gallic acid is generally found in its simple esterified forms which are polymers of (+)-catechin or (-)-epicatechin units (Haslam and Cai 1994). (+)-catechin and (-)-epicatechin are stable during oral (synthetic saliva) and gastric digestion and have an absorption rate between 40 -80% (respectively) in the presence of Caco-2 cells (Laurent et al. 2007). Within the human colon, chlorogenic acid is converted to quinic and caffeic acids by the intestinal flora and is further metabolized within the liver and kidney (Olthof et al. 2003). GA is metabolized to phenylacetic acid and *p*-hydroxy benzoic acid during *in vitro* colonic digestion (Serra et al. 2012). Polyphenols and organic acids and their beneficial effects have been observed throughout the length of the gastrointestinal tract and their metabolites appear in the bloodstream and urine (Hodgson et al. 2000; Dibner and Buttin 2002; Olthof et al. 2003). The above findings support accumulating research that at all levels of the digestive process there is extensive metabolism of phenolics that alters phenolic profiles and ultimately determines the physiological impact of ingested phenolics (Deprez et al. 2001; Donovan et al. 2001).

The acidification of a food as it is mixed with the gastric digesta also confers protection and stabilizes certain polyphenols (Tagliazucchi et al. 2010). Ascorbic acid contributes to the acidification of the gastric environment as it is a component of gastric secretions (Schorah et al. 1991; Sobala et al. 1991). In the current study, AA concentrations increased after samples were subjected to enzymatic gastric and pancreatic digestion. It is possible that the effects of pH in the initial acidification of the digested samples could have contributed to the stabilization of AA and DHAA as these compounds are stable when held at a pH of

2 (Wechtersbach and Cigic 2007). Also, an inhibition of AO at temperatures above 90˚C could have played a role in maintaining AA content (Munyaka et al. 2010), however, their was no difference in AA content between the raw and cooked samples that underwent *in vitro* digestive action in the current study.

Temperature, pH, exposure to light and oxygen, the presence of metals as well as enzymatic action all affect the oxidation of AA to DHAA (Novakova et al. 2008; Baardseth et al. 2010) and this process is most prominent when a vegetable is in the raw state and at temperatures of 30˚C – 60˚C (Munyaka et al. 2010). *M. charantia* AA content is stable at 5 – 10˚C (Tatsumi et al. 2006) but once cut and stored for 5 days at 5˚C, AA content decreases by about 78% (Wang et al. 2007) underlining the importance of purchasing and consuming foods as close to harvest as possible.

In the current study the major phenolic compound in *M. charantia*, GA, increased significantly after gastric and pancreatic digestion in both the raw and cooked samples suggesting that GA would be available for further digestion by the colonic microflora. GA is extensively metabolized in the colon, as several of its metabolites (4-*O*-methylgallic acid, 3-*O*-methylgallic acid, and 3,4-*O*-dimethylgallic acid) appear in the urine of healthy subjects after tea consumption (Hodgson et al. 2000). Further studies are needed to determine whether there is significant systemic appearance of GA metabolites following ingestion of *M. charantia*. Although the thesis studies indicate digestive enzymatic processes appear to alter phenolic profiles of *M. charantia*, further studies can examine the impact of phenolic profiles on digestive enzymes. For example, GA can repress the activity of α-amylase as well as trypsin *in vitro* (Rohn et al. 2002). The repression of α-amylase and α-glucosidase has also been reported with addition of *M. charantia* extracts to foods, thus inhibiting carbohydrate metabolism *in vitro*, and contributing to its hypoglycemic effects *in vivo* (Fonseka et al. 2007). Hence it is possible that the increased release of GA following digestion of *M. charantia* seen in the present work, could thereby contribute as one of the multiple mechanisms associated with the hypoglycemic action of *M. charantia* (Chen et al. 2003; Fonseka et al. 2007; Roffey et al. 2007; Shih et al. 2008; Tan et al. 2008).

Soil composition significantly affects cultivation of *M. charantia*, with poultry manure yielding the most fruit, *Azospirillum* manure maximizing AA content and vermicompost contributing best to keeping quality, therefore, a combination is reported to be ideal (Meerabai et al. 2007). The above factors require further study in terms of their impact on the phenolic and organic acid profile and antioxidant capacity measures of *M. charantia*. Since it is easy to grow and needs little care, *M. charantia* would be an excellent crop for the home gardener as an addition to market foods. It is also found growing wild (Wu and Ng 2008) and is readily available at Asian markets throughout the year.

Future studies could involve the Dynamic Human Gastrointestinal Model that can assess the release of polyphenols at the various digestion compartments of intestinal digestion, which includes colonic bacterial digestion to determine the phenolic release from *M. charantia*. In addition to the phenolic content of *M. charantia*, future studies involving the study of *in vivo* and *in vitro* digestion could examine the impact of the high fibre content of *M. charantia* on colonic metabolism as it contains approximately 44% fibre on a dry weight basis (Bakare et al. 2010; Horax et al. 2010). Dietary fibre also changes the microbial environment of the colon, producing more beneficial short chain fatty acids (SCFA) that could improve insulin sensitivity (Jenkins et al. 1978; Jenkins et al. 1987; Saura-Calixto et al. 2010; Serra et al. 2012). Sun dried *M. charantia* has a low glycemic index (GI; 53.4) and an inverse relationship was found between the fibre content and GI (Karthika 2006). Soluble dietary fibre may play a larger role in glucose metabolism than insoluble fibre, providing dietary bulk and prolonging intestinal transit time and absorption (Jenkins et al. 1978). *M. charantia* contains about 14% soluble fibre (Horax et al. 2010). The fibre content of *M. charantia*, coupled with its phenolics to inhibit α-amylase and α-glucosidase (Fonseka et al. 2007) may function synergistically to enable *M. charantia* to exert beneficial effects for T2D.

The most important finding of the current study is the impact of digestion on total as well as individual polyphenolic content in *M. charantia*. As a widely eaten cooked vegetable, the physiological value of *M. charantia* fruit is not

correctly evaluated in studies utilizing either raw or undigested extracts in TP measurements or antioxidant status. GA was found to be the major phenolic after digestion of *M. charantia* and it is the most widely reported phenolic in the literature. Pure GA is a potent ABTS radical scavenger and there are likely synergistic effects between it and other dietary phenolics physiologically (Tagliazucchi et al. 2010). Another unique finding is the improved concentration of ascorbic acid and fumaric acid after digestion. GA, AA and FA contribute to the health of the gastrointestinal tract and coupled with the high total fibre content of *M. charantia* (Bakare et al. 2010; Horax et al. 2010) could contribute to overall gastrointestinal tract health as well as enhanced digestion. The importance of eating *M. charantia* fruit near to harvest is also indicated as AA concentrations decline with storage and processing (Wang et al. 2007; Myojin et al. 2008).

Cultivar affects phenolic concentration and *M. charantia* cultivars India White and China White had higher phenolic concentrations than either of the green varieties (India Green and China Green) examined (Horax et al. 2005). However, Fonseka et al. (2007) found that a green variety (*Matale Green*) exhibited more potent glucose lowering effects. Green *M. charantia* cultivars (H1, H14 hybrid, *Matale green*) also possessed stronger inhibitory activity on both α-amylase and α-glucosidase (Fonseka et al. 2007). It is prudent to conclude that cultivar significantly alters the nutritional content of *M. charantia* but specific cultivar is not necessarily reported in the literature. In the current study, a China green phenotype was utilized (cultivar unknown) and TP content was high as well as some individual phenolics. Nutritional benefits would be indicated for those who are able to grow this vegetable in a home garden. The major benefits of eating your medicine, as Hypocrates once wisely suggested, include reduced financial impact on health care systems as well as the individual as well as enhanced good health. Regular consumption of cooked *M. charantia* can help to ameliorate the symptoms of chronic disease, including T2D.

VI. REFERENCES

Akbari, C. M., Saouaf, R., Barnhill, D. F., Newman, P. A., LoGerfo, F. W. and Veves, A. (1998). "Endothelium-dependent vasodilatation is impaired in both microcirculation and macrocirculation during acute hyperglycemia." Journal of Vascular Surgery **28**(4): 687-694.

Ansari, N. M., Houlihan, L., Hussain, B. and Pieroni, A. (2005). "Antioxidant activity of five vegetables traditionally consumed by South-Asian migrants in Bradford, Yorkshire, UK." Phytotherapy Research **19**(10): 907-911.

Arranz, S., Silvan, J. M. and Saura-Calixto, F. (2010). "Nonextractable polyphenols, usually ignored, are the major part of dietary polyphenols: A study on the Spanish diet." Molecular Nutrition & Food Research **54**(11): 1646-1658.

Arts, I. C. and Hollman, P. C. (2005). "Polyphenols and disease risk in epidemiologic studies." Am J Clin Nutr **81**(1): 317S-325.

Asahi, K., Ichimori, K., Nakazawa, H., Izuhara, Y., Inagi, R., Watanabe, T., Miyata, T. and Kurokawa, K. (2000). "Nitric oxide inhibits the formation of advanced glycation end products." Kidney International **58**(4): 1780-1787.

Ashcroft, F. M., Harrison, D. E. and Ashcroft, S. J. H. (1984). "Glucose induces closure of single potassium channels in isolated rat pancreatic beta-cells." Nature **312**(5993): 446-448.

Baardseth, P., Bjerke, F., Martinsen, B. K. and Skrede, G. (2010). "Vitamin C, total phenolics and antioxidative activity in tip-cut green beans (Phaseolus vulgaris) and swede rods (Brassica napus var. napobrassica) processed by methods used in catering." Journal of the Science of Food and Agriculture **90**(7): 1245-1255.

Bakare, R. I., Magbagbeola, O. A., Akinwande, A. I. and Okunowo, O. W. (2010). "Nutritional and chemical evaluation of Momordica charantia." Journal of Medicinal Plants Research **4**(21): 2189-2193.

Barcelo, A., Aedo, C., Rajpathak, S. and Robles, S. (2003). "The cost of diabetes in Latin America and the Caribbean." Bulletin of the World Health Organization **81**(1): 19-27.

Baron, A. D. (1994). "Hemodynamic actions of insulin." American Journal of Physiology **267**(2): E187-E202.

Baron, A. D., Laakso, M., Brechtel, G. and Edelman, S. V. (1991). "Reduced capacity and affinity of skeletal-muscle for insulin-mediated glucose uptake in noninsulin dependent diabetic subjects - effects of insulin therapy." Journal of Clinical Investigation **87**(4): 1186-1194.

Bashan, N., Kovsan, J., Kachko, I., Ovadia, H. and Rudich, A. (2009). "Positive and Negative Regulation of Insulin Signaling by Reactive Oxygen and Nitrogen Species." Physiological Reviews **89**(1): 27-71.

Beckman, J. A., Goldfine, A. B., Gordon, M. B. and Creager, M. A. (2001). "Ascorbate restores endothelium-dependent vasodilation impaired by acute hyperglycemia in humans." Circulation **103**(12): 1618-1623.

Benzie, I. F. F. and Strain, J. J. (1996). "The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": The FRAP assay." Analytical Biochemistry **239**(1): 70-76.

Benzie, I. F. F. and Strain, J. J. (1999). "Ferric reducing antioxidant power assay: Direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration." Oxidants and Antioxidants, Pt A **299**: 15-27.

Bermudez-Soto, M. J., Tomas-Barberan, F. A. and Garcia-Conesa, M. T. (2007). "Stability of polyphenols in chokeberry (Aronia melanocarpa) subjected to in vitro gastric and pancreatic digestion." Food Chemistry **102**(3): 865-874.

Blank, R., Sauer, W. C., Mosenthin, R., Zentek, J., Huang, S. and Roth, S. (2001). "Effect of fumaric acid supplementation and dietary buffering capacity on the concentration of microbial metabolites in ileal digesta of young pigs." Canadian Journal of Animal Science **81**(3): 345-353.

Bloch-Damti, A. and Bashan, N. (2005). "Proposed mechanisms for the induction of insulin resistance by oxidative stress." Antioxidants & Redox Signaling **7**(11-12): 1553-1567.

Bondurant, S. and Sox, H. C. (2005). "Mainstream and alternative medicine: Converging paths require common standards." Annals of Internal Medicine **142**(2): 149-150.

Bouayed, J., Hoffmann, L. and Bohn, T. (2011). "Total phenolics, flavonoids, anthocyanins and antioxidant activity following simulated gastro-intestinal digestion and dialysis of apple varieties: Bioaccessibility and potential uptake." Food Chemistry **128**(1): 14-21.

Bravo, L. (1998). "Polyphenols: Chemistry, dietary sources, metabolism, and nutritional significance." Nutrition Reviews **56**(11): 317-333.

Bravo, L., Abia, R., Eastwood, M. A. and Sauracalixto, F. (1994). "Degradation of polyphenols (catechin and tannic-acid) in the rat intestinal tract - Effect on colonic fermentation and fecal output " British Journal of Nutrition **71**(6): 933-946.

Brodsky, S. V., Gao, S. J., Li, H. and Goligorsky, M. S. (2002). "Hyperglycemic switch from mitochondrial nitric oxide to superoxide production in endothelial cells." American Journal of Physiology-Heart and Circulatory Physiology **283**(5): H2130-H2139.

Brownlee, M., Vlassara, H. and Cerami, A. (1984). "Nonenzymatic glycosylation and the pathogenesis of diabetic complications." Annals of Internal Medicine **101**(4): 527-537.

Budrat, P. and Shotipruk, A. (2008). "Extraction of phenolic compounds from fruits of bitter melon (Momordica charantia) with subcritical water extraction and antioxidant activities of these extracts." Chiang Mai Journal of Science **35**(1): 123-130.

Cai, H. and Harrison, D. G. (2000). "Endothelial dysfunction in cardiovascular diseases - The role of oxidant stress." Circulation Research **87**(10): 840-844.

Cao, G. H., Booth, S. L., Sadowski, J. A. and Prior, R. L. (1998). "Increases in human plasma antioxidant capacity after consumption of controlled diets high in fruit and vegetables." American Journal of Clinical Nutrition **68**(5): 1081-1087.

Carbonaro, M., Grant, G. and Pusztai, A. (2001). "Evaluation of polyphenol bioavailability in rat small intestine." European Journal of Nutrition **40**(2): 84-90.

Carrington, S. (2007). Wild Plants of Barbados. Carribean, Macmillan Education.

Carro, M. D., Ranilla, M. J., Giraldez, F. J. and Mantecon, A. R. (2006). "Effects of malate on diet digestibility, microbial protein synthesis, plasma metabolites, and performance of growing lambs fed a high-concentrate diet." Journal of Animal Science **84**(2): 405-410.

Chandra, A., Mahdi, A. A., Singh, R. K., Mahdi, F. and Chander, R. (2008). "Effect of Indian herbal hypoglycemic agents on antioxidant capacity and trace elements content in diabetic rats." Journal of Medicinal Food **11**(3): 506-512.

Chandra, D., Jackson, E. B., Ramana, K. V., Kelley, R., Srivastava, S. K. and Bhatnagar, A. (2002). "Nitric oxide prevents aldose reductase activation and sorbitol accumulation during diabetes." Diabetes **51**(10): 3095-3101.

Chao, C. Y. and Huang, C. J. (2003). "Bitter gourd (Momordica charantia) extract activates peroxisome proliferator-activated receptors and upregulates the expression of the acyl CoA oxidase gene in H4IIEC3 hepatoma cells." Journal of Biomedical Science **10**(6): 782-791.

Chen, Q. X., Chan, L. L. Y. and Li, E. T. S. (2003). "Bitter melon (Momordica charantia) reduces adiposity, lowers serum insulin and normalizes glucose tolerance in rats fed a high fat diet." Journal of Nutrition **133**(4): 1088-1093.

Chuah, A. M., Lee, Y. C., Yamaguchi, T., Takamura, H., Yin, L. J. and Matoba, T. (2008). "Effect of cooking on the antioxidant properties of coloured peppers." Food Chemistry **111**(1): 20-28.

Chung, S. S. M., Ho, E. C. M., Lam, K. S. L. and Chung, S. K. (2003). "Contribution of polyol pathway to diabetes-induced oxidative stress." Journal of the American Society of Nephrology **14**: S233-S236.

Cooper, R. S., Rotimi, C. N., Kaufman, J. S., Owoaje, E. E., Fraser, H., Forrester, T., Wilks, R., Riste, L. K. and Cruickshank, J. K. (1997). "Prevalence of NIDDM among populations of the African diaspora." Diabetes Care **20**(3): 343-348.

Cox, P. A. and Balick, M. J. (1994). "The ethnobotanical approach to drug discovery." Scientific American **270**(6): 82-87.

Cruz, A. B., Grande, F., Hay, L. J. and Amatuzio, D. S. (1961). "Effect of intra arterial insulin on tissue cholesterol and fatty acids in alloxan-diabetic dogs." Circulation Research **9**(1): 39-43.

Cunningham-Murie, C., Reid, M. and Forrester, T. E. (2008). "A Comparative Study of the Quality and Availability of Health Information used to Facilitate Cost Burden Analysis of Diabetes and Hypertension in the Caribbean." West Indian Medical Journal **57**(4): 383-392.

Daisy, P., Balasubramanian, K., Rajalakshmi, M., Eliza, J. and Selvaraj, J. (2010). "Insulin mimetic impact of catechin isolated from Cassia fistula on the glucose oxidation and molecular mechanisms of glucose uptake on streptozotocin-induced diabetic wistar rats." Phytomedicine **17**(1): 28-36.

Davey, M. W., Van Montagu, M., Inze, D., Sanmartin, M., Kanellis, A., Smirnoff, N., Benzie, I. J. J., Strain, J. J., Favell, D. and Fletcher, J. (2000). "Plant L-ascorbic acid: chemistry, function, metabolism, bioavailability and effects of processing." Journal of the Science of Food and Agriculture **80**(7): 825-860.

Delgoda, R., Ellington, C., Barrett, S., Gordon, N., Clarke, N. and Younger, N. (2004). "The practice of polypharmacy involving herbal and prescription medicines in the treatment of diabetes mellitus, hypertension and gastrointestinal disorders in Jamaica." West Indian Medical Journal **53**(6): 400-405.

Deprez, S., Mila, I., Huneau, J. F., Tome, D. and Scalbert, A. (2001). "Transport of proanthocyanidin dimer, trimer, and polymer across monolayers of human intestinal epithelial Caco-2 cells." Antioxidants & Redox Signaling **3**(6): 957-967.

Dibner, J. J. and Buttin, P. (2002). "Use of organic acids as a model to study the impact of gut microflora on nutrition and metabolism." Journal of Applied Poultry Research **11**(4): 453-463.

Donovan, J. L., Crespy, V., Manach, C., Morand, C., Besson, C., Scalbert, A. and Remesy, C. (2001). "Catechin is metabolized by both the small intestine and liver of rats." Journal of Nutrition **131**(6): 1753-1757.

Dowman, J. K., Tomlinson, J. W. and Newsome, P. N. (2010). "Pathogenesis of non-alcoholic fatty liver disease." Qjm-an International Journal of Medicine **103**(2): 71-83.

Durazo-Arvizu, R. A., Luke, A., Cooper, R. S., Cao, G. C., Dugas, L., Adeyemo, A., Boyne, M. and Forrester, T. (2008). "Rapid increases in obesity in Jamaica, compared to Nigeria and the United States." Bmc Public Health **8**.

El Batran, S. A. S., El-Gengaihi, S. E. and El Shabrawy, O. A. (2006). "Some toxicological studies of Momordica charantia L. on albino rats in normal and alloxan diabetic rats." Journal of Ethnopharmacology **108**(2): 236-242.

Eliasson, B., Bjornsson, E., Urbanavicius, V., Andersson, H., Fowelin, J., Attvall, S., Abrahamsson, H. and Smith, U. (1995). "Hyperinsulinemia impairs gastrointestinal motility and slows carbohydrate absorption." Diabetologia **38**(1): 79-85.

Esaka, M., Uchida, M., Fukui, H., Kubota, K. and Suzuki, K. (1988). "Marked increase in ascorbate oxidase protein in pumpkin callus by adding copper." Plant Physiology **88**(3): 656-660.

Esposito, K., Nappo, F., Giugliano, F., Guigliano, G., Marfella, R. and Giugliano, D. (2003). "Effect of dietary antioxidants on postprandial endothelial dysfunction induced by a high-fat meal in healthy subjects." American Journal of Clinical Nutrition **77**(1): 139-143.

Ferrannini, E., Buzzigoli, G., Bonadonna, R., Giorico, M. A., Oleggini, M., Graziadei, L., Pedrinelli, R., Brandi, L. and Bevilacqua, S. (1987). "Insulin resistance in essential hypertension." New England Journal of Medicine **317**(6): 350-357.

Fitzgibbons, J. F., Koler, R. D. and Jones, R. T. (1976). "Red cell age related changes of hemoglobins A1A + B and A1C in normal and diabetic subjects." Journal of Clinical Investigation **58**(4): 820-824.

Fonseka, H. H., Chandrasekara, A., Fonseka, R. M., Wickramasinghe, P., Kumara, P. and Wickramarachchi, W. N. C. (2007). Determination of anti-amylase and anti-glucosidase activity of different genotypes of bitter gourd (Momordica charantia L.) and thumba karavila (Momordica dioica L.). Proceedings of the 1st International Conference on Indigenous Vegetables and Legumes Prospectus for Fighting Poverty, Hunger and Malnutrition. M. L. Chadha, G. Kuo and C. L. L. Gowda**:** 131-136.

Foster, C., Rotimi, C., Fraser, H., Sundarum, C., Liao, Y., Gibson, E., Holder, Y., Hoyos, M., & Mellanson-King, Rosalind (1993). "Hypertension, diabetes, and obesity in Barbados: Findings from a recent population based survey." Ethnicity and Disease **3**(4): 404-412.

Francisco, M., Velasco, P., Moreno, D. A., Garcia-Viguera, C. and Cartea, M. E. (2010). "Cooking methods of Brassica rapa affect the preservation of glucosinolates, phenolics and vitamin C." Food Research International **43**(5): 1455-1463.

Fridovich, I. (2008). Superoxide dismutase. Redox biochemistry. R. Banerjee, D. Becker, M. Dickman, V. Gladyshev and S. Ragsdale. Hoboken, John Wiley & Sons Inc.**:** 55-59.

Fujii, H., Kono, K., Nakai, K., Goto, S., Komaba, H., Hamada, Y., Shinohara, M., Kitazawa, R., Kitazawa, S. and Fukagawa, M. (2010). "Oxidative and nitrosative stress and progression of diabetic nephropathy in type 2 diabetes." American Journal of Nephrology **31**(4): 342-352.

Fung, T. T., Rimm, E. B., Spiegelman, D., Rifai, N., Tofler, G. H., Willett, W. C. and Hu, F. B. (2001). "Association between dietary patterns and plasma biomarkers of obesity and cardiovascular disease risk." American Journal of Clinical Nutrition **73**(1): 61-67.

Furchgott, R. F., Cherry, P. D., Zawadzki, J. V. and Jothianandan, D. (1984). "Endothelial cells as mediators of vasodilation of arteries." Journal of Cardiovascular Pharmacology **6**: S336-S343.

Gallardo-Guerrero, L., Gandul-Rojas, B. and Minguez-Mosquera, M. I. (2008). "Digestive stability, micellarization, and uptake by Caco-2 human intestinal cell of chlorophyll derivatives from different preparations of pea (Pisum sativum L.)." Journal of Agricultural and Food Chemistry **56**(18): 8379-8386.

Getachew, G., Pittroff, W., Putnam, D. H., Dandekar, A., Goyal, S. and DePeters, E. J. (2008). "The influence of addition of gallic acid, tannic acid, or quebracho tannins to alfalfa hay on in vitro rumen fermentation and microbial protein synthesis." Animal Feed Science and Technology **140**(3-4): 444-461.

Goodwin, B. L., Ruthven, C. R. J. and Sandler, M. (1975). "Gas chromatographic assay of phenylacetic acid in biological fluids." Clinica Chimica Acta **62**(3): 443-446.

Goodwin, B. L., Ruthven, C. R. J. and Sandler, M. (1994). "Gut flora and the origin of some urinary aromatic phenolic compounds." Biochemical Pharmacology **47**(12): 2294-2297.

Green, L. C., Wagner, D. A., Glogowski, J., Skipper, P. L., Wishnok, J. S. and Tannenbaum, S. R. (1982). "Analysis of nitrate, nitrite and N-15 labelled nitrate in biological fluids." Analytical Biochemistry **126**(1): 131-138.

Halliwell, B. (1994). "Free radicals and antioxidants - A personal view." Nutrition Reviews **52**(8): 253-265.

Halliwell, B. and Gutteridge, J. M. C. (1995). "The definition and measurement of antioxidants in biological systems." Free Radical Biology and Medicine **18**(1): 125-126.

Hambleton, I. R., Jonnalagadda, R., Davis, C. R., Fraser, H. S., Chaturvedi, N. and Hennis, A. J. (2009). "All-Cause Mortality After Diabetes-Related Amputation in Barbados A prospective case-control study." Diabetes Care **32**(2): 306-307.

Han, S. J., Ryu, S. N., Trinh, H. T., Joh, E. H., Jang, S. Y., Han, M. J. and Kim, D. H. (2009). "Metabolism of Cyanidin-3-O-beta-D-Glucoside Isolated from Black Colored Rice and Its Antiscratching Behavioral Effect in Mice." Journal of Food Science **74**(8): H253-H258.

Han, X. Z., Shen, T. and Lou, H. X. (2007). "Dietary polyphenols and their biological significance." International Journal of Molecular Sciences **8**(9): 950-988.

Harinantenaina, L., Tanaka, M., Takaoka, S., Oda, M., Mogami, O., Uchida, M. and Asakawa, Y. (2006). "Momordica charantia constituents and antidiabetic screening of the isolated major compounds." Chemical & Pharmaceutical Bulletin **54**(7): 1017-1021.

Haslam, E. and Cai, Y. (1994). "Plant polyphenols (vegetable tannins) - gallic acid metabolism." Natural Product Reports **11**(1): 41-66.

Hennis, A. and Fraser, H. S. (2004). "Diabetes in the English-speaking Caribbean." Revista Panamericana De Salud Publica-Pan American Journal of Public Health **15**(2): 90-93.

Hennis, A., Fraser, H. S., Jonnalagadda, R., Fuller, J. and Chaturvedi, N. (2004). "Explanations for the high risk of diabetes-related amputation in a Caribbean population of black African descent and potential for prevention." Diabetes Care **27**(11): 2636-2641.

Hennis, A., Wu, S. Y., Nemesure, B., Li, X. W. and Leske, M. C. (2002). "Diabetes in a Caribbean population: epidemiological profile and implications." International Journal of Epidemiology **31**(1): 234-239.

Hertog, M. G. L., Feskens, E. J. M., Hollman, P. C. H., Katan, M. B. and Kromhout, D. (1993). "Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen elderly study." Lancet **342**(8878): 1007-1011.

Hodgson, J. M., Morton, L. W., Puddey, I. B., Beilin, L. J. and Croft, K. D. (2000). "Gallic acid metabolites are markers of black tea intake in humans." Journal of Agricultural and Food Chemistry **48**(6): 2276-2280.

Horax, R., Hettiarachchy, N. and Chen, P. Y. (2010). "Extraction, quantification, and antioxidant activities of phenolics from pericarp and seeds of bitter melons (Momordica charantia) harvested at three maturity stages (immature, mature, and ripe)." Journal of Agricultural and Food Chemistry **58**(7): 4428-4433.

Horax, R., Hettiarachchy, N. and Islam, S. (2005). "Total phenolic contents and phenolic acid constituents in 4 varieties of bitter melons (Momordica charantia) and antioxidant activities of their extracts." Journal of Food Science **70**(4): C275-C280.

Horax, R., Hettiarachchy, N., Kannan, A. and Chen, P. Y. (2010). "Proximate composition and amino acid and mineral contents of Mormordica charantia L. pericarp and seeds at different maturity stages." Food Chemistry **122**(4): 1111-1115.

Huang, C. J. and Wu, M. C. (2002). "Differential effects of foods traditionally regarded as 'heating' and 'cooling' on prostaglandin E-2 production by a macrophage cell line." Journal of Biomedical Science **9**(6): 596-606.

Huang, D. J., Ou, B. X. and Prior, R. L. (2005). "The chemistry behind antioxidant capacity assays." Journal of Agricultural and Food Chemistry **53**(6): 1841-1856.

Ibrahim, T. A., El-Hefnawy, H. M. and El-Hela, A. A. (2010). "Antioxidant potential and phenolic acid content of certain cucurbitaceous plants cultivated in Egypt." Natural Product Research **24**(16): 1537-1545.

Jaganath, I. B. and Crozier, A. (2010). Dietary flavonoids and phenolic compounds. Plant phenolics and human health: Biochemistry, nutrition and pharmacology. C. G. Fraga. Hoboken, John Wiley & Sons, Inc.**:** 1-49.

Jaganath, I. B. and Crozier, A. (2010). Dietary flavonoids and phenolic compounds. Plant phenolics and human health. C. G. Fraga. Hoboken, John Wiley & Sons Inc. **:** 1-39.

Jagetia, G. C. and Baliga, M. S. (2004). "The evaluation of nitric oxide scavenging activity of certain Indian medicinal plants in vitro: A preliminary study." Journal of Medicinal Food **7**(3): 343-348.

Jakobs, S., Fridrich, D., Hofem, S., Pahlke, G. and Eisenbrand, G. (2006). "Natural flavonoids are potent inhibitors of glycogen phosphorylase." Molecular Nutrition & Food Research **50**(1): 52-57.

Jalc, D., Kisidayova, S. and Nerud, F. (2002). "Effect of plant oils and organic acids on rumen fermentation in vitro." Folia Microbiologica **47**(2): 171-177.

Jenkins, D. J. A., Jenkins, A. L., Wolever, T. M. S., Collier, G. R., Rao, A. V. and Thompson, L. U. (1987). "Starchy foods and fiber - reduced rate of digestion and improved carbohydrate metabolism." Scandinavian Journal of Gastroenterology **22**: 132-141.

Jenkins, D. J. A., Wolever, T. M. S., Leeds, A. R., Gassull, M. A., Haisman, P., Dilawari, J., Goff, D. V., Metz, G. L. and Alberti, K. (1978). "Dietary fibers, fiber analogs and glucose tolerance - importance of viscosity." British Medical Journal **1**(6124): 1392-1394.

Juvekar, A. R., Hule, A. K., Sakat, S. S. and Chaughule, V. A. (2009). "In vitro and in vivo evaluation of immunomodulatory activity of methanol extract of Momordica charantia fruits." Drug Invention Today **1**(2): 6.

Kahkonen, M. P., Hopia, A. I., Vuorela, H. J., Rauha, J. P., Pihlaja, K., Kujala, T. S. and Heinonen, M. (1999). "Antioxidant activity of plant extracts containing phenolic compounds." Journal of Agricultural and Food Chemistry **47**(10): 3954-3962.

Kang, K. S., Kim, H. Y., Pyo, J. S. and Yokozawa, T. (2006). "Increase in the free radical scavenging activity of ginseng by heat-processing." Biological & Pharmaceutical Bulletin **29**(4): 750-754.

Karamac, M., Kosinska, A. and Pegg, R. B. (2005). "Comparison o radical scavenging activities for selected phenolic acids." Polish Journal of Food and Nutrition Sciences **14/55**(2): 165-170.

Karthika, B. (2006). Physical characters and glycemic response of fresh and dehydrated underutilized curcurbits. Department of Food Science and Nutrition. Dharwad, University of Agricultural Sciences. **Master of Home Sciences:** 69.
Kilpatrick, E. S. (2000). "Glycated haemoglobin in the year 2000." Journal of Clinical Pathology **53**(5): 335-339.

Kim, J. A., Montagnani, M., Koh, K. K. and Quon, M. J. (2006). "Reciprocal relationships between insulin resistance and endothelial dysfunction - Molecular and pathophysiological mechanisms." Circulation **113**(15): 1888-1904.

Kim, M. J. and Rhee, S. J. (2004). "Green tea catechins protect rats from microwave-induced oxidative damage to heart tissue." Journal of Medicinal Food **7**(3): 299-304.

King, H., Aubert, R. E. and Herman, W. H. (1998). "Global burden of diabetes, 1995-2025 - Prevalence, numerical estimates, and projections." Diabetes Care **21**(9): 1414-1431.

Kljak, K., Kis, G. and Grbesa, D. (2009). "In vitro digestibility of phenolics in grain of maize hybrids." Italian Journal of Animal Science **8**: 166-168.

Knarreborg, A., Miquel, N., Granli, T. and Jensen, B. B. (2002). "Establishment and application of an in vitro methodology to study the effects of organic acids on coliform and lactic acid bacteria in the proximal part of the gastrointestinal tract of piglets." Animal Feed Science and Technology **99**(1-4): 131-140.

Knowler, W. C., Pettitt, D. J., Savage, P. J. and Bennett, P. H. (1981). "Diabetes incidence in Pima Indians - contributions of obesity and parental diabetes." American Journal of Epidemiology **113**(2): 144-156.

Koksal, A. H., Artik, N., Simsek, A. and Gunes, N. (2006). "Nutrient composition of hazelnut (Corylus avellana L.) varieties cultivated in Turkey." Food Chemistry **99**(3): 509-515.

Kosanovic, M., Hasan, M. Y., Petroianu, G., Marzouqi, A., Abdularhman, O. and Adem, A. (2009). "Assessment of essential and toxic mineral elements in bitter gourd (Momordica charantia) fruit." International Journal of Food Properties **12**(4): 766-773.

Kotikova, Z., Lachman, J., Hejtmankova, A. and Hejtmankova, K. (2011). "Determination of antioxidant activity and antioxidant content in tomato varieties and evaluation of mutual interactions between antioxidants." Lwt-Food Science and Technology **44**(8): 1703-1710.

Kubola, J. and Siriamornpun, S. (2008). "Phenolic contents and antioxidant activities of bitter gourd (Momordica charantia L.) leaf, stem and fruit fraction extracts in vitro." Food Chemistry **110**(4): 881-890.

Kurowska, E. M. (2002). "Nitric oxide therapies in vascular diseases." Current Pharmaceutical Design **8**(3): 155-166.

Laakso, M., Edelman, S. V., Brechtel, G. and Baron, A. D. (1990). "Decreased effect of insulin to stimulate skeletal muscle blood flow in obese man - A novel mechanism for insulin resistance." Journal of Clinical Investigation **85**(6): 1844-1852.

Laakso, M., Edelman, S. V., Brechtel, G. and Baron, A. D. (1992). "Impaired insulin-mediated skeletal muscle blood flow in patients with NIDDM. ." Diabetes **41**(9): 1076-1083.

Lans, C. (2003). "Struggling over the direction of Caribbean medicinal plant research." Futures **35**(5): 473-491.

Laurent, C., Besancon, P. and Caporiccio, B. (2007). "Flavonoids from a grape seed extract interact with digestive secretions and intestinal cells as assessed in an in vitro digestion/Caco-2 cell culture model." Food Chemistry **100**(4): 1704-1712.

Lii, C. K., Chen, H. W., Yun, W. T. and Liu, K. L. (2009). "Suppressive effects of wild bitter gourd (Momordica charantia Linn. var. abbreviata ser.) fruit extracts on inflammatory responses in RAW 264.7 macrophages." Journal of Ethnopharmacology **122**(2): 227-233.

Long, F. (1982). "The food crisis in the Caribbean." Third World Quarterly **4**(4): 758-770.

Longuefosse, J. L. and Nossin, E. (1996). "Medical ethnobotany survey in Martinique." Journal of Ethnopharmacology **53**(3): 117-142.

Luiking, Y., Engelen, M.P.K.J., Deutz, N.E.P. (2009). "Regulation of nitric oxide production in health and disease." Current Opinion in Clinical Nutrition and Metabolic Care **12**.

Mahomoodally, M. F., Gurib-Fakim, A. and Subratty, A. H. (2007). "Effect of exogenous ATP on Momordica charantia Linn. (Cucurbitaceae) induced inhibition of D-glucose, L-tyrosine and fluid transport across rat everted intestinal sacs in vitro." Journal of Ethnopharmacology **110**(2): 257-263.

Manach, C., Scalbert, A., Morand, C., Remesy, C. and Jimenez, L. (2004). "Polyphenols: food sources and bioavailability." American Journal of Clinical Nutrition **79**(5): 727-747.

Marinova, E. M. and Yanishlieva, N. V. (2003). "Antioxidant activity and mechanism of action of some phenolic acids at ambient and high temperatures." Food Chemistry **81**(2): 189-197.

Marles, R. J. and Farnsworth, N. R. (1995). "Antidiabetic plants and their active constituents." Phytomedicine **2**(2): 137-189.

Masella, R., Scazzocchio, B., Pucciarmati, A., Filesi, C., Vari, R., D'Archivio, M., Santangelo, C., Iacovelli, A., Galvano, F. and Giovannini, C. (2010). "Cyanidin-3-O-beta-glucoside and protocatechuic acid stimulate GLUT4 mediated glucose uptake by up regulating PPAR gamma activity in human omental adipocytes " Atherosclerosis Supplements **11**(2): P118.

McCue, P., Kwon, Y. I. and Shetty, K. (2005). "Anti-amylase, anti-glucosidase and anti-angiotensin I-converting enzyme potential of selected foods." Journal of Food Biochemistry **29**(3): 278-294.

McLaughlin, T., Abbasi, F., Lamendola, C., Liang, L., Reaven, G., Schaaf, P. and Reaven, P. (2002). "Differentiation between obesity and insulin resistance in the association with C-reactive protein." Circulation **106**(23): 2908-2912.

Meerabai, M., Jayachandran, B. K. and Asha, K. R. (2007). Biofarming in bitter gourd (Momordica charantia L.). Proceedings of the 1st International Conference on Indigenous Vegetables and Legumes Prospectus for Fighting Poverty, Hunger and Malnutrition. M. L. Chadha, G. Kuo and C. L. L. Gowda**:** 349-352.

Meigs, J. B., Dagostino, R. B., Wilson, P. W. F., Cupples, L. A., Nathan, D. M. and Singer, D. E. (1997). "Risk variable clustering in the insulin resistance syndrome - The Framingham Offspring Study." Diabetes **46**(10): 1594-1600.

Min, S. W., Ryu, S. N. and Kim, D. H. (2010). "Anti-inflammatory effects of black rice, cyanidin-3-O-beta-D-glycoside, and its metabolites, cyanidin and protocatechuic acid." International Immunopharmacology **10**(8): 959-966.

Miura, Y., Chiba, T., Tomita, I., Koizumi, H., Miura, S., Umegaki, K., Hara, Y., Ikeda, M. and Tomita, T. (2001). "Tea catechins prevent the development of atherosclerosis in apoprotein E-deficient mice." Journal of Nutrition **131**(1): 27-32.

Moncada, S. and Higgs, E. A. (2006). "The discovery of nitric oxide and its role in vascular biology." British Journal of Pharmacology **147**: S193-S201.

Mroz, Z., Jongbloed, A. W., Partanen, K., Vreman, K., van Diepen, J. T. M., Kemme, P. A., Kogut, K. and (1997). The effect of dietary buffering capacity and organic acid supplementation (formic, fumaric, or n-butyric acid) on digestibility of nutrients (protein, amino acids, energy and minerals), water intake and excreta production in growing pigs. I. f. A. S. a. Health. Branch Runderweg, Lelystad, The Netherlands.

Munyaka, A. W., Makule, E. E., Oey, I., Van Loey, A. and Hendrickx, M. (2010). "Thermal Stability of L-Ascorbic Acid and Ascorbic Acid Oxidase in Broccoli (Brassica oleracea var. italica)." Journal of Food Science **75**(4): C336-C340.

Murdiati, T. B., McSweeney, C. S. and Lowry, J. B. (1992). "Metabolism in sheep of gallic acid, tannic acid and hydrolyzable tannin from *Terminalia oblongata*." Australian Journal of Agricultural Research **43**(6): 1307-1319.

Myojin, C., Enami, N., Nagata, A., Yamaguchi, T., Takamura, H. and Matoba, T. (2008). "Changes in the radical-scavenging activity of bitter gourd (Momordica charantia L.) during freezing and frozen storage with or without blanching." Journal of Food Science **73**(7): C546-C550.

Nakamura, T., Makino, N. and Ogura, Y. (1968). "Purification and properties of ascorbate oxidase from cucumber." Journal of Biochemistry **64**(2): 189.

Nemesure, B., Wu, S. Y., Hennis, A., Leske, M. C. and Barbados Eye Study, G. (2007). "Prevalence of obesity and associated sex-specific factors in an African-origin population." Ethnicity & Disease **17**(3): 508-514.

Nemesure, B., Wu, S. Y., Hennis, A., Leske, M. C. and Grp, B. E. S. S. (2008). "The relationship of body mass index and waist-hip ratio on the 9-year incidence of diabetes and hypertension in a predominantly African-origin population." Annals of Epidemiology **18**(8): 657-663.

Ng, Z. X., Chai, J. W. and Kuppusamy, U. R. (2011). "Customized cooking method improves total antioxidant activity in selected vegetables." International Journal of Food Sciences and Nutrition **62**(2): 158-163.

Nishikawa, T., Edelstein, D., Du, X. L., Yamagishi, S., Matsumura, T., Kaneda, Y., Yorek, M. A., Beebe, D., Oates, P. J., Hammes, H. P., Giardino, I. and Brownlee, M. (2000). "Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage." Nature **404**(6779): 787-790.

Novakova, L., Solich, P. and Solichova, D. (2008). "HPLC methods for simultaneous determination of ascorbic and dehydroascorbic acids." Trac-Trends in Analytical Chemistry **27**(10): 942-958.

Olefsky, J. M. and Kolterman, O. G. (1981). "Mechanisms of insulin resistance in obesity and noninsulin dependent (TYPE II) diabetes." American Journal of Medicine **70**(1): 151-168.

Olthof, M. R., Hollman, P. C. H., Buijsman, M., van Amelsvoort, J. M. M. and Katan, M. B. (2003). "Chlorogenic acid, quercetin-3-rutinoside and black tea phenols are extensively metabolized in humans." Journal of Nutrition **133**(6): 1806-1814.

Opara, E. C. (2002). "Oxidative stress, micronutrients, diabetes mellitus and its complications." Journal of the Royal Society for the Promotion of Health **122**(1): 28-34.

Oubre, A. Y., Carlson, T. J., King, S. R. and Reaven, G. M. (1997). "From plant to patient: An ethnomedical approach to the identification of new drugs for the treatment of NIDDM." Diabetologia **40**(5): 614-617.

Padh, H. (1990). "Cellular functions of ascorbic acid." Biochemistry and Cell Biology-Biochimie Et Biologie Cellulaire **68**(10): 1166-1173.

Parr, A. J. and Bolwell, G. P. (2000). "Phenols in the plant and in man. The potential for possible nutritional enhancement of the diet by modifying the phenols content or profile." Journal of the Science of Food and Agriculture **80**(7): 985-1012.

Pedersen, D. and Baruffati, V. (1985). "Health and traditional medicine cultures in Latin-America and the Caribbean." Social Science & Medicine **21**(1): 5-12.

Perez-Vicente, A., Gil-Izquierdo, A. and Garcia-Viguera, C. (2002). "In vitro gastrointestinal digestion study of pomegranate juice phenolic compounds, anthocyanins, and vitamin C." Journal of Agricultural and Food Chemistry **50**(8): 2308-2312.

Peters, C. M., Green, R. J., Janle, E. M. and Ferruzzi, M. G. (2010). "Formulation with ascorbic acid and sucrose modulates catechin bioavailability from green tea." Food Research International **43**(1): 95-102.

Peterson, C. M., Jones, R. L., Koenig, R. J., Melvin, E. T. and Lehrman, M. L. (1977). "Reversible hematologic sequelae of diabetes mellitus." Annals of Internal Medicine **86**(4): 425-429.

Pieper, G. M., Langenstroer, P. and Siebeneich, W. (1997). "Diabetic-induced endothelial dysfunction in rat aorta: Role of hydroxyl radicals." Cardiovascular Research **34**(1): 145-156.

Piva, A., Casadei, G. and Biagi, G. (2002). "An organic acid blend can modulate swine intestinal fermentation and reduce microbial proteolysis." Canadian Journal of Animal Science **82**(4): 527-532.

Prabhakar, P. K. and Doble, M. (2009). "Synergistic effect of phytochemicals in combination with hypoglycemic drugs on glucose uptake in myotubes." Phytomedicine **16**(12): 1119-1126.

Pulido, R., Bravo, L. and Saura-Calixto, F. (2000). "Antioxidant activity of dietary polyphenols as determined by a modified ferric reducing/antioxidant power assay." Journal of Agricultural and Food Chemistry **48**(8): 3396-3402.

Quinlan, M. B. and Quinlan, R. J. (2007). "Modernization and medicinal plant knowledge in a Caribbean horticultural village." Medical Anthropology Quarterly **21**(2): 169-192.

Racine, E. F., Jemison, K., Huber, L. R. and Arif, A. A. (2009). "The well-being of children in food-insecure households: results from The Eastern Caribbean Child Vulnerability Study 2005." Public Health Nutrition **12**(9): 1443-1450.

Ramirez-Sanchez, I., Maya, L., Ceballos, G. and Villarreal, F. (2010). "(-)-Epicatechin Activation of Endothelial Cell Endothelial Nitric Oxide Synthase, Nitric Oxide, and Related Signaling Pathways." Hypertension **55**(6): 1398-U1198.

Randle, P. J., Garland, P. B., Newsholme, E. A. and Hales, C. N. (1963). "The glucose fatty acid cycle: its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus." Lancet **1**(728): 785.

Ranilla, L. G., Genovese, M. I. and Lajolo, F. M. (2009). "Effect of Different Cooking Conditions on Phenolic Compounds and Antioxidant Capacity of Some Selected Brazilian Bean (Phaseolus vulgaris L.) Cultivars." Journal of Agricultural and Food Chemistry **57**(13): 5734-5742.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M. and Rice-Evans, C. (1999). "Antioxidant activity applying an improved ABTS radical cation decolorization assay." Free Radical Biology and Medicine **26**(9-10): 1231-1237.

Renaudin, C., Michoud, E., Rapin, J. R., Lagarde, M. and Wiernsperger, N. (1998). "Hyperglycaemia modifies the reaction of microvessels to insulin in rat skeletal muscle." Diabetologia **41**(1): 26-33.

Ribiere, C., Jaubert, A. M., Gaudiot, N., Sabourault, D., Marcus, M. L., Boucher, J. L., DenisHenriot, D. and Giudicelli, Y. (1996). "White adipose tissue nitric oxide synthase: A potential source for NO production." Biochemical and Biophysical Research Communications **222**(3): 706-712.

Rice-Evans, C. A., Miller, N. J., Bolwell, G. P., Bramley, P. M. and Pridham, J. B. (1995). "The relative antioxidant activities of plant derived polyphenolic flavonoids." Free Radical Research **22**(4): 375-383.

RiceEvans, C. A., Miller, N. J. and Paganga, G. (1996). "Structure-antioxidant activity relationships of flavonoids and phenolic acids." Free Radical Biology and Medicine **20**(7): 933-956.

Roden, M., Price, T. B., Perseghin, G., Petersen, K. F., Rothman, D. L., Cline, G. W. and Shulman, G. I. (1996). "Mechanism of free fatty acid-induced insulin resistance in humans." Journal of Clinical Investigation **97**(12): 2859-2865.

Roffey, B. W. C., Atwal, A. S., Johns, T. and Kubow, S. (2007). "Water extracts from Momordica charantia increase glucose uptake and adiponectin secretion in 3T3-L1 adipose cells." Journal of Ethnopharmacology **112**(1): 77-84.

Rohn, S., Rawel, H. M. and Kroll, J. (2002). "Inhibitory effects of plant phenols on the activity of selected enzymes." Journal of Agricultural and Food Chemistry **50**(12): 3566-3571.

Rotimi, C. N., Cooper, R. S., Ataman, S. L., Osotimehin, B., Kadiri, S., Muna, W., Kingue, S., Fraser, H. and McGee, D. (1995). Distribution of Anthropometric Variables and the Prevalence of Obesity in populations of West-African Origin - The International Collaborative Study on Hypertension in Blacks (ICSHIB).

Sabboh-Jourdan, H., Valla, F., Epriliati, I. and Gidley, M. J. (2011). "Organic acid bioavailability from banana and sweet potato using an in vitro digestion and Caco-2 cell model." European Journal of Nutrition **50**(1): 31-40.

Sahib, N. G., Hamid, A. A., Kitts, D., Purnama, M., Saari, N. and Abas, F. (2011). "The effects of *Morinda citrifolia, Momordica charantia* and *Centella asiatica* extracts on lipoprotein lipase and 3T3-L1 preadipocytes." Journal of Food Biochemistry **35**(4): 1186-1205.

Sarang, A., Donnelly, D. and Kubow, S. (2011). Identification of nutritional profiles associated with lower predicted glycemic load among potato cultivars. School of Dietetics and Human Nutrition. Sainte-Anne-de-Bellevue, McGill University. **Master of Science:** 97.

Sato, E. F., Choudhury, T., Nishikawa, T. and Inoue, M. (2008). "Dynamic aspect of reactive oxygen and nitric oxide in oral cavity." Journal of Clinical Biochemistry and Nutrition **42**(1): 8-13.

Saura-Calixto, F., Garcia-Alonso, A., Goni, I. and Bravo, L. (2000). "In vitro determination of the indigestible fraction in foods: An alternative to dietary fiber analysis." Journal of Agricultural and Food Chemistry **48**(8): 3342-3347.

Saura-Calixto, F., Perez-Jimenez, J., Tourino, S., Serrano, J., Fuguet, E., Torres, J. L. and Goni, I. (2010). "Proanthocyanidin metabolites associated with dietary fibre from in vitro colonic fermentation and proanthocyanidin metabolites in human plasma." Molecular Nutrition & Food Research **54**(7): 939-946.

Saura-Calixto, F., Serrano, J. and Goni, I. (2007). "Intake and bioaccessibility of total polyphenols in a whole diet." Food Chemistry **101**(2): 492-501.

Sayago-Ayerdi, S. G., Arranz, S., Serrano, J. and Goni, I. (2007). "Dietary fiber content and associated antioxidant compounds in roselle flower (Hibiscus sabdariffa L.) beverage." Journal of Agricultural and Food Chemistry **55**(19): 7886-7890.

Scalbert, A., Morand, C., Manach, C. and Remesy, C. (2002). "Absorption and metabolism of polyphenols in the gut and impact on health." Biomedicine & Pharmacotherapy **56**(6): 276-282.

Schorah, C. J. (1992). "The transport of vitamin C and effects of disease." Proceedings of the Nutrition Society **51**(2): 189-198.

Schorah, C. J., Sobala, G. M., Sanderson, M., Collis, N. and Primrose, J. N. (1991). "Gastric juice ascorbic acid: effects of disease and implications for gastric carcinogenesis." American Journal of Clinical Nutrition **53**(1): S287-S293.

Schwartz, J. H., Greenspun, R. B. and Porter, W. L. (1962). "Potato acids, identification and determination of the major acids of the white potato." Journal of Agricultural and Food Chemistry **10**(1): 43-46.

Sekiya, J., Hamade, R., Kimura, O., Mizuno, K. and Shimose, N. (1990). "Purification and properties of ascorbate oxidase from cucumber fruit." Soil Science and Plant Nutrition **36**(1): 1-7.

Serafini, M., Maiani, G. and Ferro-Luzzi, A. (1998). "Alcohol-free red wine enhances plasma antioxidant capacity in humans." Journal of Nutrition **128**(6): 1003-1007.

Serra, A., Macia, A., Romero, M. P., Reguant, J., Ortega, N. and Motilva, M. J. (2012). "Metabolic pathways of the colonic metabolism of flavonoids (flavonols, flavones and flavanones) and phenolic acids." Food Chemistry **130**(2): 383-393.

Shahrzad, S. and Bitsch, I. (1998). "Determination of gallic acid and its metabolites in human plasma and urine by high-performance liquid chromatography." Journal of Chromatography B **705**(1): 87-95.

Shakya, R. and Navarre, D. A. (2006). "Rapid screening of ascorbic acid, glycoalkaloids, and phenolics in potato using high-performance liquid chromatography." Journal of Agricultural and Food Chemistry **54**(15): 5253-5260.

Sharma, S., Cao, X., Harris, R., Hennis, A. J. M., Leske, M. C., Wu, S. Y. and Barbabos Natl Canc Study, G. (2007). "Dietary intake and development of a quantitative food-frequency questionnaire for the Barbados National Cancer Study." Public Health Nutrition **10**(5): 464-470.

Shibib, B. A., Khan, L. A. and Rahman, R. (1993). "Hypoglyemic activity of coccinia indica and momordica charantia in diabetic rats - depression of the hepatic gluconeogenic enzymes glucosoe-6-phosphatase and fructose-1,6-bisphosphatase and elevation of both liver and red cell shunt enzyme glucose-6-phosphate dehydrogenase " Biochemical Journal **292**: 267-270.

Shih, C. C., Lin, C. H. and Lin, W. L. (2008). "Effects of Momordica charantia on insulin resistance and visceral obesity in mice on high-fat diet." Diabetes Research and Clinical Practice **81**(2): 134-143.

Shih, C. C., Lin, C. H., Lin, W. L. and Wu, J. B. (2009). "Momordica charantia extract on insulin resistance and the skeletal muscle GLUT4 protein in fructose-fed rats." Journal of Ethnopharmacology **123**(1): 82-90.

Singleton, V. L., Orthofer, R. and Lamuela-Raventos, R. M. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. Oxidants and Antioxidants, Pt A. L. Packer. **299:** 152-178.

Sitasawad, S. L., Shewade, Y. and Bhonde, R. (2000). "Role of bittergourd fruit juice in stz-induced diabetic state in vivo and in vitro." Journal of Ethnopharmacology **73**(1-2): 71-79.

Slater, T. F. (1984). "Free radical mechanisms in tissue injury." Biochemical Journal **222**(1): 1-15.

Sobala, G. M., Pignatelli, B., Schorah, C. J., Bartsch, H., Sanderson, M., Dixon, M. F., Shires, S., King, R. F. G. and Axon, A. T. R. (1991). "Levels of nitrite, nitrate, N-nitroso compounds, ascorbic acid and total bile acids in gastric juice of patients with and without precancerous conditions of the stomach." Carcinogenesis **12**(2): 193-198.

Srivastava, S. K., Ramana, K. V., Chandra, D., Srivastava, S. and Bhatnagar, A. (2003). "Regulation of aldose reductase and the polyol pathway activity by nitric oxide." Chemico-Biological Interactions **143**: 333-340.

Srivastava, Y., Venkatakrishnabhatt, H., Verma, Y., Venkaiah, K. and Raval, B. H. (1993). "Antidiabetic and adaptogenic properties of Momordica-charantia extract: an experimental and clinical evaluation." Phytotherapy Research **7**(4): 285-289.

Stamler, J., Pick, R. and Katz, L. N. (1960). "Effect of insulin in the induction and regression of atherosclerosis in the chick." Circulation Research **8**(3): 572-576.

Stuehr, D. J. (1999). "Mammalian nitric oxide synthases." Biochimica Et Biophysica Acta-Bioenergetics **1411**(2-3): 217-230.

Sullivan, J. C. and Pollock, J. S. (2006). "Coupled and uncoupled NOS: Separate but equal? Uncoupled NOS in endothelial cells is a critical pathway for intracellular signaling." Circulation Research **98**(6): 717-719.

Tagliazucchi, D., Verzelloni, E., Bertolini, D. and Conte, A. (2010). "In vitro bio-accessibility and antioxidant activity of grape polyphenols." Food Chemistry **120**(2): 599-606.

Tagliazucchi, D., Verzelloni, E. and Conte, A. (2005). "Effect of some phenolic compounds and beverages on pepsin activity during simulated gastric digestion." Journal of Agricultural and Food Chemistry **53**(22): 8706-8713.

Tan, M. J., Ye, J. M., Turner, N., Hohnen-Behrens, C., Ke, C. Q., Tang, C. P., Chen, T., Weiss, H. C., Gesing, E. R., Rowland, A., James, D. E. and Yel, Y. (2008). "Antidiabetic activities of triterpenoids isolated from bitter melon associated with activation of the AMPK pathway." Chemistry & Biology **15**(3): 263-273.

Tatsumi, Y., Isogai, M., Sei, S. and Srilaong, V. (2006). Changes in ascorbic acid content and ascorbate metabolism-related enzyme activities during storage in cucumber (Cucumis sativus L.) and balsam pear (Momordica charantia L.). Proceedings of the IVth International Conference on Managing Quality in Chains, Vols 1 and 2: THE INTEGRATED VIEW ON FRUITS AND VEGETABLES QUALITY, Acta Horticulturae.

Teoh, S. L., Latiff, A. A. and Das, S. (2009). "A histological study of the structural changes in the liver of streptozotocin-induced diabetic rats treated with or without Momordica charantia (bitter gourd)." Clinica Terapeutica **160**(4): 283-286.

Thompson, D., Pepys, M. B. and Wood, S. P. (1999). "The physiological structure of human C-reactive protein and its complex with phosphocholine." Structure **7**(2): 169-177.

Ting, H. H., Timimi, F. K., Boles, K. S., Creager, S. J., Ganz, P. and Creager, M. A. (1996). "Vitamin C improves endothelium-dependent vasodilation in patients with non-insulin-dependent diabetes mellitus." Journal of Clinical Investigation **97**(1): 22-28.

Tongia, A., Tongia, S. K. and Dave, M. (2004). "Phytochemical determination and extraction of Momordica charantia fruit and its hypoglycemic potentiation of oral hypoglycemic drugs in diabetes mellitus (NIDDM)." Indian Journal of Physiology and Pharmacology **48**(2): 241-244.

Torel, J., Cillard, J. and Cillard, P. (1986). "Antioxidant activity of flavonoids and reactivity with peroxy radical." Phytochemistry **25**(2): 383-385.

Tripathi, U. N. and Chandra, D. (2009). "The plant extracts of Momordica charantia and Trigonella foenum graecum have antioxidant and anti-hyperglycemic properties for cardiac tissue during diabetes mellitus." Oxidative Medicine and Cellular Longevity **2**(5): 290-296.

van den Oever, I. A. M., Raterman, H. G., Nurmohamed, M. T. and Simsek, S. (2010). "Endothelial dysfunction,inflammation, and apoptosis in diabetes mellitus." Mediators of Inflammation.

Virdi, J., Sivakami, S., Shahani, S., Suthar, A. C., Banavalikar, M. M. and Biyani, M. K. (2003). "Antihyperglycemic effects of three extracts from Momordica charantia." Journal of Ethnopharmacology **88**(1): 107-111.

Virgili, F. and Marino, M. (2008). "Regulation of cellular signals from nutritional molecules: a specific role for phytochemicals, beyond antioxidant activity." Free Radical Biology and Medicine **45**(9): 1205-1216.

Vuksan, V. and Sievenpiper, J. L. (2005). "Herbal remedies in the management of diabetes: Lessons learned from the study of ginseng." Nutrition Metabolism and Cardiovascular Diseases **15**(3): 149-160.

Wachtel-Galor, S., Wong, K. W. and Benzie, I. F. F. (2008). "The effect of cooking on Brassica vegetables." Food Chemistry **110**(3): 706-710.

Wahyudiono, Sasaki, M. and Goto, M. (2008). "Recovery of phenolic compounds through the decomposition of lignin in near and supercritical water." Chemical Engineering and Processing **47**(9-10): 1609-1619.

Walters, T. W. and Deckerwalters, D. S. (1988). "Balsam pear (Momordica charantia, cucurbitaceae)." Economic Botany **42**(2): 286-288.

Wang, J. and Mazza, G. (2002). "Inhibitory effects of anthocyanins and other phenolic compounds on nitric oxide production in LPS/IFN-gamma-activated RAW 264.7 macrophages." Journal of Agricultural and Food Chemistry **50**(4): 850-857.

Wang, L., Li, Q., Cao, J., Cai, T. and Jiang, W. (2007). "Keeping quality of fresh-cut bitter gourd (Momordica charantia L.) at low temperature of storage." Journal of Food Processing and Preservation **31**(5): 571-582.

Wang, L. Z., Wang, M., Li, Q. H., Cai, T. Y. and Jiang, W. B. (2008). "Partial properties of an aspartic protease in bitter gourd (Momordica charantia L.) fruit and its activation by heating." Food Chemistry **108**(2): 496-502.

Wechtersbach, L. and Cigic, B. (2007). "Reduction of dehydroascorbic acid at low pH." Journal of Biochemical and Biophysical Methods **70**(5): 767-772.

WHO (2006). Country Health Profile, World Health Organization Statistical Information System.

Wild, S., Roglic, G., Green, A., Sicree, R. and King, H. (2004). "Global prevalence of diabetes - Estimates for the year 2000 and projections for 2030." Diabetes Care **27**(5): 1047-1053.

Wu, S. J. and Ng, L. T. (2008). "Antioxidant and free radical scavenging activities of wild bitter melon (Momordica charantia Linn. var. abbreviata Ser.) in Taiwan." Lwt-Food Science and Technology **41**(2): 323-330.

Xia, Y., Dawson, V. L., Dawson, T. M., Snyder, S. H. and Zweier, J. L. (1996). "Nitric oxide synthase generates superoxide and nitric oxide in arginine-depleted cells leading to peroxynitrite-mediated cellular injury." Proceedings of the National Academy of Sciences of the United States of America **93**(13): 6770-6774.

Zhang, M., Hettiarachchy, N. S., Horax, R., Chen, P. Y. and Over, K. F. (2009). "Effect of Maturity Stages and Drying Methods on the Retention of Selected Nutrients and Phytochemicals in Bitter Melon (Momordica charantia) Leaf." Journal of Food Science **74**(6): C441-C448.

Zhu, Q. Y., Holt, R. R., Lazarus, S. A., Ensunsa, J. L., Hammerstone, J. F., Schmitz, H. H. and Keen, C. L. (2002). "Stability of the flavan-3-ols epicatechin and catechin and related dimeric procyanidins derived from cocoa." Journal of Agricultural and Food Chemistry **50**(6): 1700-1705.

Zimmet, P., Alberti, K. and Shaw, J. (2001). "Global and societal implications of the diabetes epidemic." Nature **414**(6865): 782-787.