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Effect of *in vitro* simulated gastro-duodenal digestion on the antioxidant and anti-inflammatory activity of South African Fynbos honey

By

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Department: Anatomy

University of Pretoria

Supervisor: **Prof MJ Bester**

Co-supervisor: **Ms JC Serem**

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EFFECT OF *IN VITRO* SIMULATED GASTRO-DUODENAL DIGESTION ON THE ANTIOXIDANT AND ANTI-INFLAMMATORY ACTIVITY OF SOUTH AFRICAN FYNBOS HONEY

By

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Abstract

Honey has been shown to have bioactivity. Fynbos (FB) honey was investigated for its bioactivity as this vegetation type is from a unique bio diverse region in the Cape Floristic Kingdom.

Six FB and one medical grade Manuka (MAN) UMF 15+ honeys that were of quality grade (Codex Alimentarius) were used. Each honey sample was subjected to *in vitro* simulated gastro-duodenal digestion and the antioxidant and anti-inflammatory activity of each fraction was determined. These fractions were undigested/raw honey (UD), gastric digest (GD) and gastro-duodenal digest (GDD). Included were pH and digestive enzyme controls. The total polyphenol and the flavonoid content (TPC and TFC) were determined with the Folin-Ciocalteu (F-C) and aluminium chloride methods respectively. Antioxidant activity was measured with the trolox equivalent antioxidant capacity (TEAC) and oxygen radical absorbance capacity (ORAC) assays. Cellular antioxidant activity (CAA) in the Caco-2 and SC-1 cell lines using the dichloroflourescein diacetate (DCFH DA) assay was investigated. Nitric oxide (NO) scavenging activity was determined with the sodium nitroprusside (SNP) assay. Pro-inflammatory and anti-inflammatory effects of honey were evaluated in non-stimulated and stimulated with LPS/IFN–γ murine macrophage RAW 264.7 cells, respectively. Cell viability using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was done.

TPC and TFC of MAN were higher than that of FB honeys. With GD, TPC and TFC of MAN increased and following GDD, TPC decreased and TFC remained unchanged. In contrast TPC and TFC of FB honey were maintained with GD and GDD.

TEAC assay revealed activity by MAN being higher than that of FB honeys. With GD digestion, the antioxidant activity of MAN was unchanged but following GDD, activity was reduced. For FB honeys, TEAC was maintained with GD and GDD. ORAC assay revealed that the activity of MAN was similar to that of FB. Digestion had no effect on activity of both MAN and FB honeys.

CAA in the Caco-2 and SC-1 cell line was higher for MAN compared to FB honey. In both cell lines a similar trend was observed where with GD, CAA was unchanged while with GDD, CAA was reduced. This loss of CAA following GDD was found to be due to H_2O_2 formation as a result

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of polyphenol degradation in an alkaline environment containing sodium bicarbonate and pancreatin.

NO scavenging activity of MAN was greater than FB. For both types of honey with GD, NO scavenging activity was unchanged and with GDD for MAN was reduced and for FB unchanged. Digestion showed an increased pro-inflammatory effect for MAN, FB1, FB2 and FB3. The UD fractions of MAN, FB1 and FB6 had anti-inflammatory effects. FB5 and FB6 honeys showed increased anti-inflammatory activity after GD and GDD. All honey fractions did not show any cytotoxicity.

In conclusion, FB honey has antioxidant, pro- and anti-inflammatory properties. With digestion, GD activity was either increased or unchanged while with GDD activity was reduced, lost or unchanged. Observed effects were either due to pH and/or digestive enzyme activity. FB honey with its shown bioactivity could be an important local nutraceutical product.

Keywords: Fynbos, Manuka, honey, physicochemical, antioxidant activity, anti-inflammatory activity, *in vitro* simulated digestion, cellular antioxidant activity, LPS/IFN-γ, nitric oxide (NO).

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Declaration

I, Innocentia Botlhale Magoshi hereby declare that this research dissertation is my own work. It is being submitted for the degree of Masters of Science in Anatomy with specialization in Human Cell Biology at the University of Pretoria, South Africa. This dissertation has not been submitted before for any degree at any University.

_________________________ _________________________

IB Magoshi **Date**

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List of abbreviations

%	Percentage
˚C	Degree centigrade
±	Plus or minus
≤	Less than or equal to
µg/ml	Microgram per millilitre
µl	Microlitre
µM	Micromolar
µmol	Micromole
µmol/ml NO_2^-	Micromole per millilitre nitrite
µmol/L	Micromol per litre
AA	Arachidonic acid
AAPH	2,2'-Azobis(2-amidino-propane) dihydrochloride
ABS_{450}	Colour intensity at 450 nm
ABTS	2,2'-Azo-bis(3-ethylbenzothiazoline-6-sulfuric acid) diammonium salt
AEAC	Ascorbic acid equivalent antioxidant content
AGP	Arabinogalactans
$AlCl_3$	Aluminium chloride
ANOVA	Analysis of variance
AU	Absorbance units
AUC	Area under the decay curve
Av	Average
BC	Before Christ
BSA	Bovine serum albumin
$C_1 – C_5$	Carbon 1 to 5
CAA	Cellular antioxidant activity
Caco-2	Human colon adenocarcinoma cell line
CE	Catechin equivalents
CH_2	Ethylene
cm^2	Centimeters square

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COOH	Carboxylic acid
COX	Cyclooxygenase
DCFH-DA	Dichloroflourescein diacetate
ddH_2O	Double distilled water
DMEM	Dulbecco's Modified Essential Medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-2-picrylhydrazyl
ECACC	European collection of cell cultures
EDTA	Ethylene diamine tetra acetic acid
eNOS	Endothelial nitric oxide synthase
ET	Electron transfer
FB	Fynbos
FB1 - FB6	Fynbos 1 to 6
F-C reagent	Folin-Ciocalteu reagent
FCS	Foetal calf serum
FL	Fluorescein sodium salt
FRAP	Ferric reducing antioxidant potential
Fru	Fructose
g	Gram
g/kg	Gram per kilogram
GAE	Gallic equivalents
GD	Gastric digest
GDD	Gastro-duodenal digest
GIT	Gastrointestinal tract
Glc	Glucose
h	Hour or hours
H_2O	Water
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulphuric acid

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H_3PO_4	Phosphoric acid
HAT	Hydrogen atom transfer
HCA	Hydroxycinnamic acid
HCl	Hydrochloric acid
HEAE	Honey ethyl acetate extract
HETes	Hydroxyeicosatetraenoic acids
HIV	Human immunodeficiency virus
HME	Methanol extract of honey
HMF	Hydroxymethylfurfural content
HNO_2	Nitrous acid
HORAC	Hydroxyl radical averting capacity
HpETEs	Hydroperoxyeicosatetraenoic acids
HPLC-DAD-MS/MS	High liquid chromatography-diode array detection-tandem mass spectrometry
IL-1	Interleukin-1
IL-1β	Interleukin-1beta
IFN-α	Interferon-alpha
IFN-β	Interferon-beta
IFN–γ	Interferon-gamma
iNOS	Inducible NOS
J774	Murine macrophage cell line
$K_2S_2O_8$	Potassium peroxodisulfate
$K_3FE[CN]_6$	Potassium ferricyanide
KCl	Potassium chloride
KH_2PO_4	Potassium dihydrogen phosphate
L929	Murine fibrosarcoma cell line
LAL	Kinetic limulus amebocyte lysate assay
LOX	Lipoxygenase
LPS	Lipopolysaccharide
LSD	Least significant difference
LTs	Leukotrienes

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MAN	Manuka
MAPK	Mitogen-activated protein kinases
Max	Maximum
mg	Milligram
mg/ml	Milligram per millilitre
MGO	Methylglyoxal
Min	Minimum
min	Minutes
ml	Milliliter
mM	Millimolar
MM6	MonoMac-6 cell line
MMP-9	Matrix metalloproteinases-9
MMPs	Matrix metalloproteinases
mRNA	Messenger RNA
MRPs	Maillard reaction products
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Na_2CO_3	Sodium carbonate anhydrous
Na_2HPO_4	Disodium hydrogen phosphate
$NaH_2PO_4.H_2O$	Sodium dihydrogen phosphate monohydrate
$Na_2HPO_4.2H_2O$	Sodium phosphate dibasic dihydrate
NaCl	Sodium chloride
NaH_2PO_4	Sodium dihydrogen phosphate
$NaHCO_3$	Sodium hydrogen carbonate
$NaNO_2$	Sodium nitrite
$NaNO_3$	Sodium nitrate
NaOH	Sodium hydroxide
NED	N-1-naphthylethylenediamine dihydrochloride
NFκB	Nuclear factor-κB
ng/g	Nanogram per gram
ng/ml	Nanogram per millilitere

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nm	Nanometer
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
NSAIDs	Non-steroidal anti-inflammatory drugs
NY	New York
O_2	Oxygen
O_2^-	Superoxide anion
OD	Oxidative damage
$^{\cdot}OH$	Hydroxyl radical
ORAC	Oxygen radical absorbance capacity
PBS	Phosphate buffered saline
PGs	Prostaglandins
pH	Measurement of acidity or alkalinity on the logarithmic scale
PLA_2	Phospholipase A_2
PMNs	Polymorphonuclear neutrophils
ppb	Parts per billion
ppm	Parts per million
P-value	Probability value
QEAC	Quercetin equivalent antioxidant content
R/r	Correlation coefficient
RAW 264.7	Mouse murine macrophage cell line
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RT-PCR	Quantitative real-time polymerase chain reaction
SA	South Africa
SC-1	Mouse fibroblast cell line
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Standard error of mean

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SGDD	*In vitro* simulated gastro-duodenal digestion
SNP	Sodium nitroprusside
SOD	Superoxide dismutase
STAT-1	Signal transducer and activator of transcription 1
TE	Trolox equivalent
TEAC	Trolox equivalent antioxidant capacity
TFC	Total flavonoid content
TH1	Type 1 helper T cells
TNF-α	Tumour necrosis factor alpha
TNF-β	Tumour necrosis factor beta
TPC	Total polyphenol content
TX A_2	Thromboxane A_2
U/ml	Units per millilitre
UD	Undigested sample
UMF	Unique Manuka Factor
USA	United States of America
v/v	Volume to volume
VEGF	Vascular endothelial growth factor
w/v	Weight to volume
X	Times

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Chapter 1: Introduction

Honey is a natural sweet substance that is consumed worldwide. It has been used as a food source and a therapeutic agent since the earliest of times. The first reference to be documented on the usage of honey as an ointment and a drug was dated back to 2100 - 2000BC (Crane, 1975). Honey presently is considered as a natural functional food because of its nutritional benefits and its medicinal properties (Gómez-Caravaca *et al*., 2006; Viuda-Martos *et al*., 2008). Functional foods are foods that not only provide sufficient nutrients but improve and promote health, well-being and reduce the risk of development of some chronic diseases (Viuda-Martos *et al*., 2008). These foods also hinder the progression of manifested illness (Viuda-Martos *et al*., 2008) e.g. inflammatory diseases, heart disease, cataracts and cancer (Al-Mamary *et al*., 2002). Honey possesses antimicrobial, antioxidant, wound-healing properties, anti-mutagenic (Alvarez-Suarez *et al*., 2010a) and anti-proliferative properties (Jaganathan and Mandal, 2009). These physiological effects are attributed to the presence of phytochemicals which include polyphenols that act as antioxidants (Jaganathan and Mandal, 2009).

Medical-grade Manuka honey is derived from the Manuka bush, *Leptospermum scoparium* native to New Zealand (Adams *et al*., 2009) and has been identified to have antioxidant (Inoue *et al*., 2005), anti-inflammatory and wound-healing activities (Simon *et al*., 2009). In addition, Manuka honey is renowned for its non-peroxide antibacterial activity (Kwakman and Zaat, 2012). Based on these properties Manuka (MAN) honey is sold as a functional food or is used as an ingredient for wound care products. The scientific characterization of MAN honey has led to the development of an $80 million per year industry in New Zealand. Manuka based products (honey and wound care products) are exported throughout the world and are available in South Africa. These products are expensive and the question rose whether local South African honey types are available that have functional food properties equivalent or even better than that of MAN honey. Serem and Bester (2012) screened 13 honey samples from the southern Africa region and identified that three honey samples from the Western cape of South Africa had high levels of antioxidant activity however these honeys were not compared to MAN honey.

The South African Cape region has been well recognized due to its endemism and richness in plant species (Ojeda *et al*., 2001). The most prevalent vegetation type within this region is Fynbos (Bond and Goldblatt, 1984; Cowling and Hilton-Taylor, 1994). Fynbos (FB) is a local name for a shrub or heathland-like vegetation (Moll, 1990). The main features of FB are that, it grows in mild or semi-Mediterranean climate, is prone to re-occurring fires and the substrata is

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acidic sandstone resulting in poor nutrition sandy soils (Cowling, 1992). FB needs fire for germination and the low nutrient soils and low water-holding capacity, gives it treeless, low-growing and sclerophyllic characteristics (Perrings *et al.*, 2000). Dominant floral species that have been found are the Cape reeds (*Restionaceae*), heath or Erica (*Ericaceae*) family and the Protea (*Proteaceae*) family (Perrings *et al.*, 2000). FB contains about 8700 species, 68% of which is endemic (Cowling, 1992). Due to this biodiversity the Cape Floristic Kingdom, is ranked as the sixth floristic kingdom in the world. Thus honey from this region may be potentially a unique functional food that may possess health promoting properties.

Studies have documented that the beneficial therapeutic effects of honey are its antioxidant activity, antimicrobial and anti-inflammatory properties. However most of these studies have been done on European honeys and very few studies on African honeys exist and even less on FB honey. An anti-fungal study conducted by Theunissen *et al.*, (2001) focused on 3 South African honeys namely the FB, Bluegum and Wasbessie. Results obtained revealed a partial inhibitory effect of all 3 honeys against *Candida albicans*. Wassbessie was the highest and FB was the lowest. Related to antimicrobial activity, FB provided some inhibitory effects against a selection of oral strains of *Streptococci* however not enough to qualify it as a medical-grade product (Basson and Grobler, 2008). Serem and Bester (2012) evaluated the antioxidant properties of 3 FB honey samples and found that these honeys had antioxidant activity and protected cells *in vitro* against oxidative damage. No studies about the anti-inflammatory effects of FB honey could be found.

As a functional food, knowledge on the effect of gastro-intestinal (GIT) digestion on the bioactivity of honey is limited. A study done by O'Sullivan *et al.*, (2013) evaluated the effect of GIT digestion on the antioxidant activity and the DNA protective effects of 4 commercial honeys. The study revealed that antioxidant content of the honeys was maintained throughout *in vitro* simulated GIT digestion, however the antioxidant activity decreased. These honeys following digestion protected Caco-2 cells against oxidative damage. No studies have investigated the effect of digestion on the anti-inflammatory properties of honey.

The aim of this study was to evaluate compared to MAN honey, the physicochemical, antioxidant and anti-inflammatory properties of FB honey. In addition, the effect of simulated *in vitro* GIT digestion on these properties was also investigated.

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Chapter 2: Literature review

2.1 Introduction

Honey is a supersaturated sugar solution (Ball, 2007; Aurongzeb and Azim, 2011) which is produced by different bee *Apis* species (Bogdanov *et al*., 2008). *Apis mellifera* species that are imported from Europe are often used for production of honey in most honey farms (Aurongzeb and Azim, 2011). The composition of honey depends primarily on the nectar (Al-Mamary *et al*., 2002). Nectar is defined as an aqueous solution of proteins, amino acids, enzymes, sugars, lipids, vitamins, minerals, organic acids, trace elements and other components such as aroma compounds and polyphenols (Ball, 2007; Alvarez-Suarez *et al*., 2010a). The sugars are the major components while the bioactive constituents of honey are the minor components. Floral source and environmental conditions (Al-Mamary *et al*., 2002; Ball, 2007) determine the composition of honey and also have a direct impact on the quality and flavour of the honey (Ball, 2007). In numerous nectars, sucrose is the dominant sugar, whilst in some; fructose, sucrose and glucose are present in more or less equal quantities (Ball, 2007).

Bees collect nectar from different floral species and then it is processed into honey. The processing of honey involves the worker bee, collecting nectar from various floral species and storing it in the stomach or honey sac (Jaganathan and Mandal, 2009). During the collection of nectar, pollen can also be collected and is often found in honey. The composition of pollen as well as pollen-derived amino acids depends also on the floral species (Alvarez-Suarez *et al*., 2010a). Processing of nectar involves secretions of enzymes from two glands, the salivary and hypopharyngeal glands. The specific enzymes that are involved are invertase, diastase and glucose oxidase. These enzymes are responsible for breaking down the sugars into monosaccharides (Graham, 1992). In the beehive the worker bee, disgorges or regurgitates the nectar several times before it can be stored in the honeycomb (Jaganathan and Mandal, 2009). The maturing of the nectar into honey, involves two processes which are the conversion of sucrose to fructose and glucose and evaporation of excess water from the nectar in order to prevent the fermentation of the honey (Ball, 2007; Jaganathan and Mandal, 2009). The composition of 300 USA honey samples was determined and the average composition was 79.6% sugars and 17.2% water. The remaining 3.2% accounts for the minor constituents present in honey (Graham, 1992).

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Some of the characteristics of honey, include, high osmotic pressure, acidity (Küçük *et al*., 2007) and low moisture content (Al-Mamary *et al*., 2002; Ball, 2007; Alvarez-Suarez *et al*., 2010a). This is beneficial as it makes honey highly resistant to spoilage triggered by micro-organisms (Al-Mamary *et al*., 2002; Ball, 2007). In addition this high osmolarity is known to be beneficial in inhibiting the growth of bacteria thus promotes wound healing. The acidity of honey is attributed to the presence of organic acids, specifically pyruvic acid, gluconic acid, citric acid and malic acid, in equilibrium with esters or lactones and inorganic ions such as chloride and phosphate (Anklam, 1998). Other characteristics of honey are that it has a high refractive index and viscosity (Graham, 1992).

Research done on honey, has shown that it contains about 181 components and is considered a traditional medicine due to the medicinal properties it possesses (Gómez-Caravaca *et al*., 2006; Viuda-Martos *et al*., 2008). It has been shown to be efficacious in GIT-related ailments e.g. gastric lesions and healing of burns and wounds (Postmes *et al*., 1993; Ladas *et al*., 1995; Molan, 2001). In addition it possesses anti-inflammatory, antioxidant, antimicrobial, anti-mutagenic and anti-proliferative properties (Jaganathan and Mandal, 2009; Alvarez-Suarez *et al*., 2010a).

2.2 Physicochemical properties of honey

The quality of honey is dependent on the physical and chemical properties. The revised version of CODEX Alimentarius (CODEXSTAN12-1981 revision 2001) documents the global regulatory standards for physicochemical properties. Properties that are included are the sugar, moisture and ash contents, proline, hydroxymethylfurfural content (HMF), pH, diastase activity, water insoluble solids content and electrical conductivity. It has been shown that the most prevalent monosaccharides in honey are fructose and glucose and the disaccharide, sucrose is only present in small amounts (Iglesias *et al*., 2004; Ball, 2007). Other, disaccharides that are present in honey in minute quantities are isomaltose, maltose, turanose, nigerose and maltulose. More complex sugars, dextrins and oligosaccharides are reported to be present in honey but only in very small quantities (Sanz *et al*., 2004). Honey with a high sucrose content means it has been harvested early, as sucrose has not been converted to fructose and glucose by the invertase enzyme (Küçük *et al*., 2007). In addition the ratio of reducing sugars (Fru:Glc) in honey is primarily dependent on the nectar source and it indicates how easily the honey will crystallise (Finola *et al*., 2007).

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The moisture content is an important parameter, which determines the amount of water present in honey (Moniruzzaman *et al*., 2013). It is indicative of the source of the plant's floral type (Abu-Tarboush *et al*., 1993) and contributes to the stability of honey against fermentation and granulation during storage (Küçük *et al*., 2007). In addition it is a function of season and climatic factors of the region where the honey is harvested (Finola *et al*., 2007). The quality of honey according to the regulatory standards stipulates the minimum value of the moisture content to be ≤ 20% (CODEXSTAN12-1981 revision 2001). Moisture content and quantification of proline levels are used as a measure of sugar adulteration (Silva *et al*., 2009).

Other physicochemical properties like the diastase activity and HMF content are used as an indicator of the freshness of honey (Küçük *et al*., 2007). HMF can be produced by Maillard reactions or by hexose dehydration in acid media. Storage duration and heating temperature cause an increase in the HMF level (Tosi *et al*., 2002). In addition, the diastase activity is closely related to the heat treatment (Anklam *et al*., 1998). A high quality honey is reported to contain low HMF content and a high diastase activity (Küçük *et al*., 2007).

The ash content indicates the mineral content present in honey. It also relates to the botanical origin of honey and is used to determine if the honey is of a floral, honeydew or mixed origin (Tosi *et al*., 2002; Silva *et al*., 2009). Trace elements found in honey include lithium, nickel, chromium, zinc, lead and tin. Vitamins such as ascorbic acid, niacin, thiamine, riboflavin and pyridoxin are also present in minute quantities (Graham, 1992).

Other enzymes present in honey apart from invertase, diastase and gluocose oxidase are catalase and acid phosphatase (Ball, 2007). Antioxidant activity of honey is mainly due to the enzymatic and non-enzymatic antioxidants (Meda *et al*., 2005). The enzymatic component is due to catalase activity and the non-enzymatic component is due to the polyphenols present. The biological function of catalase is to convert or degrade hydrogen peroxide (H_2O_2) to water (H_2O) and oxygen (O_2). This conversion is ideal in protecting cells against excessive H_2O_2 oxidative damage (Ou *et al*., 1996). Thus measurement of catalase activity indicates the protective ability of honey against H_2O_2-induced oxidative damage. The roles of enzymatic and non-enzymatic antioxidants will be discussed in greater detail in section 2.3.2.

The flavour and the aroma of honey are due to the sugars, acids and other volatile components of honey (Ball, 2007). These volatile components include alcohols and various C_1 – C_5

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aldehydes. Methyl and ethyl formate have been found in honey (Ball, 2007) and many phenylacetic esters are reported to have honey-like tastes and aromas (Guyot *et al*., 1999).

Chemicals that are responsible for colour in honey remain unknown. However some researchers have suggested that the colour is due to the presence of carotenoids and polyphenols. Also the chemical caramelization of saccharides that is catalyzed by acids and a Malliard reaction between amino acids and sugars contributes to honey colour (Ball, 2007). It has been reported that the colour of honey correlates with the concentration of polyphenol content. The darker the honey the greater the polyphenol content and therefore antioxidant activity (Abu-Tarboush *et al*., 1993).

2.3 Bioactivity of honey

2.3.1 Antioxidant activity of honey

Honey has been well documented to have antioxidant activity and therefore a potential therapy for diseases related to oxidative stress (Storz and Imlayt, 1999; Zheng and Wang, 2001; Aljadi and Kamaruddin, 2004; Beretta *et al*., 2005). As mentioned previously, the antioxidant activity is attributed to enzymatic (catalase) and non-enzymatic (polyphenols) components. The polyphenols that are present in honey depend upon the climatic conditions and the geographical area (Jaganathan and Mandal, 2009). Polyphenols have been found to possess anti-thrombotic, anti-carcinogenic, anti-inflammatory, anti-atherogenic as well as antioxidative properties (Salah *et al*., 1995; Cook and Samman, 1996) and the structure of about 8000 polyphenols have been described (Bravo,1998).

Phenolic compounds can be classified into 10 types depending on their basic structure and these are stilbenes, flavonoids, lignins, phenolic acids, simple phenols, xanthones, naphthoquinones, isocoumarins, coumarins and anthraquinones (Jaganathan and Mandal, 2009). The structure associated with each polyphenol class is presented in Figure 2.1. More than 5000 flavonoid compounds have been described and this type of phenolic compound has been considered the most important polyphenolic class. Flavonoids are natural antioxidants that possess numerous biological activities, such as anti-allergic, anti-thrombotic, antibacterial, vasodilatory actions and anti-inflammatory activities (Cook and Samman, 1996). This study will focus on the antioxidant and anti-inflammatory activity of honey and therefore these processes and the role of polyphenols in preventing oxidative damage and inflammation will be described in greater detail.

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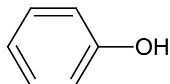
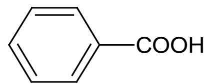
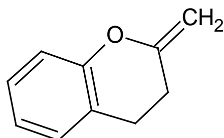
Simple phenols



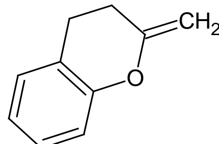
Phenolic acids



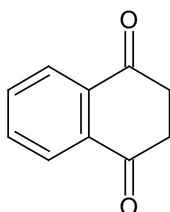
Coumarins



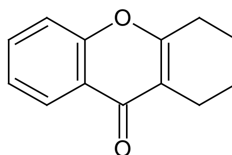
Isocoumarins



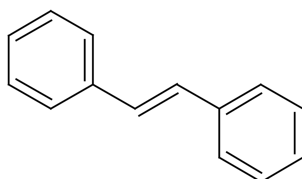
Naphthoquinones



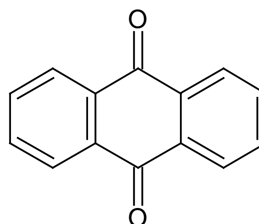
Xanthones



Stilbenes



Anthraquinones



Lignins

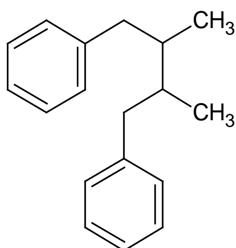


Figure 2.1: Main classes of polyphenolic compounds. Adapted from Vladimir-Knežević *et al.*, (2012)

2.3.2 Oxidative stress and antioxidants

Oxidative stress is an imbalance between the production of free radicals and reactive metabolites which include oxidants or reactive oxygen species (ROS) and their elimination. An imbalance induces damage to cellular biomolecules including DNA, proteins, lipids and carbohydrates present in cells or tissues. This damage results in membrane impairment and random cross linking of biomolecules such as structural proteins, enzymes and DNA. Such

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events can lead to cell death due to lipid peroxidation and DNA fragmentation. Oxidative stress has been implicated as a causative agent in chronic diseases like diabetes, cancer, neurodegenerative disorders, autoimmune disorders and cardiovascular diseases (Vladimir-Knežević *et al.*, 2012).

Formation of ROS, at normal levels plays a vital role in cellular systems. ROS products stimulate cellular signalling pathways when there are changes in the extracellular and intracellular environmental conditions. Most ROS are produced by the mitochondrial respiratory chain. In addition aerobic cells also produce ROS during endogenous metabolic reactions. These are H_2O_2, superoxide anion (O_2^-), hydroxyl radical ($^{\cdot}OH$) and organic peroxidases as a result of oxygen reduction. The electron transfer to oxygen occurs in the electron transfer chains, localized in the mitochondrial membrane. Under hypoxic conditions, mitochondrial respiratory chains tend to generate nitric oxide (NO), which produces reactive nitrogen species (RNS). ROS/RNS can increase the generation of oxidants or reactive species leading to increased lipid peroxidation. Antioxidant defences prevent or delay further damage by ROS/RNS, either by involving endogenous enzymatic antioxidants (glutathione reductase, glutathione peroxidase, superoxide dismutase (SOD) and catalase) or by non-enzymatic defences (glutathione, vitamins, polyphenolics) which are mostly acquired through the diet (Vladimir-Knežević *et al.*, 2012). Antioxidants can be defined as any substances that have the ability to significantly delay or prevent the oxidation of substrates when present at lower concentrations compared to the oxidizable substrate (Halliwell, 1990). Antioxidants can inhibit, prevent or delay the oxidation of oxidizable substrates by scavenging free radicals, suppressing the formation of radicals and by chelating metal ions that are involved in the production of radicals (Vladimir-Knežević *et al.*, 2012). Therefore antioxidants in general are essential in combating oxidative stress and preventing chronic diseases.

Enzymatic antioxidants are present in all eukaryotic organisms. Major classes of these enzymatic antioxidants are SOD, catalases and glutathione peroxidases (Sies, 1997). The main roles of these antioxidants are to break down and remove radicals. SOD, found in extracellular fluids breaks down O_2^- (causative of lipid peroxidation) into H_2O_2 and O_2, a process in which zinc, copper, iron and manganese are important co-factors. As mentioned above catalases scavenge radicals by degrading H_2O_2 into H_2O and O_2. Glutathione peroxidase and glutathione reductase contain selenium and these enzymes are responsible for the degradation of H_2O_2 as

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well as organic peroxides into alcohols. Levels of these enzymatic antioxidants vary according to cell type and subcellular compartments (Soboll *et al*., 1995).

Non-enzymatic antioxidants, intercept radicals that are formed so as to avoid deleterious reactions (Sies, 1997). The interaction of radicals with non-enzymatic antioxidants yields non-reactive and non-radical endpoints (Sies, 1997). Another mechanism that has been noted is that these non-enzymatic compounds have the ability to shift the radical function away from the most delicate target sites of the cells to other compartments, resulting in a minimal deleterious effect (Sies, 1997). For example, in the blood the deleterious radical effects can be directed away from targeted cellular lipoproteins to the plasma which often depending on the diet has a high beneficial antioxidant status. Alternatively the deleterious radical effects can be directed away from the cellular membrane to the cytosol, demonstrating a shift from the targeted hydrophobic phases to aqueous phases thus producing minimal effects (Sies, 1997). Such chain-breaking antioxidants include carotenoids, plant polyphenols, vitamins C and E.

Natural antioxidants are vitamins C and E, carotenoids, ascorbic acid, phenolic compounds (phenolic acids and flavonoids) and nitrogen compounds which include the amines, amino acids, alkaloids and chlorophyl derivatives (Larson, 1988; Hall and Cuppet, 1997). These natural antioxidants as well as elements like peroxidases, products of the Maillard reaction and catalases are associated with the antioxidant activity of honey (Gheldof and Engesth, 2002). The greatest influence on the antioxidant activity of honey is the botanical origin while the handling, processing and the storage has a minor influence on activity (Frankel *et al*., 1998; Al-Mamary *et al*., 2002; Gheldof and Engesth, 2002; Gheldof *et al*., 2002; Beretta *et al*., 2005). The potential and the ability of honey in reducing oxidative reactions within food systems and human health is termed or referred to as the antioxidant activity or antioxidant capacity of honey (Alvarez-Suarez *et al*., 2010a).

2.3.2.1 Honey antioxidant activity or antioxidant capacity studies

There are numerous antioxidant activity studies done on honey most of them on European honeys and very little on African honeys. Table 2.1 gives a summary of several recent studies. In these studies irrespective of origin and type there is a strong correlation between antioxidant content namely the total polyphenolic content (TPC), total flavonoid content (TFC) and antioxidant activity determined using different types of antioxidant assays and these include the

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DPPH (2,2-diphenyl-2-picrylhydrazyl), TEAC (Trolox equivalent antioxidant capacity), ORAC (Oxygen radical absorbance capacity) and FRAP (Ferric reducing antioxidant potential) assays.

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Table 2.1: Antioxidant activity studies on honey

Honey origin	Sample number; type	Assays	Correlations	Reference
Malaysian	2; Gelam and coconut	TPC, FRAP	**FRAP** vs. TPC; r = 0.87	Aljadi & Kamaruddin (2004)
Romanian	24; Acacia, lime, sunflower and honey dew flavours	TPC, TFC, DPPH	**TPC** vs. TFC, r = 0.84, vs. DPPH; r = 0.97 **TFC** vs. DPPH; r = 0.91	Al *et al.,* (2009).
Slovenian	7; Dark (fir, spruce, forest, chestnut), light (acacia, lime, multifloral)	Colour, TPC, FRAP, DPPH	**TPC** vs. FRAP; r = 0.97, vs. DPPH; r = 0.93 **FRAP** vs. DPPH; r = 0.89, **Colour** vs. TPC; r = 0.91, vs. FRAP; r = 0.85 vs. DPPH; r = 0.43	Bertoncelj *et al.,* (2007)
Different geographical and floral origin	14; Burkinabe, honeydew, buckwheat, strawberry tree, clover, sulla, chestnut, acacia, chicory, dandelion and mutli-flora	Colour, TPC, FRAP, DPPH, ORAC	**Colour** vs. TPC r = 0.93, vs. FRAP r = 0.92, vs. DPPH r = 0.88 vs. ORAC r = 0.73 **TPC** vs. FRAP r = 0.89, vs. DPPH r = 0.92 vs. ORAC r = 0.87, **FRAP** vs. DPPH r = 0.89 vs. ORAC r = 0.72 **DPPH** vs. ORAC r = 0.86	Beretta *et al.,* (2005)
Burkinabe (Burkina Faso)	27, Multifloral, acacia, vitellaria, honeydew, combretaceae and lannae	TPC, TFC, DPPH, AEAC, QEAC	**TPC** vs. TFC r = 0.11, **DPPH** vs. vs. TPC r = 0.50, vs. QEAC r = 0.95, vs. AEAC r = 0.95, **AEAC** vs. QEAC r = 0.99	Meda *et al.,* (2005)
Southern African	13; Agricultural, South East Mozambique, Eastern and Western Cape	Colour, TFC, TPC, DPPH, TEAC, ORAC	**Colour** vs. TPC r = 0.89, vs. TFC r = 0.89, vs. TEAC r = 0.91, vs. DPPH r = 0.62 vs. ORAC r = 0.89. **TPC** vs. TFC r = 0.87, vs. TEAC r = 0.95, vs. DPPH r = 0.72 vs. ORAC r = 0.94, **TFC** vs. TEAC r = 0.89, vs. DPPH r = 0.64 vs. ORAC r = 0.88, **DPPH** vs. TEAC r = 0.67 ORAC r = 0.72, **TEAC** vs. ORAC r = 0.89	Serem and Bester (2012)

AEAC (ascorbic acid equivalent antioxidant content), QEAC (quercetin equivalent antioxidant content)

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2.3.2.2 Effect of digestion on the antioxidant activity

A functional food is one which improves health physiologically beyond inherent nutrition (Viuda-Martos *et al*., 2008). Once a food has been researched and shown to have potential functionality, it is important to further test its efficacy post GIT digestion. This is to ascertain that the physiological activities are maintained and that the food is bioactive. A study done by Tagliazucchi *et al.,* (2010) investigated the *in vitro* bioaccessibility (the release of compounds from a food matrix) of the major polyphenolic classes as well as the changes in antioxidant activity of grape polyphenols. Using the FRAP and ABTS (2,2'-Azo-bis(3-ethylbenzothiazoline-6-sulfuric acid) diamonium salt)/TEAC assay it was found that with gastric digestion the amount of bio-accessible polyphenols, flavonoids and anthocyanins increased. However transition to the intestinal environment caused a decrease in measured activity. Of the polyphenol classes the anthocyanins were the most unstable at a neutral or slightly basic pH. Differences in the stability of different polyphenols were also reported where under pancreatic conditions, the duodenal phase of digestion, resveratrol and the phenolic acids were degraded whereas quercetin and catechin remained unchanged (Tagliazucchi *et al*., 2010).

Wootton-Beard *et al.,* (2011) evaluated the total antioxidant activity, total phenolic content and the stability of the antioxidant activity of 23 vegetable commercial juices pre- and post *in vitro* simulated digestion. Analysis by the FRAP and DPPH assays, revealed a pattern, whereby the antioxidant activity significantly increased with gastric digestion and decreased with duodenal digestion, however the decrease still left the samples with higher antioxidant activity relative to the undigested or pre-digested juice samples. Scavenging of the ABTS radical, was shown to increase for 18 juices after gastric digestion. In the duodenal digestion, most of the juices showed a small decline in the activity although, some had an increase of activity. Phenolic content increased post gastric and duodenal digestion for a majority of samples. Noted results for most vegetable juices showed that the total antioxidant capacity was stable or even enhanced for the majority of vegetable juices throughout digestion.

A honey study done by O'Sullivan *et al.,* (2013) investigated antioxidant activity of commercial honeys pre and post *in vitro* simulated digestion. In this study the total polyphenol content remained unchanged, however the antioxidant activity of honeys decreased significantly after gastroduodenal digestion.

Changes in antioxidant activity during GIT digestion are due to increased extraction of polyphenols at low pH, the specific structure and consequently the instability of these

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polyphenols at a neutral or slightly basic pH. Bioavailability is another important consideration when evaluating the effects of digestion on antioxidant activity.

In vitro studies try to mimic *in vivo* studies as closely as possible however, it does not always represent systemic interactions (Youdim *et al*., 2000). To address these differences Youdim *et al.,* (2000) evaluated the antioxidant protective effects of blueberry polyphenols. The *in vitro* results showed that blueberry hydroxycinnamic acids (HCA) and anthocyanins significantly protected erythrocytes against H_2O_2-induced oxidative damage. Observed effects were time and concentration-dependent and HCA was the most potent. *In vivo* results with oral supplementation of HCA and anthocyanins to the Sprague-Dawley male rats, showed a significant increase of both HCA and anthocyanins in plasma. This implies these polyphenols are absorbed, are bioavailable and based on *in vitro* studies protect rat erythrocytes against oxidative damage.

2.3.3. Inflammation

Chronic diseases like diabetes, cancer, neurodegenerative disorders, autoimmune disorders and cardiovascular diseases (Vladimir-Knežević *et al*., 2012) develop due to increased oxidative stress. A hallmark of these diseases is inflammation. Inflammation is a physiological response that is triggered by infection and tissue injury such as that induced by oxidative stress (Majno and Joris, 1996).

The acute inflammatory reaction involves the delivery of plasma and leukocytes to the site of injury (Majno and Joris, 1996). This initial recognition is mediated by the resident mast cells and macrophages which in turn trigger the production of inflammatory mediators including cytokines, chemokines, products of proteolytic cascades, vasoactive amines and eicosanoids which causes the migration of plasma cells and leukocytes (neutrophils) from the blood vessels to the extravascular tissue at the site of injury. Neutrophils at the affected tissue become activated by either the cytokines or the invading pathogen. This then, triggers the production and release of ROS/RNS species, elastase, proteinase 3 and cathepsin G (Nathan, 2006). A successful acute inflammatory response will then eliminate invading agents and the repair phase will start and this involves lipoxins that inhibit neutrophils and recruit monocytes which remove dead cells and commence tissue remodelling (Serhan and Savill, 2005). If however the acute inflammatory response fails in eliminating infectious pathogens then the neutrophil infiltrate becomes replaced by macrophages and T cells (in a case of infection). Further failure to eliminate invading agents results in chronic inflammation and the associated formation of tertiary lymphoid tissues and granulomas (Drayton *et al*., 2006). Besides the persistent pathogens, chronic inflammation can also be triggered by

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undegradable foreign bodies and autoimmune response due to the persistence of self-antigens. Inflammation involves several biochemical pathways and includes the arachidonic acid (AA) pathway, activation of nitric oxide synthase (NOS) enzymes, production and release of cytokines, the nuclear factor-κB (NFκB) and mitogen-activated protein kinases (MAPK) pathways.

2.3.3.1 Inflammatory mechanisms and polyphenols

2.3.3.1.1 Arachidonic acid (AA) pathway

In the arachidonic pathway, AA which is released from the membrane phospholipids by the phospholipase A_2 (PLA_2) enzyme can then be metabolised by the cyclooxygenase (COX) and lipoxygenase (LOX) pathways to yield, molecules such as prostaglandins and leukotrienes which are also known as eicosanoids (Santangelo *et al*., 2007). Anti-inflammatory actions of dietary polyphenols have been shown to inhibit COX, LOX and PLA_2 enzymes, thus reducing the production of AA, prostaglandins and leukotriens as shown in Figure 2.2 (Yoon and Baek, 2005).

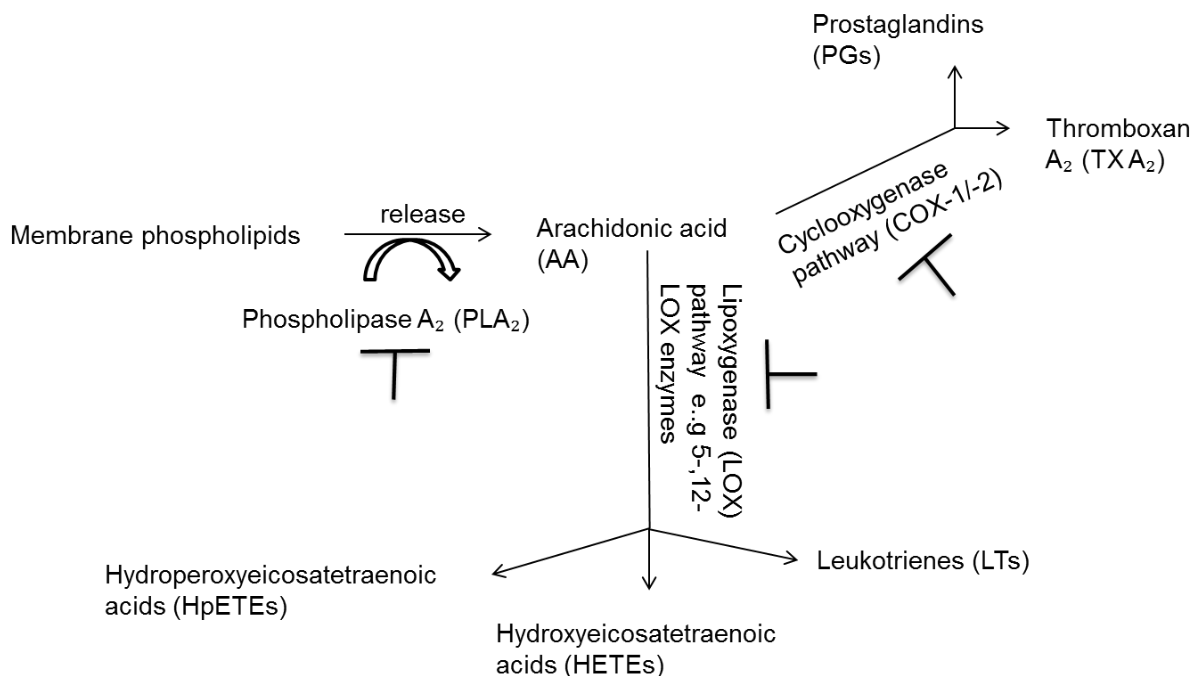

Figure 2.2: The arachidonic pathway. The potential inhibitory sites of polyphenols are shown by inhibiting PLA_2, COX-1/-2 and 5-, 12-LOX enzymes, therefore reducing the formation of AA, PGs and LTs. Adapted from Santangelo *et al.,* (2007).

2.3.3.1.2 Nitric oxide synthase (NOS) family

Nitric oxide is a gaseous free radical and a cellular mediator (Tsai *et al*., 2007). It Is an important inter and intracellular regulatory molecule (Bor *et al*., 2006). Enzymatically, NO is formed through the oxidation of the terminal guanidine nitrogen atom of L-arginine by the

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nitric oxide synthase (NOS) enzyme. There are 4 forms of NOS enzymes (Tsai *et al*., 2007). Neuronal nitric oxide synthase (nNOS) is found in the neuronal tissue and acts as neurotransmitter. Another form of NOS is known as the endothelial nitric oxide synthase (eNOS), which is found in the endothelium and has a regulatory role in vasodilation. Both of these enzymes (nNOS and eNOS) are constitutive isoforms of NOS which produce NO at very low levels (Tsai *et al*., 2007). The third isoform is the mitochondrial NOS, which is less understood (Ghafourifar and Richter, 1997) and the fourth isoform is known as inducible NOS (iNOS). This form of NOS can be induced by various cytokines including interferon gamma (IFN–γ) and tumour necrosis factor alpha (TNF-α) in endothelial cells, macrophages and hepatocytes. It can also be induced by the bacterial lipopolysaccharide (LPS) and it is involved in host immune defence system against invading pathogens (Mayer and Hemmens, 1997). Overproduction of NO by iNOS, increases the production of RNS and promotes cellular damage by having a detrimental effect on cellular macromolecules. Additionally the excess NO production by iNOS has been implicated in many diseases such as, diabetes, atherosclerosis, renal disease and cancer (Beckman and Koppenol, 1996; Cooke and Dzau, 1997). Thus the scavenging of NO or the suppressing of the iNOS enzyme by the phenolic compounds present in honey can therefore illustrate its anti-inflammatory property (Liang *et al*., 1999; Kim *et al*., 2004; Tsai *et al*., 2007).

2.3.3.1.3 Cytokines

Cytokines are small proteins that are triggered by inflammation. These molecules regulate immune response and are released by macrophages, lymphocytes and other types of cells such as glial cells, neurons and endothelial cells (Barrett *et al*., 2010). Once released, these molecules can act in a paracrine or autocrine manner. There are more than 100 documented cytokines and some relevant to this study are summarised in Table 2.2.

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Table 2.2: Examples of cytokines and their clinical relevance. Adapted from Delves and Roitt (2000)

Cytokine	Cellular sources	Major activities	Clinical relevance
Interleukin-1 (IL-1)	Macrophages	Activation of macrophages and T-cells which promote inflammation	Involved in the pathogenesis of rheumatoid arthritis, septic shock and atherosclerosis
Interleukin-8 (IL-8)	Macrophages and T-cells	Chemotaxis of basophils, neutrophils and T-cells	Increased levels in diseases that are accompanied by neutrophils therefore a potential disease marker
TNF-α	Mast cells, natural killer cells, macrophages, T and B cells	Promotes inflammation	Relevant in the treatment of rheumatoid arthritis
Lymphotoxin (tumour necrosis beta (TNF-β)	B cells and type 1 helper T cells (TH1)	Promotes inflammation	Involved in the pathogenesis of insulin-dependent diabetes mellitus and multiple sclerosis
Interferon-alpha (IFN-α)	Cells that are virally infected	Induces resistance of cells to viral infection	Used to treat HIV related Kaposi sarcoma, chronic hepatitis B and C infections and melanoma
Interferon-beta (IFN-β)	Cells that are virally infected	Induces resistance of cells to viral infection	Used to reduce the severity and frequency of relapses in multiple sclerosis
IFN–γ	Natural killer cells and TH1 cells	Activates macrophages and inhibits type 2 helper T cells (TH2)	In chronic granulomatous disease, it enhances the killing of phagocytised bacteria

There are pro-inflammatory and anti-inflammatory cytokines. Pro-inflammatory cytokines are usually released by macrophages and play a role in up-regulating inflammatory reactions. Examples of pro-inflammatory cytokines include TNF-α, IL-6 and IL-1β. Anti-inflammatory cytokines regulate the response of the pro-inflammatory cytokines. These include IL-4, IL-10, IL-13 (Zhang and An, 2007). Cytokines have been implicated in many inflammatory diseases and the balance between the effects of anti-inflammatory and pro-inflammatory cytokines is thought to be involved in the outcome of the disease (Dinarello, 2000; Gabay, 2006). Flavonoids have been shown to alter the production of these cytokines by decreasing their expression (Santangelo *et al.*, 2007). This implies that molecules such as specific polyphenols have very specific protein or gene targets. More importantly honey has been shown as an immunomodulator in wound healing (Majtan, 2014). During acute inflammation, honey has been shown to trigger the release of pro-inflammatory cytokines. A study performed by Tonks *et al.*, (2003) illustrated the effect of honey on the release of pro-inflammatory cytokines (TNF-α, IL-6 and IL-1β) from the MM6 cell line and the human peripheral monocytes. Of the three assayed honeys (Manuka, pasture and jellybush honey) it was found that jellybush honey triggered the maximal release of cytokines. Another similar

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study by Timm *et al*., (2008), further confirmed that the effect of natural honeys including Manuka, induced the release of IL-6 cytokine from MM6 cell line. Therefore according to Matjan (2014), the pro-inflammatory effect of honey is ideal for acute wound healing. Also the immunomodulatory effect of honey has also been seen in chronic inflammation. During chronic inflammation, honey can hinder the release of pro-inflammatory cytokines and other factors such as ROS. A schematic diagram showing the immunomodulatory effect of honey during acute and chronic inflammation is shown in Figure 2.3.

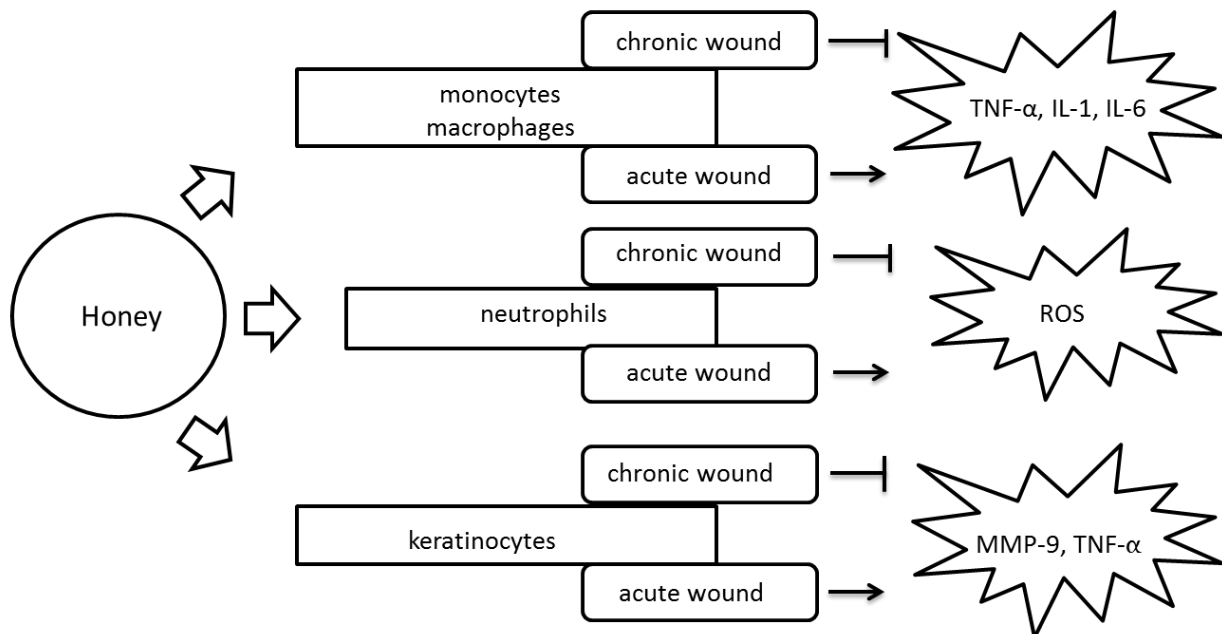

Figure 2.3: The immunomodulatory property of honey, shown on immune (monocytes, macrophages and neutrophils) and cutaneous, keratinocytes. During the inflammatory and proliferative wound healing phases, honey can stimulate the release of pro-inflammatory cytokines, ROS and MMP-9 respectively. These inflammatory factors are necessary for acute wound healing. However in chronic wounds, honey can also reduce the levels of ROS, MMP-9 and pro-inflammatory cytokines. Adapted from Majtan (2014).

2.3.3.1.3 NFκB pathway and mitogen-activated protein kinases (MAPK) pathway

NFκB/Rel transcription factors have been implicated in many acute and chronic inflammatory diseases (Santangelo *et al*., 2007). NFκB is involved in the proliferative, immune, stress, apoptotic and inflammatory responses of cells to various different stimuli (Karin and Ben-Neriah, 2000). It co-ordinates the induction of various genes that encode for chemokines, pro-inflammatory cytokines, inducible enzymes such as COX-2 and vascular endothelial growth factor (VEGF), immune receptors, iNOS, growth factors, matrix metalloproteinases (MMPs) and acute-phase proteins (Nam, 2006). A way of treating inflammatory disorders is to inhibit the activation of NFκB and flavonoids have been found to inhibit this pathway at various steps (Rahman *et al*., 2004; Rahman *et al*., 2006). As much as NFκB activates most of the inflammatory genes, it also depends on other sequence transcription factors such as

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MAPK (Karin, 1995; Karin 2005). MAPK regulate cellular processes at transcriptional level. These cellular processes include cell death, proliferation, differentiation and growth. Additionally these protein kinases are involved in the synthesis of inflammatory cytokines at a transcriptional and translation level. The MAPK pathway consists of a three-tiered cascade and polyphenols have been shown to hinder this pathway at various steps (Soobrattee *et al.*, 2005; Santangelo *et al.*, 2007).

2.3.3.2 Anti-inflammatory studies

Honey has been shown to promote wound healing and reduce inflammation in GIT disorders. Kassim *et al.,* (2010a) evaluated the anti-inflammatory effects of organic extracts of Malaysian honey. Phenolic compounds of honey were extracted using ethyl acetate (HEAE) and methanol (HME). The ability of both extracts to inhibit NO production in a stimulated mouse macrophage (RAW 264.7) cell line was evaluated. In addition the ability of each extract to protect the murine fibrosarcoma (L929) cells against TNF-α was also determined. Ellagic acid was the most abundant polyphenol and other polyphenols present in the honey extracts were gallic acid, ferulic acid, caffeic acid and chlorogenenic acid and myricetin. Extracts inhibited NO formation with median maximal concentrations of 37.5 µg/ml (HEAE) and 271.7 µg/ml (HME). Protection against TNF-α cytotoxicity was at 168.1 µg/ml (HEAE) and 235.4 µg/ml (HME). The observed anti-inflammatory effects were attributed to the presence of the phenolic compounds.

The human complement pathway is part of an innate defence system that responds to invading pathogens. This complex pathway involves complement factors such as C3b and C5a which activate the polymorphonuclear neutrophils (PMNs) and attract them to the wound site (van den Berg *et al.*, 2008). The PMNs then produce a cascade of ROS species ideal for wound repair. Excessive and continuous ROS formation leads to chronic inflammation and associated cellular and tissue damage. van den Berg *et al.,* (2008), evaluated the antioxidant and anti-inflammatory activities of several different types of honey in order to identify a honey type that can be developed as a wound dressing. Using *in vitro* assays the ability of each honey to prevent ROS and the O_2^- anion formation as well as activation of the human complement pathway was determined.

Honeys that were used this study were, Manuka honey (*Leptospermum scoparium*), honey from the Maule region of Chile, two samples of dark-brown American buckwheat honey *(Fagopyrum esculentum*) from New York (NY) and North Dakota, 2 honey samples from Hawaii: rare kiawe (*Prosopis pallida*) and a brown macadamia honey (*Macadamia integrifolia)* and a Canadian mixture of alfalfa and clover honeys. Honeys were compared to

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Manuka and the Chilean honeys, because both of these honeys are medical grade honeys used commercially for wound-healing (van den Berg *et al*., 2008).

All honeys inhibited ROS and activated PMNs with buckwheat honey (NY), showing the greatest inhibition among all honeys. Subsequently this honey was used as a wound dressing in a clinical study involving 21 burn patients with open wounds. An application of MelMax (a wound dressing product that contains NY buckwheat honey and an ointment that had synthetic metal ions and citric acid) resulted in a spontaneous 94% full wound closure. The mean healing time was 25.6 days and there was reduced exudates and slough. In addition recruitment of granulation tissue with rapid epithelialization from the edges of wounds was seen as well as reduced microbial growth. In conclusion, buckwheat honey (NY) showed higher antioxidant and anti-inflammatory activities *in vitro* as well as wound healing effects (van den Berg *et al*., 2008).

An *in vivo* study done by Owoyele *et al.,* (2011), investigated the effects of honey on acute and chronic inflammation using animal models as well as the effect of honey on the NO production in Wister rats. The carregeenan-induced oedema model (acute inflammation), the cotton-pellet model (chronic inflammation), formaldehyde-induced arthritis models (chronic inflammation) were established and the effects on inflammation were evaluated. The anti-inflammatory effects of honey were compared to indomethacin, an established anti-inflammatory drug. In the acute model the effects of honey 10 g/kg body weight was less effective than the control, indomethacin. In the models of chronic inflammation a dose-dependent inhibition of inflammation was observed which was similar to indomethacin. Honey inhibited NO formation in all models. In these animal models of inflammation, honey reduced inflammation and NO formation and these effects were attributed to the presence of polyphenols (Soler *et al*., 1995; Busserolles *et al*., 2002).

2.3.3.3 Effect of digestion on the anti-inflammatory effects of honey

Digestion has been seen to have variable effects on antioxidant activity, therefore it is important to assess or investigate the effect of digestion on anti-inflammatory activity. An *in vivo* study performed by Al-Mazrooa and Sulaiman, (1999), investigated the effects of honey on stress-induced ulcers in rats. Administration of different doses of honey by an intraperitoneal injection was given 20 min before stress was induced. Honey was found to lower the incidence of stress-induced ulcers in a dose-dependent manner compared to the control. It was concluded that honey provides gastro-protection and that its ability to reduce ulcers is attributed to the presence of polyphenols in honey.

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2.4 Honey and gut health

Honey has been shown to be an effective remedy in wound healing and infections (Ndayisaba *et al*., 1992; Molan, 2001). Honey on wounds, reduces inflammation, exudation and oedema. It clears infection, stimulates granulation, angiogenesis and epithelialization which increases the rate of wound healing (Molan, 2001). Likewise honey effectively promotes the healing of ulcers of the GIT.

Evidence has shown that honey can provide gastro-protection against infections of bacteria and ulcers (Postmes *et al*., 1993; Ladas *et al*., 1995; Molan, 2001). An *in vivo* study done by Mahgoub *et al.,* (2001), evaluated the therapeutic effects of natural honey on induced colitis in rats. Over a period of 4 days rats were given honey and a glucose, sucrose, fructose, maltose mixture orally and rectally. On the third day colitis was induced with 3% acetic acid. Honey was found to induce a dose-dependent protective effect against colitis as well as preventing a reduction in antioxidant enzyme levels.

Ali (2003) investigated the gastro-protective role of honey against ammonia-induced gastric lesions in rats. Ammonia is formed by urease, an enzyme found in *Helicobacter pylori.* Excess ammonia formation causes damage to the mucosal lining of the GIT. Findings were that honey and sucralfate (a drug against gastric and duodenal ulcers) reduced the size of the lesions as well as the levels of non-protein sulfhydryl, which includes glutathione, an antioxidant tripeptide in the gastric mucosa. This study identified honey as a potential agent in the management of mucosal lesions associated with the *Helicobacter pylori* bacterium.

2.5 Aim and objectives

Africa is a large exporter of honey and honey is widely used as a source of sweetness and for medicinal purposes. Extensive literature regarding therapeutic properties of honey e.g. antioxidant and antimicrobial activities have been documented on European/Western honeys produced by European honey bees, *Apis mellifera*. Very little information is known about African honeys produced by African bees (*Apis mellifera scutellata*) especially from the southern Africa region which also has the Cape bee population (*Apis mellifera capensis*). One of the few studies available on the southern African honeys reviewed the antioxidant activity, biological and cellular protective effects of the honeys as well determining the physicochemical properties (Serem and Bester, 2012). Additionally, there is limited information about the profiling of anti-inflammatory activities of African honeys as well as studies investigating the effect of *in vitro* simulated GIT digestion on these bioactivities. Regarding specifically Fynbos honey all published studies have focused on the antimicrobial activity of Fynbos honey.

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The effects of pre and post *in vitro* simulated GIT digestion on the bioactivities of functional foods such as fruits, vegetables and grains has been widely researched. Only a single study by O'Sullivan *et al.,* (2013) where the authors investigated the effects of digestion on the bioactivity of honey could be found, although there is evidence based on animal studies that with digestion bioactivity is retained (section 2.3.2.2).

The aim of this study was to address the identified gaps with the aim of determining the antioxidant and anti-inflammatory activities of Fynbos honey pre and post *in vitro* simulated gastro-duodenal digestion.

The aim was achieved using the following objectives:

1. To determine the physicochemical properties of 1 MAN and 6 FB honeys in order to show that the honeys used in this study comply with several aspects of the revised version of the CODEX Alimentarius (CODEXSTAN12-1981 revision 2001).
2. Using an *in vitro* simulated GIT digestion model, for undigested and digested honey samples determine the,
    a. total polyphenol and flavonoid content,
    b. antioxidant activity (TEAC and ORAC assays),
    c. cellular antioxidant protection effects in the Caco-2 (human colon adenocarcinoma) and SC-1 (mouse fibroblast) cell lines using the dichlorofluorescein diacetate (DCFH-DA) assay,
    d. and then to evaluate the effect of pH change and digestive enzymes on the antioxidant properties of honey at each phase of digestion.
3. To further determine for undigested and digested honey the following inflammatory properties;
    a. the chemical NO scavenging activity,
    b. NO inducing and scavenging abilities in the RAW 264.7 cellular model,
    c. and finally evaluate the effect of pH change and digestive enzymes on these activities at each phase of digestion.

The results of objectives 1, 2 and 3 are presented in chapters 3, 4, and 5 respectively.

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Chapter 3: The physicochemical, antioxidant and anti-inflammatory properties of Fynbos honey

3.1 Introduction

The quality of honey is dependent on the physical and chemical properties. The revised version of CODEX Alimentarius (CODEXSTAN12-1981 revision 2001), documents the global regulatory standards for physicochemical properties of honey. Properties that are included are the sugar, moisture and ash contents as well as proline, hydroxymethylfurfural content (HMF), pH, diastase activity, water insoluble solid content and electrical conductivity.

Honey is a supersaturated solution containing the monosaccharides fructose and glucose and the disaccharide, sucrose is only present in small amounts (Ball, 2007; Iglesias *et al*., 2004). In addition the ratio of reducing sugars (Fru:Glc) in honey is primarily dependent on the nectar source and this indicates how easily the honey will crystallise (Finola *et al*., 2007). Honey also contains a variety of amino acids with proline being the most dominant (Ball, 2007) and the levels of this amino acid can be used as a measure of sugar adulteration (Silva *et al*, 2009).

Other constituents present in honey are phenolic acids, flavonoids, carotenoids, protein as well as enzymes. Especially flavonoids and phenolic compounds (Monirruzaman *et al*., 2013) contribute to the antioxidant activity of honey. Enzymes present in honey are diastase, glucose oxidase, invertase and phosphatase. Catalase found in honey is an antioxidant enzyme and contributes towards the antioxidant activity of honey (Meda *et al*., 2005). Another component present in honey is H_2O_2 which is found at low levels. It is a product that forms together with gluconic acid when glucose is oxidised by glucose oxidase (Bang *et al*., 2003; Aurongzeb and Azin, 2011). This H_2O_2 together with methylglyoxal (MGO) are responsible for the antibacterial activity of honey.

Correlations have been made between honey colour and antioxidant activity. The general rule is, the darker the honey the higher the antioxidant activity. The components that are responsible for honey colour are carotenoids, polyphenols and products from the chemical caramelization of saccharides (Ball, 2007; Bertoncelj *et al*., 2007). Caramelization of saccharides results in compounds specifically known as Maillard reaction products (MRPs) (Rice-Evans *et al*., 1997). MRPs are important as they are also known as non-nutrient antioxidants (Blasa *et al*., 2006; Turkmen *et al*., 2006; Bertoncelj *et al*., 2007).

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Prior to the analysis of the effect of digestion on honey bioactivity, it is necessary to determine whether each honey sample conforms to the CODEX regulatory standards related to pH, sugar and proline content. The antioxidant content and activity, antioxidant cellular protection and anti-inflammatory activities related to NO scavenging and suppression will also be determined, prior to simulated *in vitro* digestion.

Aim

The aim of this chapter was to determine the physicochemical properties as well as the antioxidant and anti-inflammatory properties of six FB honey samples compared to one medicinal MAN honey sample.

The objectives of this chapter are:

1. To determine the physicochemical properties of 6 undigested FB honeys in order to show that the honeys used comply with several aspects of the revised version of the CODEX Alimentarius (CODEXSTAN12-1981 revision 2001).
2. For each honey sample determine the,

a. Total polyphenol and flavonoid content with the Folin Ciocalteu and the aluminium chloride methods respectively,
b. Antioxidant activity with the trolox equivalent antioxidant capacity (TEAC) and oxygen radical absorbance capacity (ORAC) assays,
c. Cellular antioxidant protection effects in the Caco-2 and SC-1 cell lines using the dichloroflourescein diacetate (DCFH-DA) assay.
d. NO scavenging activity using the sodium nitroprusside (SNP) assay.
e. To further evaluate the pro- and anti-inflammatory activity in the RAW 264.7 cellular model.

3.2 Materials

3.2.1 Honey samples

Six FB honey samples (FB1 - FB6) were purchased from local bee keepers and farm stores in the Western Cape Province in South Africa (SA). A medicinal MAN honey with a Unique Manuka Factor (UMF) factor of 15+ was used as control for this study and was purchased from a local health shop.

3.2.2 Cell lines

The human colon adenocarcinoma cell line (Caco-2), and the mouse macrophage cell line (RAW 264.7) were obtained from the European collection of cell cultures (ECACC) through

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Sigma Aldrich (Johannesburg, SA). The mouse fibroblast cell line (SC-1) was obtained from Highveld Biological (Johannesburg, SA).

3.2.3 Reagents, equipment and laboratory facilities

Reagents that were used in this study were, fructose (Fru), glucose (Glc), sodium hydroxide (NaOH), sodium phosphate (NaH_2PO_4), sodium phosphate dibasic dihydrate ($Na_2HPO_4.2H_2O$), sodium chloride (NaCl), potassium ferricyanide ($K_3Fe[CN]_6$), L-proline, ninhydrin, hydrogen peroxide (H_2O_2), sorbitol, sulphuric acid (H_2SO_4), xylenol orange and ammonium ferrous sulphate ($(NH_4)_2Fe(SO_4)_2.6H_2O$)) were purchased from Merck Chemicals (Modderfontein, SA). Other reagents included; bovine serum albumin (BSA), Coomassie Brilliant Blue, gallic acid, catechin, Trolox (6-hydroxy-2,5,7,8–tetramethylchroman-2-carboxylic acid), Folin-Ciocalteu (FC) reagent, sodium carbonate anhydrous (Na_2CO_3), aluminium chloride ($AlCl_3$), potassium peroxodisulfate ($K_2S_2O_8$), (2,2'-Azo-bis(3-ethylbenzothiazoline-6-sulfuric acid) diamonium salt (ABTS), fluorescein sodium salt (FL) and (2,2'-Azobis(2-amidino-propane) dihydrochloride (AAPH) were obtained from Sigma-Aldrich Company, Atlasville, SA. Reagents such as the dichloroflourescein diacetate (DCFH-DA), sodium nitroprusside (SNP), sodium nitrite ($NaNO_2$), sulphanilamide, N-1-naphthylethylenediamine dihydrochloride (NED), phosphoric acid solution (H_3PO_4), potassium chloride (KCl), disodium hydrogen phosphate (Na_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were also obtained from Sigma-Aldrich Company, Atlasville, SA. All reagents and chemicals were of analytical grade. Dulbecco's Modified Essential Medium (DMEM), antibiotic solution (streptomycin, penicillin and fungizone) and foetal calf serum (FCS) were obtained from the Highveld Biological Company, Johannesburg, SA. Acetic acid, dimethyl sulphoxide (DMSO) and ethylene diamine tetra acetate (EDTA) were obtained from Merck, SA. Trypsin was obtained from Life Technologies Laboratories and was supplied by Gibco BRL products, Johannesburg, SA. Sartorius cellulose acetate membrane filters 0.22 μm were obtained from National Separations, Johannesburg, SA. Interferon gamma (IFN–γ) and lipopolysaccharide (LPS–*E. coli* 0111:B4) were obtained from Sigma-Aldrich, SA.

Equipment used in this study included a BioTek ELx800 plate reader purchased from Analytical and Diagnostic Products (ADP), Johannesburg, SA. A FLOUstar OPTIMA plate reader from the BMG labtechnologies at Offenburg, Germany as well as a Lambda LS5OB spectrophotometer from Perkin Elmar at Boston, MA, USA, supplied by Separations Scientific, Honeydew, SA. A Crison GLP21 pH meter as well as an Ecobath from LaboTec, SA was also used.

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Disposable plasticware that was used include: 96 well plates, 25 and 75 cm^2 tissue flasks, 15 and 50 ml centrifuge tubes, 10 and 5 ml pipettes and pipette tips (10, 25, 100, 200 and 1000 µl) and was obtained from either Greiner Bio-one supplied by LASEC, Cape Town, SA or NUNC ™ supplied by AEC-Amersham, Johannesburg, SA.

Laboratory facilities

All research was conducted in the Cell Biology Laboratory, Department of Anatomy as well as the Pharmacology Laboratory, Department of Pharmacology of the Faculty of Health Sciences, University of Pretoria.

Preparation of honey solutions

A 90% (v/v) of all honey stock solutions was prepared in double distilled water (ddH_2O). This was done to reduce the viscosity of the samples. From the stock solutions, 50% (v/v), 25% (v/v), 12.5% (v/v), 10% (v/v), 9% (v/v), 5% (v/v), 2,5% (v/v) and 1% (v/v) working solutions were prepared using ddH_2O. Honey solutions were then stored in the dark at -20˚C until needed.

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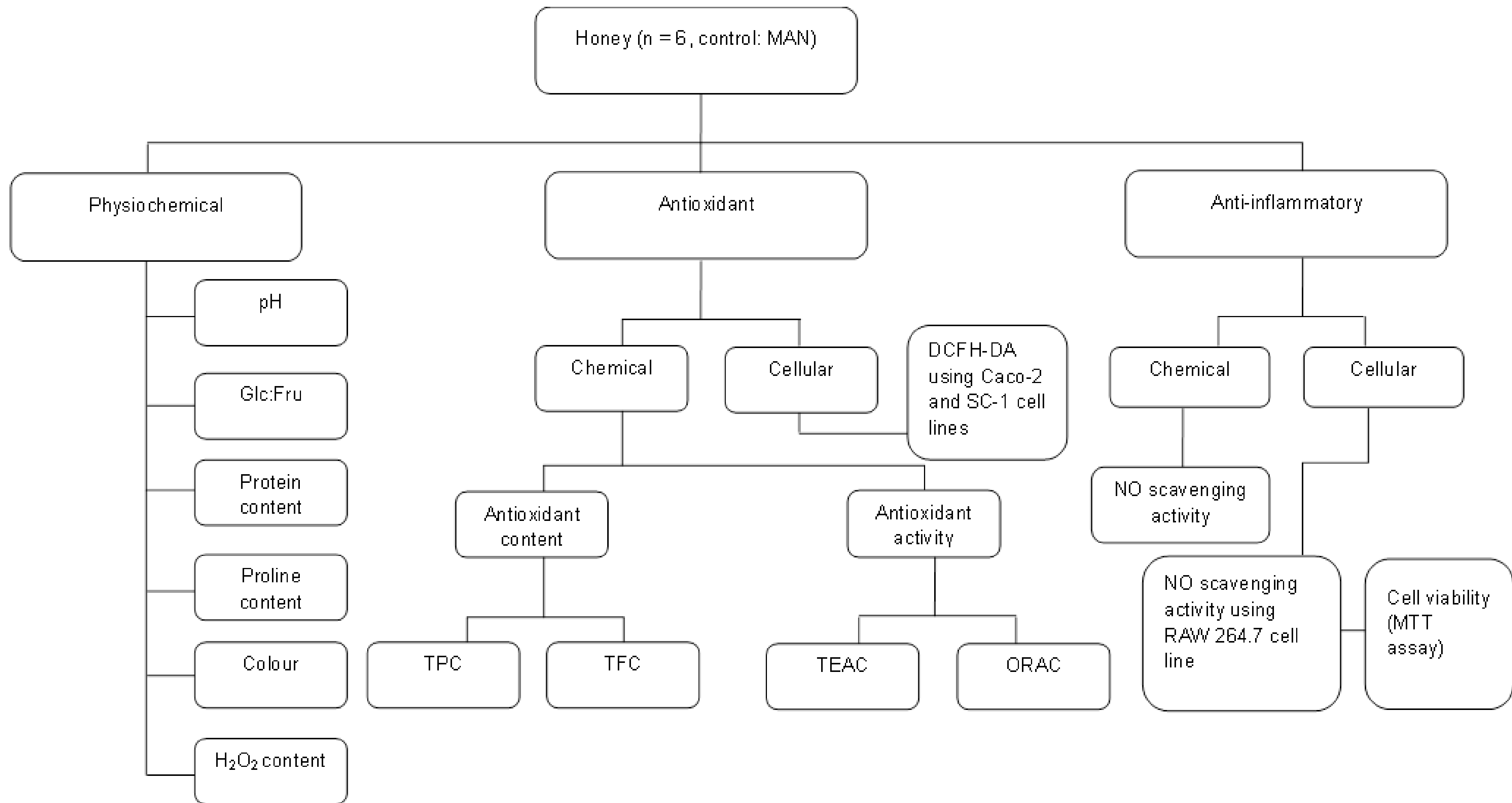

Figure 3.1: A summary of all the chemical and cellular assays performed in this study.

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3.3 Methods

All methodologies used in this study are summarised in Figure 3.1.

3.3.1 Determination of physicochemical properties

3.3.1.1 pH

The pH of each honey sample was determined by the method of Silva and co-workers (2009), whereby samples were used at a concentration of 10% (v/v) and measured using a pH meter.

3.3.1.2 Fru:Glc ratio

The potassium ferricyanide reagent also known as the Perkins test is used to detect reducing sugars. The most prevalent monosaccharides in honey are fructose and glucose (Ball, 2007). The ratio of reducing sugars (Fru:Glc) depends primarily on the nectar source and indicates how easily the honey will crystallise (Serem and Bester, 2012).

To determine the Fru:Glc ratio the method of Silva and co-workers (2009) was used. Fructose and glucose stock solutions were prepared by mixing together 1 g of Fru and Glc in 100 ml ddH_2O. These stock solutions were then diluted 10X to yield working solution of 0.1 g in 100 ml of ddH_2O. Potassium ferricyanide reagent was prepared by weighing a stock solution of 1 g of potassium ferricyanide mixed with a 20% sodium hydroxide solution. A working solution was then prepared by diluting the stock solution 10X. Two standard curves of 0 – 0.14 mM (0 – 0.025 mg/ml) of glucose and fructose each were prepared. A volume of 25 µl of 0.01 (v/v) honey solutions were then added to the 96 well plate, followed by the addition of 150 µl of the potassium ferricyanide reagent to the glucose and fructose standards as well as the honey samples. The plate was then incubated in the dark at room temperature for 30 min. The absorbance was read at 405 nm. To account for possible sample interference, blanks were prepared and consisted of 25 µl of each honey fraction with 150 µl of ddH_2O and the results were expressed as a ratio of fructose to glucose (Fru:Glc). This ratio was calculated by obtaining a glucose and fructose standard curve, and dividing the gradient of Fru with that of Glu. The Fru:Glc ratio of honey was then calculated the following equation:

$$\text{Fru:Glu} = \frac{\text{Fructose (mg/Kg)}}{\text{Glucose (mg/Kg)}}$$

3.3.1.3 Protein content

The protein content of honey is approximately 0.2% and is due to the presence of invertase, α-amylase, catalase, phosphatase and glucose oxidase which relates to nectars, pollens

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and plant origin (Anklam, 1998). The Bradford method by Ouchemoukh *et al.*, (2007) was used to determine protein content of all undigested samples. This assay makes use of a Commassie Brilliant blue reagent which turns blue upon the formation of the protein-dye complex (Bradford, 1976). The protein-dye complex possesses a high extinction coefficient, thus making it sensitive in the detection and measure of protein (Bradford, 1976).

A standard with a concentration range of BSA (0 – 0.03 mg/ml) was prepared. A 5X dilution of Bio Rad (Coomasie Brilliant Blue) dye solution was prepared. A volume of 20 µl of the 10% honey solutions were added to the 96 well plate followed by the addition of 100 µl of the dye solution. In order to avoid any colour interference, blanks consisted of 20 µl of 10% (v/v) honey and 100 µl of ddH_2O were prepared. The absorbency readings were read at 630 nm and the results were expressed as mg protein/100g of honey.

3.3.1.4 Proline content

Proline is the major free amino acid in honey (Ball, 2007). It is used as a marker for the quality of honey, as honey adulteration results in reduced proline levels. A modified method established by Ouchemoukh *et al.*, (2007), was followed for the determination of proline levels in the honey samples. Ninhydrin is used for the determination of amino acids. A reaction of ninhydrin with amino acids yields ammonia, carbon dioxide, an aldehyde and an intermediate amine forming a purple colour. However with proline, ammonia is not formed and a yellow colour product forms as a result of the reaction of ninhydrin and proline in acidic medium (Wu, 2013).

The proline content was determined by adding 50 µl of a 10% (v/v) honey solution, followed by the addition of 50 µl ninhydrin salt solution prepared by mixing 0.33 g of ninhydrin in 100 ml of a 2% acetic acid solution. A proline standard of 0 – 0.1 mM (0 – 0.01 mg/ml) was prepared and the plate was incubated in the oven at 80 – 100˚C for 30 min. In order to avoid any sample colour interference blank samples consisted of 20 µl of a 10% (v/v) honey solution and 100 µl of ddH_2O were prepared. The absorbency was read at 405 nm and the data was expressed as mg proline/100 g honey.

3.3.1.5 Ferrous ion oxidation xylenol orange (FOX) assay

Hydrogen peroxide (H_2O_2) is present in honey as a result of glucose oxidation and the levels in different honeys differ (Bang *et al.*, 2003) and the presence of H_2O_2 in honey contributes to its antibacterial activity. The H_2O_2 content was determined using a slightly modified version of FOX assay (Banerjee *et al.*, 2003).

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The FOX reagent was prepared as follows: A concentration of 100 µM of xylenol orange was mixed with 100 mM of sorbitol to a final volume of 50 ml. A volume of 65 µl sulphuric acid and 250 µM ammonium ferrous sulphate were added. A volume of 150 µl of reagent was added to a volume of 10 µl of 10% (v/v) honey sample and a H_2O_2 standard concentration series (0 – 0.036 µM) in a 96 well plate. This was then incubated at room temperature in the dark for 30 min, after which an absorbency reading was measured at 570 nm. Results are reported as µmol/ml H_2O_2.

3.3.1.6 Colour absorbance/intensity

The colour of honey is due to the presence of carotenoids, polyphenols, and chemical caramelization of saccharides that is catalyzed by acids. The Malliard reaction between amino acids and sugars also contributes to honey colour (Ball, 2007). Generally a darker honey usually has higher antioxidant activity (Frankel *et al*., 1998; Beretta *et al*., 2005). To determine the colour of the honey two different wavelengths were used.

Colour determination was done according to Beretta *et al*., (2005). The absorbency of a 10% (v/v) honey solution was measured at 450 nm and 720 nm. These two wavelengths were used because the absorbance between 420 nm and 450 nm is accounted for by the presence of polyphenols while an absorbance between 560 nm and 720 nm is due to the presence of MPRs (Brudzynski and Miotto, 2011). Thus the net colour intensity absorbance was defined as the difference between 450 nm and 720 nm (Beretta *et al*., 2005). The difference between the two wavelengths was then calculated and the results were expressed as absorbance units (AU).

3.3.2 Determination of antioxidant content

3.3.2.1 Total polyphenolic content (TPC)

The TPC of natural samples like honey and plants approximately reflects the antioxidant activity of these samples (Beretta *et al*., 2005). However other reducing molecules such as metals, amino acids and ascorbic acid may give false high values. This gives a good indication of the potential therapeutic properties of honey (Al-Mamary *et al*., 2002). Determination of TPC involves the use of the Folin-Ciocalteu (F-C) method which involves the use of the F-C reagent. The reaction of the yellow F-C reagent with polyphenols results in the formation of a blue complex which can be quantified spectrophotometrically (Schofield *et al*., 2001). The formation of the blue colour is due to the oxidation of the phenolate ion in alkaline conditions thereby reducing the phosphotungstic-phosphomolybdenum complex in the F-C reagent (Waterman and Mole, 1994).

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The TPC assay was determined by using the F-C method that was modified as described by Amin *et al*., (2006). Gallic acid was used as a standard with a concentration range of 0 – 0.18 mM (0 – 0.03 mg/ml). F-C reagent was diluted 15X with ddH_2O and a 7.5% of anhydrous $NaCO_3$ solution was prepared. A volume of 10 µl of the 10% (v/v) honey solution was added to the wells of a 96 well microplate. To this 50 µl FC reagent was then added followed by the addition of the 50 µl of 7.5% the Na_2CO_3 solution. After 10 min of incubation the microplate was read at 630 nm and the results were expressed as gallic acid equivalents (mM GAE/100g).

3.3.2.2 Total flavonoid content (TFC)

The TFC of each sample was determined using the aluminium chloride method following a modified procedure of Amaral *et al*., (2009). The method involves the formation of the acid stable complexes with either the C-3 or C-5 hyrdoxyl group or C-4 keto groups of flavonols and flavones (Chang *et al*., 2002).

Catechin was used to create a standard curve with a concentration range of 0 – 0.10 mM (0 - 0.03 mg/ml). A volume of 10 µl of the 10% (v/v) honey solution was added to the wells of a 96 well microplate, followed by the addition of 30 µl of a 2.5% $NaNO_3$ solution. Then 20 µl of a 2.5% $AlCl_3$ solution was added followed by 100 µl of a 2% NaOH solution. In order to correct any colour interference, a blank of 10 µl of a 10% (v/v) honey solution and 150 µl of ddH_2O was used. The samples were mixed well and the absorbance of the samples was measured at 450 nm and the results were expressed as catechin equivalents (mM CE/100g).

3.3.3 Determination of antioxidant activity

3.3.3.1 Trolox equivalent antioxidant capacity (TEAC) assay

$ABTS^{\cdot+}$ is a blue-green chromophore which undergoes decolourization in solution. The principle of the TEAC assay is that antioxidants are able to quench the reduction of the cationic radical $ABTS^{\cdot+}$ to ABTS which translates into a loss of colour. The degree of colour loss is correlated with the concentration of antioxidants present in the sample (Zulueta *et al*., 2009).

The TEAC assay was performed according to Awika *et al*., (2003). To prepare an $ABTS^{\cdot+}$ solution, 3 mM of $K_2S_2O_8$ was added to 8 mM of ABTS and then the mixture was left in the dark at room temperature for 12 h. A working solution, the TEAC reagent was prepared by diluting the stock solution 30 times with a 0.2 M phosphate buffer, pH 7.4. Trolox was used to prepare a concentration series of 0 – 1000 µM (0 – 0.25 mg/ml). A volume of 10 µl of a

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10% (v/v) of each honey solution and the Trolox concentration series were added to the wells of a 96 well-plate, followed by the addition of 290 µl TEAC reagent. The incubation period for the samples was 30 min and 15 min for the Trolox standards. Blank controls, honey with ddH_2O instead of TEAC reagent was included. Absorbance was measured at 630 nm and the results were expressed as µmol Trolox equivalents per gram (µmol TE/g).

3.3.3.2 Oxygen radical absorbance capacity (ORAC) assay

The principle behind the ORAC assay is that it measures a fluorescent signal from a fluorescent probe (fluorescein) that is quenched in the presence of hydroxyl radicals generated from AAPH. Trolox, a water soluble vitamin E analogue that quenches radical formation is used as a standard reference. This assay inhibits hydrogen radicals which are more biologically relevant (Ou *et al.*, 2002; Awika *et al.*, 2003; Dávalos *et al.*, 2004).

An AAPH working solution prepared fresh for each experiment consisted of 0.08 g AAPH in 4 ml ddH_2O. A stock solution of fluorescein (FL) was prepared by adding 3.20 mg to 50 ml of 0.2 M phosphate buffer (PBS) (0.2M $NaH_2PO_4.H_2O$, 0.2M Na_2HPO_4, 0.15M NaCl, pH = 7.4). This was then further diluted 4X in PBS (solution A). A volume of 140 µl was taken from solution A was added to 5 ml PBS and made up to 50 ml with ddH_2O (solution B). A working solution was then prepared by combining 4 ml of the AAPH solution with 16 ml of the solution B, yielding final concentrations of 14.8 mM AAPH and 0.0952 µM FL. Trolox (0 – 800 µM or 0 – 0.200 mg/ml) was used to generate a standard curve. Two controls were included and these were 200 µl FL and 200 µl of the working solution. Experimental wells contained 5 µl of the 1% (v/v) honey solutions or the prepared Trolox concentration series with 200 µl of the working solution. Fluorescence was read every 5 min for 4 h. The automated ORAC assay was carried out using a fluorescence filter with an excitation of 485 nm and an emission wavelength of 535 nm. The ORAC values were then calculated by using Microcal Origin 6.0 which measured the net area under the decay curve (AUC). The results were expressed as µmol Trolox equivalents per gram (µmol TE/g).

3.3.4 Determination of antioxidant protection using cellular models

Chemical based assays provide a rapid measurement of antioxidant activity however the first level of evaluation for health benefits is the use of cells in cell culture. Although a limitation of such studies that these cells are usually of one type and do not reflect the cellular and functional diversity of human tissue and organ systems (Edmondson *et al.*, 2014).

3.3.4.1 Caco-2 cell line

The Caco-2 cell line is a human colon adenocarcinoma cell line. It is an acceptable model for evaluating intestinal absorption and metabolism due to their well-differentiated microvillus

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brush borders. This cell line also has the ability to differentiate in standard cell culture conditions into enterocytes (Turner and Turner, 2010) as well as expressing nutrient transporter molecules (Meunier *et al.*, 1995). The Caco-2 cell line is a physiologically relevant cellular model as it has morphological and functional properties similar to that of the human small intestine epithelium (Meunier *et al.*, 1995).

The Caco-2 cells were maintained and cultured as follows. To establish cultures of the Caco-2 cell line vials of Caco-2 cells stored in liquid nitrogen were rapidly thawed, by placing the vial in warm water at 37°C. Then the cells in the freezing medium were mixed in 5 ml of DMEM medium that contained 10% Foetal Calf Serum (FCS) and 1% antibiotic solution (DMEM/10%FCS). The antibiotic stock solution contained 10,000 µg/ml of streptomycin, 10,000 µg/ml of penicillin and 25 µg/ml of Amphotericim B in 0.85% saline. The cells were then collected by centrifugation at 800 g for 2 min. The supernatant was then discarded and fresh DMEM/FCS was added to the cells. After resuspending the cells, the cells were plated at a concentration of $4x10^4$ cells per ml in a 25 cm^2 culture flasks.

Once the Caco-2 cells were confluent the medium was removed from the 25 cm^2 culture flasks. The monolayer was then rinsed with 5 ml of a 0.53 mM EDTA/PBS solution. This solution was prepared by mixing together 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4 and 0.53 mM EDTA. Thereafter 1 ml of a 5% trypsin solution prepared in PBS was added and then the flask was placed in an incubator at 37°C for 1 – 2 min. After the incubation period, 5 ml of DMEM/FCS medium was added to the flask to collect the detached cells. The content within the flask was then transferred to a 15 ml tube and centrifuged at 800 g for 2 min. The supernatant was then removed and the cells were resuspended in 5 ml of DMEM/FCS medium. The number of cells was determined by counting 10 µl aliquot of cells with a hemocytometer. Trypan blue dye was used to exclude non-viable cells and the cells were plated according to the desired concentration.

3.3.4.2 SC -1 cell line

The SC-1 cell line is a mouse fibroblastic cell. Fibroblasts are important component of the mucosa of the gastrointestinal tract. Excessive inflammation and oxidative damage causes fibrosis which results disruption of normal mucosa structure and function leading to disease associated with oxidative damage. The establishment and maintenance of SC-1 cell cultures were similar to that of the Caco-2 cell line as described in section 3.3.4.1.

3.3.5 Cellular antioxidant activity (CAA): Caco-2 and SC-1 cell lines

To measure the cellular antioxidant effects the dichlorofluorescein diacetate (DCFH-DA) assay was used. This assay measures or detects oxidative damage caused by RNS and

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ROS in a cellular environment. When cells are exposed to the nonpolar, nonionic DCFH-DA which diffuses into the cells, this compound becomes hydrolyzed enzymatically by the action of the intracellular esterases to DCFH which is non-fluorescent (LeBel *et al*., 1992). When DCFH is exposed to radicals generated by AAPH, it becomes oxidized into a fluorescent probe (DCF) that is used as a measure of radical production as shown in Figure 3.2.

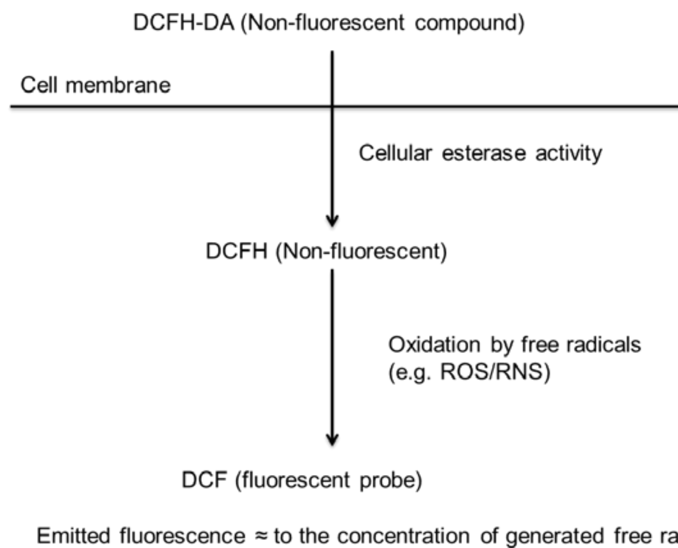

Figure 3.2: Principle of the DCFH-DA assay. The emitted fluorescence by the DCF probe is proportional to the concentration of the free radicals. Adapted and modified from Crow (1997).

The Caco-2 and SC-1 cells were plated at a concentration of 2 X 10^4 cells per ml in a 96 well plate, 100 µl per well. Then the cells were cultured for 24 h at 37ºC at 5% CO_2 prior to the determination of CAA. For CAA, 50 µl of a 75 µM DCFH-DA solution was added to each wells of the plate (final concentration = 25 µM), followed by a 1h incubation period at 37ºC. Thereafter the medium was removed and the cells were washed once in PBS. A volume of 50 µl of 10 % (v/v) honey was then added, followed the addition of 50 µl of a 7.5 mM AAPH solution. The change in fluorescence was measured every 2 min for 60 min at excitation wavelength of 485 nm and emission wavelength of 520 nm. The gradient change of fluorescence was measured and the percentage oxidative damage was calculated as follows:

$$\text{\% Oxidative damage (OD)} = (\text{sample} - \text{PBS})/(\text{AAPH} - \text{PBS})*100$$

The change in fluorescence for AAPH, no honey added was 100%. A significant decrease in fluorescence with the addition of honey indicates CAA. Significant levels of fluorescence greater than 100% indicates a pro-oxidant effect.

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3.3.6 Determination of anti-inflammatory activity

3.3.6.1 Chemical NO scavenging assay

Sodium nitroprusside (SNP) in an aqueous solution at a physiological pH, spontaneously generates NO (Green *et al.*, 1982; Marcocci *et al.*, 1994a). NO in an aqueous environment forms nitrite ions that can be quantified using the Griess reagent. Scavengers of NO prevent the formation of nitrite ions (Marcocci *et al.*, 1994b) and consequently the amount of nitrite quantified with the Griess reagent is reduced.

A modified method of Kim *et al.*, (1999) was used. A 5 mM SNP solution was prepared in PBS. The amount of NO formed was quantified using a $NaNO_2$ standard curve with a concentration range of 0 – 0.05 mM (0 – 0.003 mg/ml). The Griess reagent was used to quantify the formed NO and to determine NO levels in the honey samples. To determine, if antioxidants would effectively scavenge NO generated by SNP, a Trolox concentration range of 0 – 1 mM (0 – 0.25 mg/ml) was used.

For the quantification of the NO scavenging activity of honey, to a 20 µl volume of a 10% (v/v) honey solution, 80 µl of 5 mM SNP was added. After 1 h incubation at room temperature, NO levels were quantified with 50 µl Griess reagent.

Griess reagent is a 1% (w/v) sulphanilamide (SA) solution prepared by dissolving 1 g SA in 100 ml of a 2.5% H_3PO_4 solution, followed by an incubation period of 10 min. Thereafter 50 µl of a 0.1% NED solution (w/v) prepared by dissolving 0.1 g NED in 100 ml of 2.5% H_3PO_4 was added. The samples were mixed well and the absorbency was read at 550 nm and NO scavenging activity was expressed as a percentage of the control, no antioxidant added.

3.3.6.2 Cellular NO scavenging activity: RAW 264.7 cell model

3.3.6.2.1 RAW 264.7 cell line

The RAW 264.7 cell line is a murine macrophage cell line that has been used as a model for inflammation as it synthesizes and releases inflammatory markers such as NO (McClain *et al.*, 2002; Nakao *et al.*, 2008). The cell line can be used to determine if molecules in complex mixtures such as honey can induce or inhibit NO formation i.e. has either a pro-inflammatory or anti-inflammatory effect.

RAW 264.7 cells were thawed and cultured as described for the Caco-2 cells. Once these cells were confluent, the medium was removed and the monolayer of cells was scraped using a cell scrapper. The cells were collected in a 5 ml volume of DMEM/FCS medium and the cells were collected by centrifugation at 800 g for 2 min. The supernatant was then removed and then fresh DMEM/FCS medium was added. Cell number was determined in a

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10 µl aliquot of cells with a hemocytometer and cell viability was determined using trypan blue dye.

3.3.6.2.2 Cellular pro-inflammatory anti-inflammatory effects

NO is an important inflammatory mediator (Kassim *et al*., 2010a). Increased NO formation indicates a pro-inflammatory effect that is important for acute wound healing whereas a reduction in NO levels is essential for the healing of chronic wounds. To determine if the honey samples had a pro-inflammatory effect, RAW 264.7 cells were not stimulated with LPS and IFN-γ prior to the addition of the honey samples. To determine if the honey samples had an anti-inflammatory effect, RAW 264.7 cells were stimulated with LPS and IFN-γ prior to the addition of the honey samples. For both experiments NO levels were determined in an aliquot of the supernatant with the Griess assay. To the RAW 264.7 cells *in vitro*, MTT was added to determine cell viability. In the MTT assay, cellular dehydrogenases reduces the water soluble tetrazolium salt into an insoluble purple formazan product (Fotakis and Timbrell, 2006), which is solubilised and spectrometrically quantified. Cellular viability is measured to ensure that increased NO formation is not the result of cellular death.

In this study, 70 µl of 1.5 $X10^6$/ml RAW 264.7 cells were exposed to honey samples 10 µl 10% (v/v), without (pro-inflammatory effect) and with (inflammatory effect) 10 µl 1 µg/ml LPS and 10 µl 250 U/ml IFN-γ, yielding final concentrations of $1X10^6$ cells, 100 ng/ml LPS and 25 U/ml IFN-γ (Kassim *et al*., 2010a). This mixture was then incubated for 24 h at 37˚C, 5% CO_2. After incubation a 50 µl volume the supernatant was used to determine NO levels with 100 µl of the Greiss reagent as described in Section 3.3.6.1. For the NO quantification, controls were colour control (medium only), cells without LPS and IFN-γ, cells with LPS and IFN-γ and cells with honey only.

The cells with the remaining 50 µl of medium were assayed for cell viability to ensure that the changes in NO production were not due to cell death. This involved the addition of 5 µl of 1 mg/ml MTT solution to the supernatant and incubating the plate for 3 h at 37˚C, 5% CO_2. After incubation, the medium was removed and the plate blotted dry. The purple insoluble formazan formed by viable cells was solubilised with 25% DMSO in ethanol and the absorbance was measured at 570 nm. Results for NO production were reported as µmol/ml NO_2^- and cell viability as percentage of the untreated cellular control.

3.3.7 Data management and statistical analysis

All data is an average of at least three experiments with each measurement performed in triplicates thus generating at least 9 data points. The results are expressed as mean ± standard error of mean (SEM) of three experiments. Several parameters such as colour,

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antioxidant content, antioxidant activity assays as well as the chemical and cellular NO scavenging activity were correlated. Data was statistically evaluated using analysis of variance (ANOVA), using samples as independent variables and the values determined as dependent variables. Fisher's least significant difference (LSD) and Tukey tests were used for comparison of means using Statistica software Version 9.0 (StatSoft, Tulsa, OK) and Graphpad Prism 6.01 (GraphPad Software, San Diego, CA). Correlation analysis was also run with the same statistical package and Microsoft Excel 2013.

3.4 Results and discussion

3.4.1 Physicochemical properties

The physicochemical properties of honey are important in the characterization of the honey used in this study as this ensures all honeys fulfil the CODEX Alimentarius requirements. Honey is a supersaturated sugar solution that contains vitamins, enzymes, proteins, minerals, trace elements, organic acids, aromatic compounds and polyphenols. Also present in honey is H_2O_2, methylglyoxal (MGO) and bee defensin peptides which are responsible for the antibacterial activity of honey, while the polyphenols are mainly responsible for the antioxidant and anti-inflammatory activities of honey.

The measured therapeutic effect of honey is due to the presence of a unique molecule at high concentrations and due to a combinational effect between individual components. An example of the former would be MAN honey from New Zealand, where high MGO levels are responsible for the antibacterial activity of this honey and accordingly MGO levels determine the unique Manuka factor (UMF) of this honey. Due to these properties, MAN is also classified as a nutraceutical product with medicinal properties. Southern Africa specifically the Fynbos biome (Figure 3.3) is a region of unique biodiversity with many endemic plant species of over 8920 flowering plant species. Within this biosphere honey is produced commercially and sold in farm stalls and supermarkets (Cowling, 1983; Goldblatt and Manning, 2000) and these honeys due to their different floral origin may have different physicochemical characteristics, levels of H_2O_2 and polyphenol content, which could translate into unique therapeutic properties.

Serem and Bester determined the physicochemical properties of honey from the southern Africa region (Serem and Bester, 2012). A limitation of this study was the small number of honey samples from the FB region that was evaluated and measured activity was not compared to a honey type that has been well characterized such as MAN honey. In the present study six region specific samples were collected and the physicochemical properties of these undigested honeys were compared to MAN honey.

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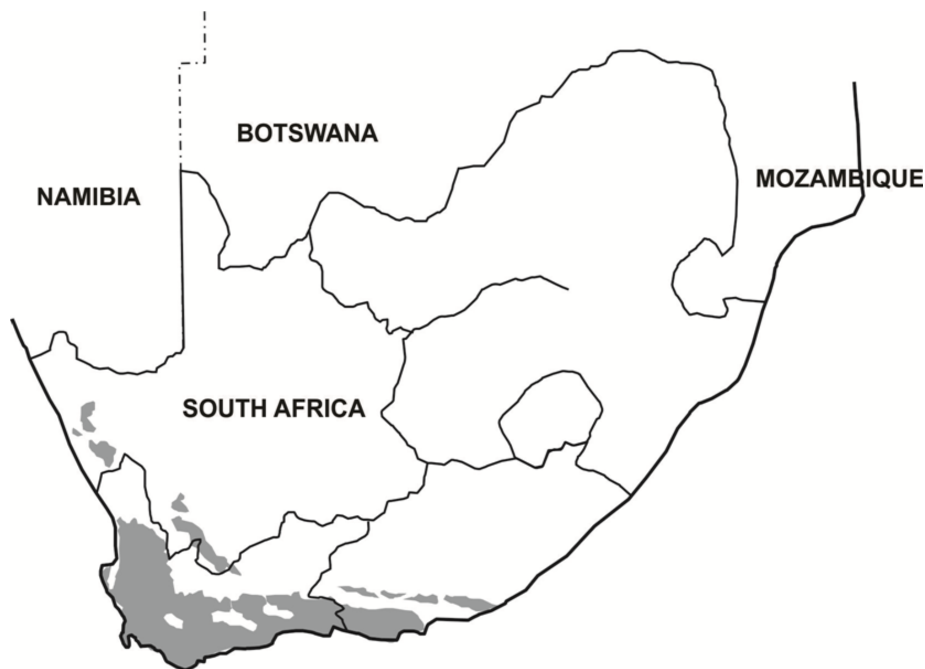

Figure 3.3: Fynbos biome region along the Western Cape of South Africa.

In addition to floral and geographical source, honey type is also influenced by environmental, seasonal, processing and storage factors (Gheldof *et al*., 2002; Bertoncelj *et al*., 2007; Guler *et al*., 2007; Alvarez-Suarez *et al*., 2010b) and this contributes to the differences in the activities of different honeys. Table 3.1 gives a summary of the physicochemical properties of the six FB honey compared to MAN used in this study.

Table 3.1: Physicochemical properties of MAN and FB honeys

Samples	pH	Fru:Glc	Protein (mg/100g)	Proline (mg/100g)	H_2O_2 (µmol/ml)	Colour (AU)
MAN	3.92[b]	0.80[a]	27.79[c]	75.69[a]	0.97[c]	7.27[a]
FB1	4.41[a]	0.76[a]	86.96[a]	67.25[a]	1.67[a]	5.38[b]
FB2	4.49[a]	0.76[a]	17.94[d]	68.65[a]	0.80[c]	2.45[c]
FB3	4.04[b]	0.75[a]	28.39[c]	53.52[b]	0.77[c]	2.71[c]
FB4	4.28[ab]	0.71[a]	10.07[d]	55.12[b]	0.82[c]	2.45[c]
FB5	3.98[b]	0.74[a]	16.55[d]	49.03[b]	0.67[c]	2.19[c]
FB6	4.48[a]	0.75[a]	52.28[b]	80.91[a]	1.78[b]	5.66[b]
FB honey						
Average ± SEM	4.28 ± 0.03	0.75 ± 0.02	35.37 ± 3.35	62.41 ± 2.38	1.08 ± 0.06	3.47 ± 0.05
%SD	1.39	5.68	19.32	6.60	14.67	2.80
Min	3.98	0.71	10.07	49.03	0.67	2.19
Max	4.49	0.76	86.96	80.91	1.78	5.66

Data is an average of 3 experiments ± SEM. Different letters indicates significant difference ($p \leq 0.05$).

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3.4.1.1 pH

Naturally, honey has a low pH and this acidity is irrespective of the different geographical areas of origin (Ball, 2007; Berreta *et al.*, 2005). This low pH is generated as the result of the action of glucose oxidase when, during the ripening of the nectar into honey, gluconic acid/gluconolactone is produced thus contributing to the acidity of honey (White *et al.*, 1975). The obtained range of the FB pH values shown in Table 3.1, varied from 3.98 to 4.49 compared to MAN with a pH of 3.92. No significant difference was found between the pH of MAN, FB3, FB4 and FB5 ($p \leq 0.05$). Furthermore, FB1, FB2, FB6 were found to be similar to each other and similar to FB4 ($p \leq 0.05$). The obtained FB pH range as well as that of MAN fell within the pH range of the Codex Alimentarius (3.4 – 6.1) which indicates the freshness of honey. These results are similar to a number of studies done on the pH of honey: Indian honeys reported a range of 3.7 – 4.4 (Saxena *et al.*, 2010), 3.49 – 4.43 for Algerian (Ouchemoukh *et al.*, 2007), 3.53 – 4.03 for Malaysian honey (Moniruzzaman *et al.*, 2013) and 3.30 – 4.90 for Rubus honey from North West Spain (Escuredo *et al.*, 2011).

3.4.1.2 Fru:Glc

The Fru:Glc ratio in honey is primarily dependent on the nectar source (Anklam,1998) and it is a parameter used to evaluate the extent of crystalization (de Rodŕiguez *et al.*, 2004, Serem and Bester, 2012). The Fru:Glc ratio indicates whether the honeys are in a crystal or fluid state. So when Fru levels are higher than Glc levels, it means that the honeys are more fluid (Al *et al.*, 2009). The Fru:Glc ratio for FB honeys ranged from 0.71 to 0.76 and for MAN was 0.80 as shown in Table 3.1. Differences among the honeys were not significant ($p \leq 0.05$). In addition the similarity of the FB ratios illustrates their similar origin. These results were lower than the measured range of 0.9 – 1.7 for Rubus honey (Escuredo *et al.*, 2011), 0.81 – 1.41 as reported for the Romanian honeys. (Al *et al.*, 2009) and 0.85 – 1.31 as reported for the southern African honeys (Serem and Bester, 2012).

3.4.1.3 Protein

There is only about 0.2% protein present in honey (Anklam, 1998) and this is partly due to the presence of enzymes such as invertase, α-amylase, catalase, phosphatase and glucose oxidase, which is dependent on the nectar and pollen content as well as plant origin (Anklam, 1998). In Table 3.1, the observed protein content in all honeys ranged from 10.07 to 86.96 mg/100g. FB1 contained the significantly highest protein content. The measured least protein content was for FB4 however similar to FB5 and FB2 ($p \leq 0.05$). Comparing these results with literature done by Serem and Bester (2012), the range of the protein content found particularly in FB honey was lower than southern African honeys with a range of 620 – 1290 mg/100g (Serem and Bester, 2012), 370 – 940 mg/100g as reported for Algerian honeys (Ouchemoukh *et al.*, 2007) and 204 – 438 mg/100g reported for Malaysian

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honeys (Moniruzzaman *et al*., 2013). The honeys in this study were somewhat similar to the Indian honeys reported by Saxena *et al.,* (2010) with a range of (48 – 2290 mg/100g), 65 – 170 mg/100g as reported for honeys from Anatolia (Küçük *et al*., 2007) and 12.0 – 92.3 mg/100g as reported for Cuban honeys (Alvarez-Suarez *et al*., 2010b). According to Bogdanov (2008) the general protein content of honey ranges from 200 – 500 mg/100g. This indicates that the honeys evaluated in this study tend to have a low protein content, attributed to floral or geographical differences.

3.4.1.4 Proline

The predominant free amino acid in honey is proline which serves as a measure for the amino acid content of honey. This parameter could be used as a measure of sugar adulteration (Silva *et al*., 2009) in addition to being an indicator of ripeness. Furthermore the proline levels are primarily dependant on the floral source (Moniruzzaman *et al*., 2013). The observed results indicated a proline FB range of 49.03 – 80.91 mg/100g compared to MAN with a proline content of 75.69 mg/100g. No significant difference was found between FB6, FB2, FB1 and MAN. FB3 was similar to FB4 and FB5 ($p \leq 0.05$). The proline content of FB honey was greater than the minimum accepted value of 18 mg/100g for a genuine honey (Bogandov *et al*., 1999). The proline content of FB honey was similar to the range of 20.2 – 68 mg/100g reported for Algerian honeys (Ouchemoukh *et al*., 2007), 17.64 – 62.87 mg/100g reported for Malaysian honeys (Moniruzzaman *et al*., 2013) and 43.78 – 2169.40 mg/100g for Burkinabe honeys (Meda *et al*., 2005). The proline content of FB was found higher for southern African honeys with a range of 15.57 – 45.24 mg/100g (Serem and Bester, 2012).

3.4.1.5 Hydrogen peroxide content

Honey samples had a H_2O_2 range of 0.67 – 1.78 µmol/ml. MAN honey had a measured value of 0.97 µmol/ml. Of these values FB1 and FB6 had statistically the highest H_2O_2 values. FB2, FB3, FB4, FB5 and MAN had a similar H_2O_2 content ($p \leq 0.05$). High sugar and H_2O_2 levels and an acidic pH contribute to the antibacterial activity of honey (Kwakman and Zaat, 2012). H_2O_2 in honey is produced under aerobic conditions by glucose oxidase which converts glucose to H_2O_2 and gluconic acid (Bang *et al*., 2003). The presumed function of H_2O_2 is to prevent spoilage of unripe honey when the concentration of sugar has not yet reached levels that exhibit antimicrobial growth (Bang *et al*., 2003).

These results when compared to other studies are very variable e.g. Brudzynski (2006), reported H_2O_2 levels of 0.0294 – 0.2385 µmol·ml^{-1} (mL honey)$^{-1}$, which when compared to this study, are much lower. Also the honeys in the Brudzynski study were of different dilutions ranging from 6.25 – 50% (v/v). In a study by Chen *et al*., (2012), the H_2O_2 content of

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a 25% (w/v) honey solution ranged from 0.15 – 1.017 µmol.ml^{-1} H_2O_2, which is the closest in range to this study, however, it is uncertain if these values were for per ml or per litre of honey and also had different concentrations (w/v vs. v/v). These variations are due to the use of non-standardized methods used and this makes comparisons between studies difficult. Identified factors that make comparisons difficult are: honey concentration v/v vs. w/v (dilutions made in order to make honey easier to assay) and the different environment in which honey is assayed (temperature, time and absorbance) (Bang *et al*., 2013).

3.4.1.6 Colour absorbance/intensity

The measured difference in absorbance is due to the presence of flavonoids and carotenoids which contribute to the antioxidant activity of honey (Beretta *et al*., 2005, Monirruzaman *et al*., 2013). The colour absorbance of the FB honeys ranged from 2.19 – 5.66 AU compared to MAN with an absorbance of 7.27 AU, which was statistically the darkest of all honeys. FB6 and FB1 were similar and were the darkest of the FB honeys. The honey that appeared with the lowest colour absorbance value was FB5, although, statistically not different from FB2, FB3 and FB4 ($p \leq 0.05$). The colour values obtained in this study were higher than 0.32 – 2.16 AU reported for southern African honeys (Serem and Bester, 2012), 0.52 – 1.68 AU reported for Indian honeys (Saxena *et al*., 2010), 0.170 – 0.740 AU reported for Malaysian honeys (Khalil *et al*., 2011b) and 0.312 – 0.544 AU reported for other different Malaysian honeys (Moniruzzaman *et al*., 2013).

3.4.2 Antioxidant properties

The antioxidant properties of honey are mainly due to the presence of polyphenols (phenolic acids and flavonoids) as well as peroxidases, non-peroxidal components, carotenoids and enzymes such as catalase (Bogdanov, 1997). The quantity of these constituents in honey depends upon the floral and geographical origin, processing as well as the handling and storage of the honey (Turkmen *et al*., 2006; Gheldof and Engeseth, 2002). The predominant component responsible for antioxidant activity are polyphenols.

3.4.2.1 Antioxidant content

3.4.2.1.1 TPC

The F-C method is a simple method to rapidly assess the total phenolic content of complex mixtures such as honey. However, there can be overestimation of the phenolic content as the F-C reagent also reduces non-phenolic compounds such as sugars, proteins, ascorbic acid, organic acid and aromatic amines (Prior *et al*., 2005). The mean TPC of FB honey was 53.45 mg GAE/100g compared to MAN with a value of 119.23 mg GAE/100g. The TPC of FB honeys ranged from 12.45 – 74.32 mg GAE/100g. FB1, FB2, FB5 and FB6 were similar. FB4 presented with the significantly lowest TPC value ($p \leq 0.05$) as shown in Table 3.2. The

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TPC of the average FB honey compared to the MAN honey was significantly less, 44.5% of the content measured for MAN honey. The TPC of FB honey was in a similar range of 68.85 – 167.96 mg GAE/100g as reported for the southern African honeys (Serem and Bester, 2012), 0.24 – 141.83 mg GAE reported for Rhododendron honeys (Silici *et al*., 2010), 32.59 - 114.75 mg GAE/100g reported for Burkinabe honeys (Meda *et al*., 2005), 33.90 – 154.20 mg GAE/100g reported for Rubus honey (Escuredo *et al*., 2011), 47.00 – 98.00 mg GAE/100g for Indian honeys (Saxena *et al*., 2010), 21.39 – 59.58 mg GAE/100g reported for monofloral Cuban honeys (Alvarez-Suarez *et al*., 2010b) and 16.697 – 87.658 mg GAE/100g as reported for Malaysian honeys (Khalil *et al*., 2011b). The FB range was somewhat less than the obtained range of 64.00 – 1304.00 mg GAE/100g as reported for Algerian honeys (Ouchemoukh *et al*., 2007) and higher than the other different Burkinabe honeys (Beretta *et al*., 2005) reported as 28.74 – 59.52 mg GAE/100g, higher than 1.52 – 4.23 mg GAE/100g as reported for other different Malaysian honeys (Khalil *et al*., 2011a) and 18.67 – 35.20 mg GAE/100g reported for 4 Malaysian honeys produced by the different bee species (Moniruzzaman *et al*., 2013).

3.4.2.1.2 TFC

Flavonoids are phenolic compounds that have a higher molecular weight compared to phenolic acids and contribute to the antioxidant activity as well as the aroma of honey (Moniruzzaman *et al*., 2013). Quantification of flavonoids from propolis and honey extracts by the aluminium chloride method has been previously described by Chang *et al*., (2002) and Alvarez-Suarez *et al*., (2009). It was shown that the real flavonoid content is the sum of flavones, flavonols and flavanones. The use of the aluminium chloride method only detects flavones and flavonols however not flavanones. Therefore, using the aluminium chloride method to measure flavonoid content results in an underestimation of TFC. A 2, 4 – dinitrophenylhydrazine method was developed for the detection of flavanones, however it was shown not to be so reliable (Chang *et al*., 2002). Therefore the most accurate way of quantifying TFC is by the use of HPLC analysis coupled to a mass spectrometer (Alvarez-Suarez *et al*., 2009). However Alvarez-Suarez *et al*., (2010b) further elaborated that when the aim does not involve quali-quantification of compounds, the use of the aluminium chloride method is ideal as it provides a valuable indication of a general TFC content.

In Table 3.2, the mean TFC of FB honey was 24.30 mg CE/100g which was 55.0% of the 43.51 mg CE/100g measured for MAN.

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Table 3.2: Chemical antioxidant content, activity and cellular antioxidant activity of MAN and FB honey samples

	Antioxidant content		Antioxidant activity		Cellular antioxidant activity	
	TPC (mg GAE/100g)	**TFC (mg CE/100g)**	**TEAC (µmol TE/g)**	**ORAC (µmol TE/g)**	**DCFH-DA Caco-2 (% OD)**	**DCFH-DA SC-1 (% OD)**
MAN	119.23^{a}	43.51^{a}	22.09^{a}	46.71^{b}	4.28^{d}	-10.97^{b}
FB1	74.32^{b}	19.41^{b}	17.03^{b}	59.09^{a}	37.61^{b}	10.00^{b}
FB2	53.80^{b}	29.26^{b}	14.91^{b}	39.11^{b}	19.50^{c}	25.92^{ab}
FB3	49.42^{b}	26.73^{b}	12.28^{b}	39.21^{b}	58.83^{a}	46.50^{a}
FB4	12.45^{c}	20.03^{b}	4.34^{c}	19.87^{c}	37.40^{b}	41.38^{a}
FB5	59.00^{b}	24.82^{b}	11.70^{b}	62.89^{a}	28.40^{b}	26.29^{ab}
FB6	71.73^{b}	25.55^{b}	16.49^{b}	60.08^{a}	34.80^{b}	9.20^{b}
FB honey						
Av ± SEM	**53.45 ± 5.06**	**24.30 ± 2.25**	**12.79 ± 0.76**	**46.71 ± 4.78**	**36.09 ± 3.95**	**26.55 ± 5.56**
%SD	**16.37**	**15.35**	**10.26**	**20.62**	**24.52**	**59.10**
Min	**12.45**	**19.41**	**4.34**	**19.87**	**19.50**	**9.20**
Max	**74.32**	**29.26**	**17.03**	**62.89**	**58.83**	**46.50**

Data is an average of at least 3 experiments ± SEM. Different letters indicates significant difference ($p \leq 0.05$).

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The TFC of FB honey ranged from 19.41 – 29.26 mg CE/100g. TFC differences among FB honey were not statistically significant. The FB range was similar to the range of 14.44 – 51.60 mg CE/100g as reported for southern African honeys (Serem and Bester, 2012). This range was however higher than 1.71 – 22.76 mg CE/100g reported for Malaysian honeys (Khalil *et al*., 2011b), 1.15 – 2.53 mg CE/100g reported for other Malaysian honeys (Khalil *et al*., 2011a), 2.20 – 6.57 mg CE/100g as reported for four Malaysian honeys produced by different bee species (Moniruzzaman *et al*., 2013), 2.70 – 7.10 mg CE/100g reported for Algerian honeys (Khalil *et al*., 2012) and 1.09 – 2.52 mg CE/100g reported for monofloral Cuban honeys (Alvarez-Suarez *et al*., 2010b).

3.4.2.2 Antioxidant activity

The mechanism of antioxidant activity of polyphenols differs and includes radical-scavenging, singlet oxygen quenching, hydrogen-donation, metal ion chelating properties as well as being substrates for radicals such as hydroxyl and superoxide (Kücük *et al*., 2007). Depending on the reaction mechanism, methods used to determine antioxidant activity can be classified into 2 types that is, methods based on electron transfer (ET) and on a hydrogen atom transfer (HAT) (Huang *et al*., 2005). TEAC is based on ET which measures the ability of the antioxidant in reducing compounds such as radicals by transferring an electron while ORAC (HAT-based) measures the quenching effect of antioxidants that donate hydrogen (Prior *et al*., 2005).

3.4.2.2.1 TEAC activity

The mean antioxidant activity determined by the TEAC assay for FB honey was 12.79 µmol TE/g. The measured TEAC value for MAN was 22.09 µmol TE/g and the FB range was from 4.34 – 17.03 µmol TE/g. This activity was 58.71% of that measured for MAN. Among FB honey, the antioxidant activity of FB1, FB2, FB5 and FB6 were similar while that of FB4 differed significantly. MAN showed the highest antioxidant activity compared to all honeys. The obtained FB range was similar to the range of 5.36 – 20.84 µmol TE/g reported for southern African honeys (Serem and Bester, 2012) and higher than 0.2146 – 2.945 µmol TE/g reported for Cuban honeys (Alvarez-Suarez *et al*., 2010b).

3.4.2.2.2 ORAC activity

With the ORAC assay which is a HAT-based method and is considered physiologically relevant, the mean antioxidant activity of FB honey was 46.71 µmol TE/g with a range of 19.87 – 62.89 µmol TE/g. Antioxidant activity of MAN was 46.71 µmol TE/g similar to the mean of FB honey.

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This similarity implies that in a biologically relevant environment both types of honey effectively scavenge free radicals. FB5 with the highest ORAC value was found to be similar to FB6 and FB1. FB2 was similar to FB3 and MAN while FB4 had significantly the least antioxidant activity. The FB range was comparable to the range of 3.71 – 49.26 reported for southern African honeys (Serem and Bester, 2012) and higher for the obtained range of 1.09 – 12.89 µmol TE/g reported for the Cuban honeys (Alvarez-Suarez *et al*., 2010c) and for 2.12 – 21.07 µmol TE/g as reported for honeys of different floral sources and geographical regions (Beretta *et al*., 2005).

3.4.3 Cellular antioxidant activity

Cellular antioxidant activity assays were developed for quantification of antioxidant activity in cell culture and to better represent the activity in a more biologically relevant environment (Wolfe and Liu, 2007). DCFH-DA assay has been widely used to determine cellular antioxidant activity (Wolfe and Liu, 2007; Song *et al*., 2010; Faller *et al*., 2012). It makes use of a radical generator AAPH which generates peroxyl radicals that cause cellular oxidative damage. Molecules such as polyphenols with antioxidant activity will reduce the cellular effects of AAPH from 100% to < 100%. The principle behind this assay is the same as that for ORAC assay and both involve the use of a physiologically relevant radical, however cellular systems are generally more sensitive to the effects of radicals.

3.4.3.1 Caco-2 cell line

Honey contains H_2O_2 that can oxidative damage. In the absence of AAPH the measured levels of oxidative damage in the Caco-2 cell line honey samples was minimal. With AAPH-induced oxidative damage, fluorescence levels decreased from 100% to 19.50 – 58.83% to a mean of 36.09% as presented in Table 3.2. MAN reduced oxidative damaging effects of AAPH to 4.28%. FB2 statistically showed the strongest cellular antioxidant activity amongst the FB honeys with the measured level of oxidative damage of 19.50%. FB1, FB4, FB5 and FB6 were similar to each other and FB3 had the least effect of 58.83%.

3.4.3.2 SC-1 cell line

As for the Caco-2 cell line in the SC-1 cells no oxidative effect due to the presence of H_2O_2 in the honey samples was observed. The measured levels of oxidative damage decreased from 100% for AAPH alone to 9.20 – 46.50% with the average mean of 26.55% for FB honeys. A negative effect was observed for MAN, indicating that all the AAPH radicals were scavenged and in addition had a possible protective effect greater than that observed in the controls. The

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effects of FB1 and FB6 were similar to that of MAN. Furthermore, FB2 and FB5 were statistically the same and similar to the darker honeys (MAN, FB1 and FB6). FB3 and FB4 were significantly the same and similar to both FB2 and FB5. The cellular protective effects of each honey type in the Caco-2 and SC-1 cell lines were compared. These effects were similar for most honey samples except for FB1 and FB6 (Figure 3.4). FB6 and FB1 more effectively reduced AAPH induced oxidative damage in the SC-1 than the Caco-2 cell line.

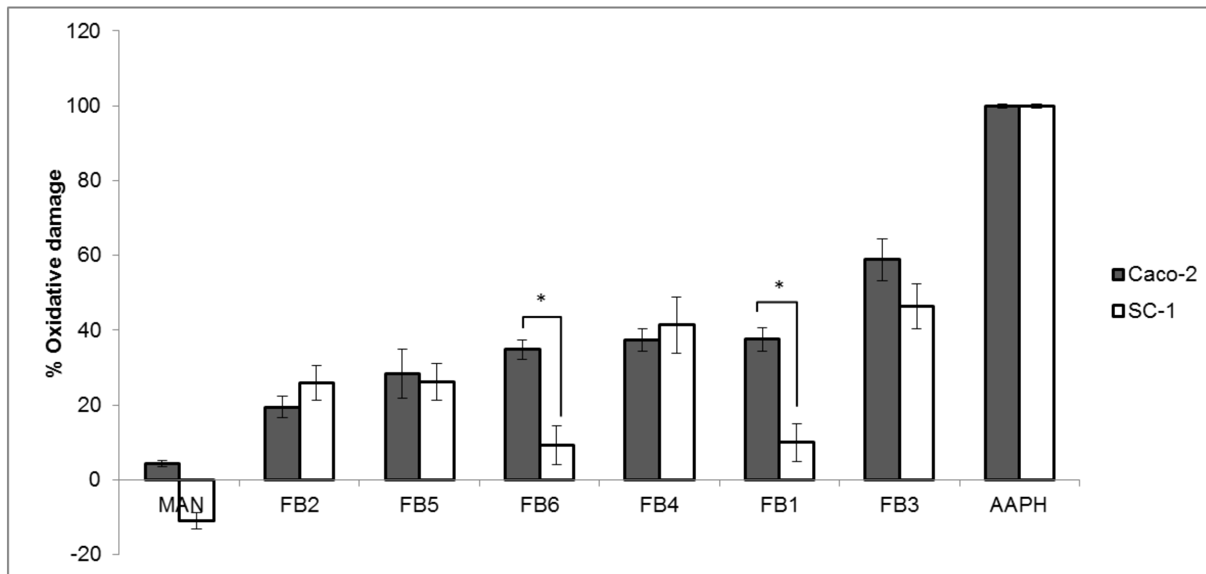

Figure 3.4: Comparison of cellular antioxidant activity between Caco-2 and SC-1 cell lines. Data is an average of 3 experiments ± SEM (in descending order of cellular protection in Caco-2 cells). *Indicates significant difference in protection against oxidative damage between cell lines ($p \leq 0.05$).

In the present study the cellular antioxidant effects of a 10% (v/v) honey solution was evaluated while in the study of Serem and Bester (2012) a 2.5% (v/v) solution was used and the protective effects in the Caco-2 cell line was 30 - 100% and in the SC-1 cell line 10 – 100%. The higher sensitivity of the Caco-2 cell line to oxidative damage is similar to the findings of Serem and Bester (2012). Differences found between the Caco-2 and SC-1 cell lines may be due to differences between species, cell type, doubling times as well as antioxidant enzyme profiles. Interestingly FB1 and FB6 have the highest H_2O_2 content of all honey samples evaluated (Table 3.1). Polyphenols in these honey samples in a cellular environment needs to scavenge radicals generated by H_2O_2 and AAPH. If a cell line such as SC-1 has higher catalase activity than the Caco-2, this may explain the differences in CAA observed for FB1 and FB6.

Ahmad *et al*., (2013) investigated the radioprotective effects of Gelam honey against gamma-irradiation on the antioxidant enzymes present in the human fibroblast diploid cells. Gamma-irradiation as a source of ROS production was found to down-regulate the gene expression and

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enzyme activity of SOD, catalase and glutathione peroxidase enzymes. Upon treatment with Gelam honey for 24 h, and pre-treatment of honey before the fibroblast cells were exposed to gamma-irradiation, significant up-regulation of gene expression and antioxidant enzyme activity were shown for all antioxidant enzymes compared to the down-regulation seen in untreated irradiated cells. Therefore this study, illustrates the protective effects of honey in the presence of ROS by directly scavenging radicals as well as by possibly up-regulation of gene expression which may be greater for the SC-1 cell line.

3.4.4 Anti-inflammatory activity

3.4.4.1 Chemical NO scavenging assay

The NO or more accurately the nitrite content of honey was determined. The nitrite content of FB honey was 0.12 – 0.47 µmol/ml and for MAN honey was 0.41 µmol/ml (Table 3.3). The residual nitrite content of FB5 was similar to that of FB4, FB3 and FB6. FB1 had the highest measured nitrite content among all honeys however was significantly similar to MAN, FB2, FB3 and FB6. Al-Waili (2003) measured residual NO metabolites in seven unprocessed honeys from different geographical regions. The residual nitrite content of these honeys was 0.0027– 0.035 µmol/ml. These levels are much lower than levels found in FB honey and this might be due to the different methods used for analysis. However in a study by Fermo *et al*., (2013), where ion chromatography was used, the nitrite levels in honey was 0.065 – 0.167 umol/ml (converted from 3.0 – 7.7 ppm), a range similar to that found for FB honey.

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Table 3.3: Chemical and cellular anti-inflammatory activity of MAN and FB honey samples

	Chemical NO scavenging activity		**Cellular NO scavenging activity**	
	Nitrite content ($\mu mol/ml\ NO_2^-$)	**NO scavenging (%)**	**Inflammatory activity in RAW 264.7 cells (μmol/ml)**	
			- LPS/IFN-γ	**+ LPS/IFN-γ**
MAN	0.40^a	85.55^a	0.21^b	7.49^b
FB1	0.47^a	59.73^b	2.48^b	10.69^b
FB2	0.32^a	56.52^b	2.46^b	14.13^b
FB3	0.29^{ab}	44.17^{bc}	5.94^a	14.24^b
FB4	0.17^b	37.49^c	7.60^a	15.88^a
FB5	0.12^b	35.67^c	0.23^b	13.89^b
FB6	0.23^{ab}	53.87^b	1.59^b	10.23^b
FB honey				
Av ± SEM	**0.27 ± 0.04**	**47.91 ± 2.44**	**3.38 ± 1.49**	**13.18 ± 5.09**
%SD	**26.03**	**8.80**	**132.17**	**35.60**
Min	**0.12**	**35.67**	**0.23**	**10.23**
Max	**0.47**	**59.73**	**7.60**	**15.88**

Data is an average of at least 3 experiments ± SEM. Different letters indicates significant difference ($p \leq 0.05$).

To determine NO scavenging activity, SNP that forms 24.41 μmol/ml NO, which is several fold greater than the residual nitrite content of honey was used. The ability of each honey sample to scavenge NO formation was evaluated. The FB honeys scavenged 35.67 – 59.73% with a mean of 47.91% (Table 3.3) of the NO generated by SNP (100%). The NO scavenging activity of MAN was 85.55% and significantly greater than all FB honey samples. For FB honeys, the NO scavenging ability of FB1, FB2 and FB6 were similar. Additionally FB3 was similar to FB1, FB2, FB3, FB4, FB5 and FB6.

3.4.4.2 Pro-inflammatory effect

Honey can either have a pro or an anti-inflammatory effect. Firstly the pro-inflammatory effect was measured i.e. the ability of honey to induce NO formation. In the absence of LPS/IFN–γ (- LPS/IFN-γ, table 3.3, Figure 3.5a), RAW 264.7 cells were exposed to a 10% (v/v) honey solution for 24 h. Levels of NO in medium were measured with the Griess assay. FB range for residual honey NO production in RAW 264.7 cells was 0.23 – 7.60 μmol/ml and MAN with 0.21 μmol/ml (Table 3.3). MAN and FB5 significantly produced the least NO and were statistically the same. These honeys were also similar to FB1, FB2 and FB6. FB3 and FB4 produced higher NO levels. When these honey samples were compared to the negative

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control (cells only) Figure 3.5a, a statistical difference was seen only for FB4, indicating that FB4 induces NO production in the RAW 264.7 cell line.

When these honey samples were compared to the positive control (cells+LPS/IFN–γ), all honeys were statistically lower with both FB5 and MAN presenting with the lowest NO content, Figure 3.5a. The release of NO in LPS and IFN–γ non-stimulated RAW 264.7 cells illustrate a pro-inflammatory effect which is necessary for acute wound healing. A study by Tonks *et al.*, (2003), showed the release of pro-inflammatory cytokines (TNF-α, IL-6 and IL-1β) from the MM6 cells and the human peripheral monocytes. The release of these pro-inflammatory cytokines was triggered by three different types of honey and these were Manuka, jellybush and pasture. Similar results were also illustrated by Timm *et al.*, (2008) when natural honeys including Manuka honey released IL-6 from the MM6 cells. Based on the results in Figure 3.5a, the honeys showed a pro-inflammatory effect with the most significant release seen for FB4.

3.4.4.3 Anti-inflammatory effect

Anti-inflammatory effect was evaluated by firstly inducing NO production with the addition of LPS/IFN–γ, after which the cells were exposed to 10% (v/v) honey solution for a further 24 h (+ LPS/IFN-γ, Table 3.3 and Figure 3.5b). NO production in LPS-stimulated RAW 264.7 cells reduced NO levels from 16.54 μmol/ml to 10.23 – 15.88 μmol/ml for FB and 7.49 μmol/ml for MAN honey. MAN, FB1 and FB6 presented with a statistically lower NO production when compared to the positive control (cells+LPS/IFN–γ). This shows that MAN is a better NO scavenger or inhibitor of NO production as compared to FB honeys while FB4 showed the lowest NO scavenging effects (values shown in Table 3.3).

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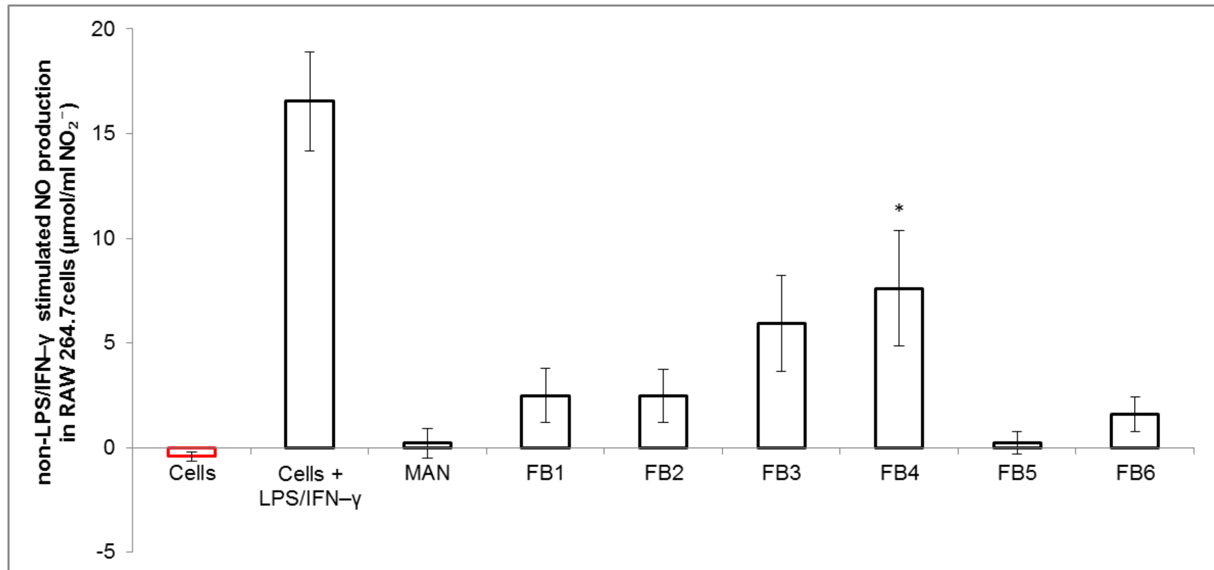

Figure 3.5a: RAW 264.7 cells induced NO formation by honey (Pro-inflammatory effect, No LPS and IFN–γ added) Cells (negative control) and cells exposed to LPS/IFN–γ (positive control). Data is expressed as mean ± SEM. * Indicates significant difference between honey samples and the negative control, p≤0.05.

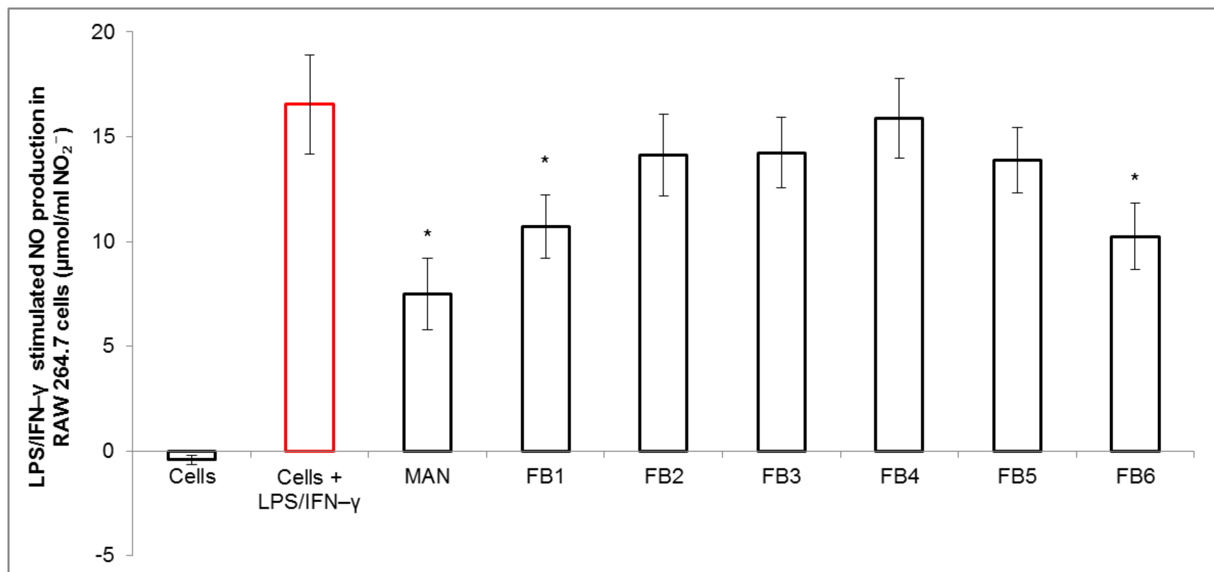

Figure 3.5b: Inhibition of LPS-induced NO production in RAW 264.7 cells (Anti-inflammatory effect, LPS and IFN–γ added) by honey. Cells alone (negative control), cells exposed to LPS/IFN–γ (positive control). Data is expressed as mean ± SEM. * Indicates significant difference between honey samples and the positive control, p≤0.05.

A cell viability assay was linked to measuring NO production in honey samples with or without LPS, to ensure that the samples that showed reduced levels of NO was due to sample scavenging or inhibition of NO formation and not cell death (Figure 3.5c). A concentration of 10% (v/v) honey samples did not show any cytotoxicity in the RAW 264.7 cell line with/without LPS and IFN–γ. There was no significant difference in cell viability between honey samples with LPS/IFN–γ and honey samples without LPS/IFN–γ (p≤0.05). A

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study by Jaganathan and Mandal (2010), evaluated the anti-proliferative effects of honey against human colon cancer cell lines (HT-29 and HCT-15). An MTT assay was used to assess cell viability of different honey concentrations. Lower concentrations of honey were not toxic towards the colon cancer cells however with higher concentrations (10% and 20%) the honeys were cytotoxic. Compared to FB honeys, although using an anti-inflammatory cell line (RAW 264.7) higher honey concentrations of 12,5% (v/v), 25% (v/v) and 50% (v/v) of FB honeys were found to be cytotoxic (data not shown).

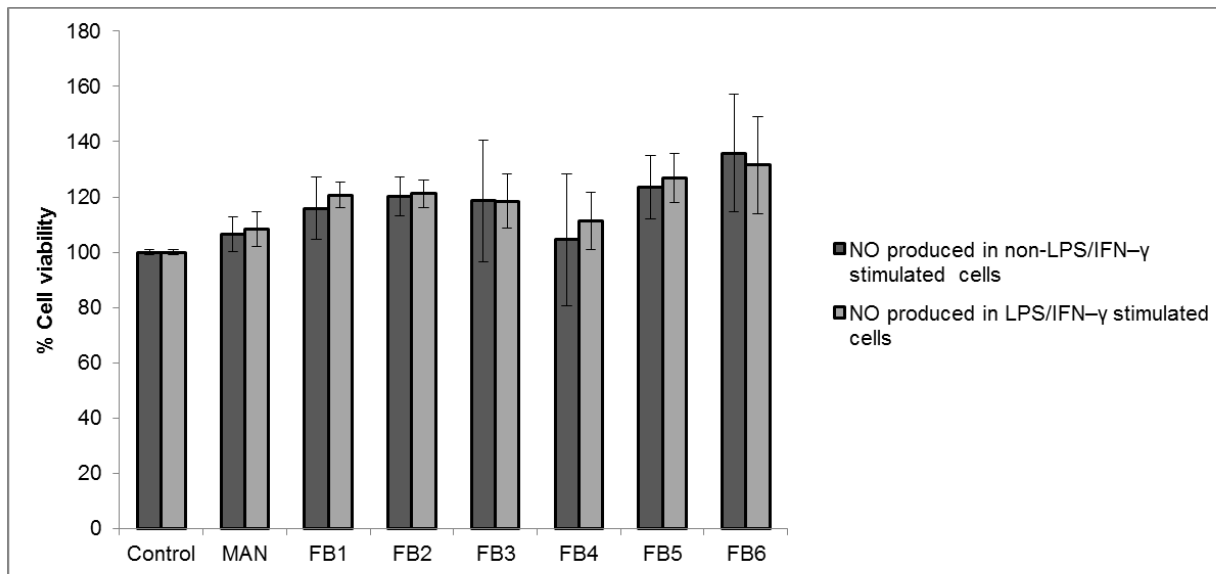

Figure 3.5c: Percentage cell viability of samples from Figure 3.5a and Figure 3.5b. Data is an average of at least three experiments ± SEM. No statistical significance was found between cells exposed to honey with and without LPS and IFN–γ.

Comparing this study to a study done by Kassim *et al.*, (2010a) where the anti-inflammatory properties of Malaysian (Gelam) honey extracted in methanol (HME) and ethyl acetate (HEAE), in the presence of LPS and IFN-γ stimulated macrophages (RAW 264.7) a higher NO inhibition of 80% (4.3 μmol/L remaining NO levels) was seen for HEAE and 40% (16 umol/L remaining NO levels) for HME. These values are higher than found in the present study where 4.00 – 38.12% inhibition was observed. This can be explained due to the fact that in the study of Kassim *et al.*, (2010a), methanol and ethyl acetate extracts represent fractions that are enriched in specific types of polyphenols. The polyphenols present in these extracts were caffeic acid, gallic acid, ellagic acid, chlorogenic acid, myricetin, ferulic acid, quercetin, hesperetin, chrysin, luteolin, kaempferol and p-coumaric acid.

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Table 3.4a: Correlations of colour, chemical antioxidant content and activity and cellular antioxidant activity of FB honeys

	Colour	**TPC**	**TFC**	**TEAC**	**ORAC**	**DCFH-DA**	
						Caco-2	**SC-1**
Colour	**1**	0.66	-0.31	0.66	0.54	0.08	**-0.80**
TPC	-	**1**	0.21	**0.96**	**0.91**	-0.13	**-0.78**
TFC	-	-	**1**	0.32	0.07	-0.18	0.14
TEAC	-	-	-	**1**	**0.76**	-0.16	**-0.75**
ORAC	-	-	-	-	**1**	**-0.75**	**-0.89**
DCFH-DA Caco-2	-	-	-	-	-	**1**	0.48
DCFH-DA SC-1	-	-	-	-	-	-	**1**

Bold values indicate high level of correlation.

Table 3.4b: Correlations of TPC, chemical and cellular NO scavenging activity of FB honeys

	NO scavenging	**RAW 264.7**	
			+ LPS/IFN – γ
TPC	0.64		**-0.87**
NO scavenging	**1**		**-0.70**

Bold values indicate high level of correlation.

Correlations

Table 3.4a presents a correlation matrix to demonstrate if there are any relationships between colour and antioxidant assays, both chemical and cellular for FB honeys. Colour has been related with compounds such as pigments and flavonoids. Furthermore literature has reported strong correlations between colour and TPC, TFC as well as antioxidant activity assayed by colorimetric assays (Saxena *et al*., 2010; Khalil *et al*., 2011a; Khalil *et al*., 2011b; Serem and Bester, 2012 and Moniruzzaman *et al*., 2013). Thus darker honeys, possess more phenolic acids, flavonoids and associated increased antioxidant activity.

In this study, colour correlations between TPC and TEAC were not high with a coefficient of r = 0.66 and low for the ORAC assay (r = 0.54). With TFC, there was no correlation (r = -0.31) and this may be attributed to the underestimation of flavonoids with the aluminium chloride method.

Strong correlations for FB honeys were found between TPC vs TEAC and TPC vs ORAC with values of r = 0.96 and r = 0.91, showing that the polyphenols contribute towards the antioxidant activity of honey. These results were similar to r = 0.96, p≤ 0.001 (TPC vs TEAC), r = 0.96, p≤ 0.001 (TPC vs ORAC) reported for monofloral Cuban honeys (Alvarez-

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Suarez *et al*., 2010c). Serem and Bester (2012) also showed a strong correlation between TPC vs TEAC and TPC vs ORAC with correlation coefficient values of r = 0.95 and r = 0.94, p< 0.001 respectively for southern African honeys.

A positive correlation of r = 0.76 was obtained for FB honeys between the ET and HAT assays, TEAC vs ORAC assays. A higher correlation value was obtained for southern African honeys (r = 0.89, p<0.001) as reported by Serem and Bester, 2012 and r = 0.96, p ≤ 0.001 for monofloral Cuban honeys reported by (Alvarez-Suarez *et al*., 2010c). This illustrates that the higher the polyphenolic content usually translates into higher antioxidant activity.

Strong correlations between TFC and other parameters such as, colour, TPC as well as antioxidant activity have been reported, for monofloral Cuban honeys (Alvarez-Suarez *et al*., 2010b), Malaysian honeys (Khalil *et al*., 2011a; Khalil *et a*l., 2011b; Moniruzzaman *et al*., 2013), Romanian honeys (Al *et al*., 2009), Rubus honey from North West of Spain (Escuredo *et al*., 2011) and for southern African honeys (Serem and Bester, 2012). In this study, no correlations between TFC and the rest of the parameters especially for colour, TEAC and ORAC were found (Table 3.4a). Generally the flavonoid content is low as compared to the phenolic content in honeys. Correlations of TFC in FB honeys illustrated that FB honeys contain less flavonoids. A study by Meda *et al*., (2005) reported similar TFC results for Burkinabe honeys with a correlation coefficient value of R = 0.11 between TPC vs. TFC. This could be explained as mentioned earlier by the underestimating property of the aluminium chloride test or possibly that these honeys are rich in phenolic acids and have a low flavonoid content.

Relative to the CAA all correlations were negative i.e. with increased antioxidant activity there is an increase in the inhibition of oxidative damage in both cell lines. For the Caco-2 cell line there was a correlation between the ORAC and DCFH-DA assays with r = - 0.75. In the SC-1 cell line there is a strong and significant correlation between DCFH-DA and colour, TPC, TEAC and ORAC assays. Similar correlations were also reported by Serem and Bester (2012) for southern African honeys.

Correlations between TPC and NO scavenging activity of FB honeys in a chemical and cellular environment will indicate that the polyphenols present in honey are responsible for its anti-inflammatory activity. In Table 3.4b, a low correlation of r = 0.64 between TPC and chemical NO scavenging activity was found, however there was a strong correlation (r = - 0.87) with the NO produced in + LPS/IFN – γ stimulated RAW 264.7 cells. Furthermore,

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indicating the inhibition of NO levels by polyphenols. A correlation of r = - 0.70 between chemical NO scavenging activity and NO levels produced in + LPS/IFN – γ stimulated RAW 264.7 cells was found. This means that honeys with high NO scavenging activity showed low NO production in + LPS/IFN – γ stimulated RAW 264.7 cells. This effect might be accounted for by the direct scavenging of NO or the inhibition of iNOS function by polyphenols.

3.5 Conclusion

All honeys are of good quality and have met the standards of CODEX Alimentarius.

In summary, the darkest honeys are, MAN, FB6 and FB1. The honeys with the highest antioxidant content are MAN FB6, and FB1. For antioxidant activity these are, MAN, FB1 and FB6 (TEAC assay) and FB5, FB6 and FB1 (ORAC assay). Therefore it can be concluded that honeys with the highest antioxidant content and activity are the darkest honeys. For CAA, in the SC-1 cells, the trend is similar to the chemical antioxidant activity whereby MAN, FB1 and FB6 honeys show the most protection. In contrast in the Caco-2 cells, MAN, FB2 and FB5 show the most protection against oxidative damage.

Regarding anti-inflammatory effects related to NO scavenging ability, MAN, FB1, FB6 and FB2 show the highest NO scavenging activity and likewise cellular NO scavenging activity is the highest for MAN, FB1 and FB6.

Correlations reveal that antioxidant and anti-inflammatory properties related to NO scavenging is due to the presence of polyphenols. For all parameters measured MAN has consistent high levels or activity except for antioxidant activity measured with the ORAC assay.

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Chapter 4: The effect of *in vitro* simulated digestion on the antioxidant properties of Fynbos honey

4.1 Introduction

Polyphenols are secondary metabolites produced by plants. These molecules or phytochemicals play an important role in the defence system of plants. Dietary polyphenols have been shown to be beneficial to health by possessing biological activities such as antioxidant and anti-inflammatory activities. These dietary polyphenols or phytochemicals are found in many foods such as vegetables, fruits, honey and grains. Various studies have reported that a higher intake of such foods is linked with a reduction of many chronic diseases such as cardiovascular diseases, chronic inflammation, diabetes, degenerative diseases (Willett, 1994; Willett, 1995; Ness and Powles, 1997; Temple, 2000) and cancer, especially in the GIT (Milner, 1994; Johnson, 2004).

The antioxidant activity of honey is attributed to the presence of phenolic compounds which have been shown to be responsible for health beneficial effects such as anti-atheriogenic, anti-carcinogenic, antioxidative properties as well as anti-inflammatory properties of honey (Cook and Samman, 1996; Al-Mamary *et al*., 2002; Alvarez-Suarez *et al*., 2010a).

One of the concerns regarding the health benefits of these phenolic compounds is their bioavailability. Bioavailability is defined as the extent to which the fraction of nutrients passes through or is absorbed by the intestinal cells to be utilised (Wood, 2005; Chandrasekara and Shahidi, 2012). Steps that are involved in determining the bioavailability of these released phenolic compounds include their bioaccessibility (release of compounds from a food matrix) in the GIT digestion, absorption and metabolic fate (Rodríguez-Roque *et al*., 2013). Several factors are known to affect the bioavailability of these dietary compounds, such as the food matrix, the extraction of these compounds, their stability throughout the GIT digestion as well as transepithelial absorption (Tagliazucchi *et al*., 2010).

The purpose of simulated gastro-duodenal digestion is to evaluate the release of phenolic compounds from the food matrix into the gut and serves as a first step in evaluating the bioavailability of these dietary compounds. Furthermore this model is also used to investigate the stability of phenolic compounds as these compounds are structurally influenced by the different pH conditions as well as the interactions with digestive enzymes. In employing *in vivo* animal models, it is difficult (in terms of experimental design, time and expense) to evaluate such changes on the phenolic compounds hence the use of *in vitro* models as alternatives for *in vivo* models is of value. *In vitro* models are cheap, easier to

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perform (Failla and Chitchumroonchokchai, 2005), and can more easily be used to monitor each stage of digestion. In addition *in vitro* models provide less ethical restrictions as compared to the *in vivo* models (You *et al*., 2010). This model has been widely used to evaluate the effect of digestion on the bioactivities of foods such as apples (Bouayed *et al*., 2011), grapes (Tagliazucchi *et al*., 2010), vegetable juices (Wootton-Beard *et al*., 2011) and millet grains (Chandrasekara and Shahidi, 2012). Although it was shown in chapter 3, that the antioxidant and anti-inflammatory activity related to NO scavenging is the highest for MAN, the effect of digestion of these properties is unknown.

Aim

The aim of this chapter is to investigate the effects of *in vitro* simulated gastro-duodenal digestion (SGDD) on the chemical and cellular antioxidant content and activity of FB honeys compared to MAN honey.

The objectives of this chapter are:

a. To determine the effect of each phase of *in vitro* SGDD on the total polyphenolic and flavonoid content of each honey.
b. To determine the effect of each phase of SGDD on the antioxidant activity (TEAC and ORAC assays) of each honey.
c. To determine the effect of each phase of SGDD on the ability of each honey to provide cellular antioxidant protection (Caco-2 and SC-1 cell lines) against AAPH induced oxidative damage (DCFH-DA assay).
d. For each phase of digestion determine the effect of pH and digestive enzymes.

4.2 Materials

All materials for this chapter have been described in chapter 3, with exception of those used for the digestion process. These include: Hydrochloric acid (HCl), sodium bicarbonate ($NaHCO_3$), pepsin enzyme from porcine gastric mucosa and pancreatin from porcine pancreas. All reagents were all obtained from Sigma-Aldrich Company, Atlasville, SA and were of analytical grade. All equipment and laboratory facilities were the same as used in chapter 3.

4.3 Methods

4.3.1 *In vitro* simulated gastro-duodenal digestion

The *in vitro* simulated digestion was performed according to a modified method of Daglia *et al*., (2013). A gastric digestion stock solution was prepared by adding 20 mg of pepsin in 1ml of 1 M HCl. For the gastro-duodenal digestion, a pancreatic stock solution was made by

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adding 4 mg of pancreatin to 1 ml of a 1 M $NaHCO_3$ solution. Both solutions were left to shake for 1 h at room temperature. Honey samples were then diluted to 90% (v/v) to achieve fluidity, and subjected to *in vitro* GIT digestion in two stages, gastric and gastro-duodenal. Gastric digestion involved decreasing the pH of the honey solution to 2. Thereafter, 5 µl of the prepared gastric digestion solution was added per ml of the honey solution and incubated in the water bath for 30 min at 37˚C. This was then followed by gastro-duodenal digestion which involved the increasing of the pH of honey samples to 7 then adding 5 µl of the prepared pancreatin stock solution per ml of honey solution. This honey solution was then incubated in the water bath for 1 h. To inactivate the enzyme reactions samples were removed from the water bath and heated in water (95˚C) for 5 min. All stages of digestion had their controls in order to rule out the effect of pH and enzyme factors on measured activity. These then yielded a total of 5 fractions for each honey sample namely: Fraction 1: Undigested sample (UD); fraction 2: pH 2 control (pH 2), fraction 3: gastric digest (GD), fraction 4: pH 7 control (pH 7) and fraction 5: gastro-duodenal digest (GDD), as summarised in Figure 4.1.

Samples of ddH_2O were also included in this study and subjected to SGDD and were processed and assayed in the same manner as the digested samples. This was done to correct for possible interference by the digestive enzymes as well as the effect of pH changes.

4.3.2 Antioxidant content and activity

Each sample and digest was diluted to 10% (v/v) and 1% (v/v) and antioxidant content and activity was determined as described in sections 3.3.2 and 3.3.3 of chapter 3, respectively.

4.3.3 Cellular antioxidant activity

Each sample and digest was diluted to 10% (v/v) and cellular antioxidant activity was determined as described in section 3.3.4 and 3.3.5 of chapter 3.

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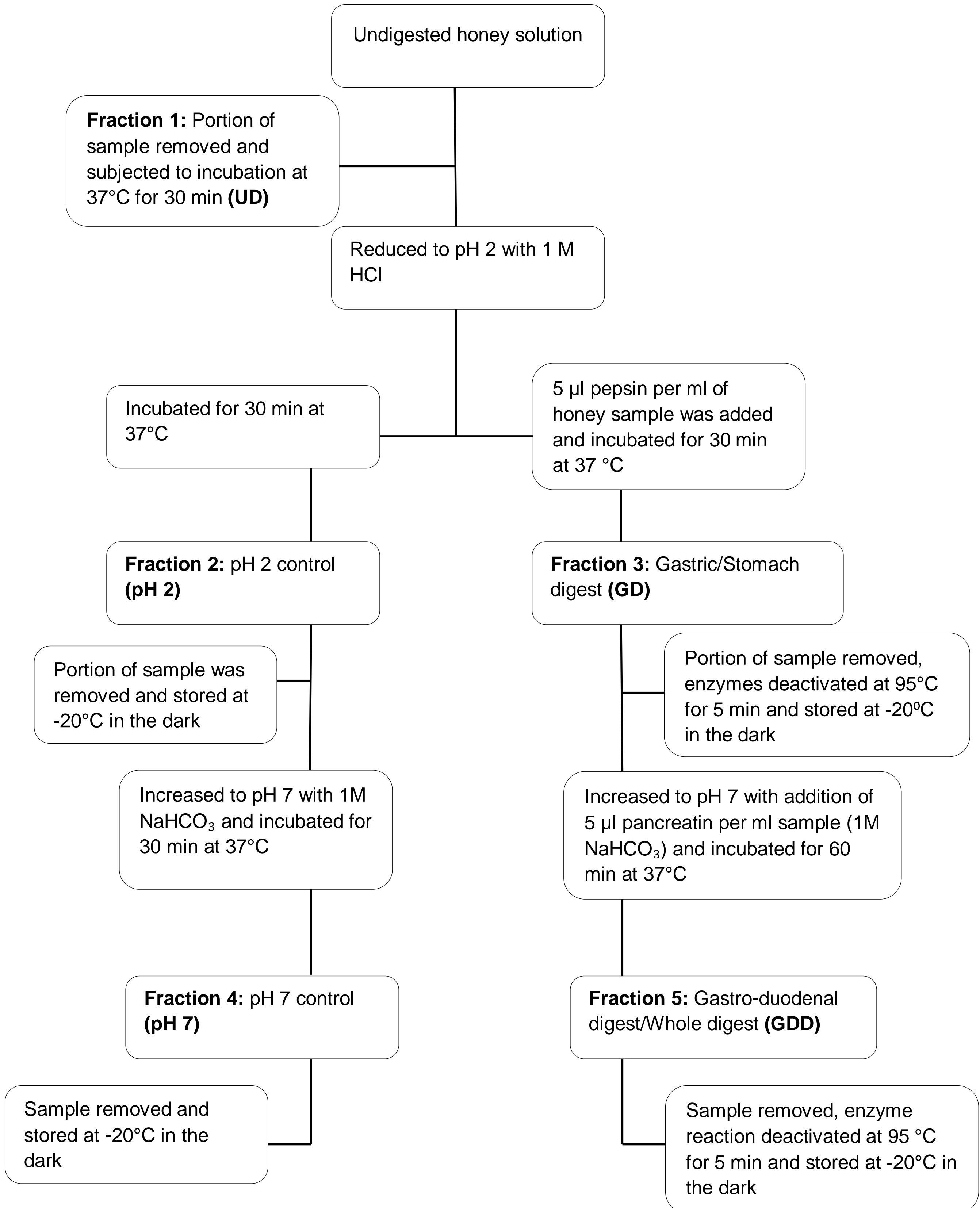

Figure 4.1: Methodology for the *in vitro* simulated gastro-duodenal digestion of the honey samples.

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4.3.4 Data management and statistical analysis

At least three sets of experiments were conducted, with each point evaluated in triplicate, thus yielding a total of 9 data points for statistical analysis. Analysis of Variance techniques were used to compare chemical antioxidant content, activity and cellular antioxidant activity of MAN and 5 FB honeys for each treatment. Interactions between different honey treatments were investigated using post-hoc pairwise tests to identify which honeys and treatments differed from one another. The statistical method employed for these analyses as well as for the correlations was the same as those used to analyse data for chapter 3.

4.4 Results and discussion

In chapter 3 the chemical antioxidant content and/or activity showed that the FB honeys were similar except for FB4, which consistently had lower antioxidant activity and content. Therefore to study the effect of SGDD honey samples with similar bioactivity FB1, FB2, FB3, FB5 and FB6 were selected.

4.4.1 Antioxidant content

4.4.1.1 TPC

The average TPC of all FB honeys compared to MAN is presented in Figure 4.2a. For MAN with gastric digestion, TPC was significantly increased compared to UD while following GDD, TPC was reduced to less than UD. For MAN, differences between GD and GDD were significant. For FB there were no differences when compared to UD, although differences between GD and GDD were significant.

Low pH associated with gastric digestion resulted in increased TPC for MAN. No effect was observed for FB (Figure 4.2b.a). For all samples, the presence of digestive enzymes had no effect on TPC (Figure 4.2b.b).

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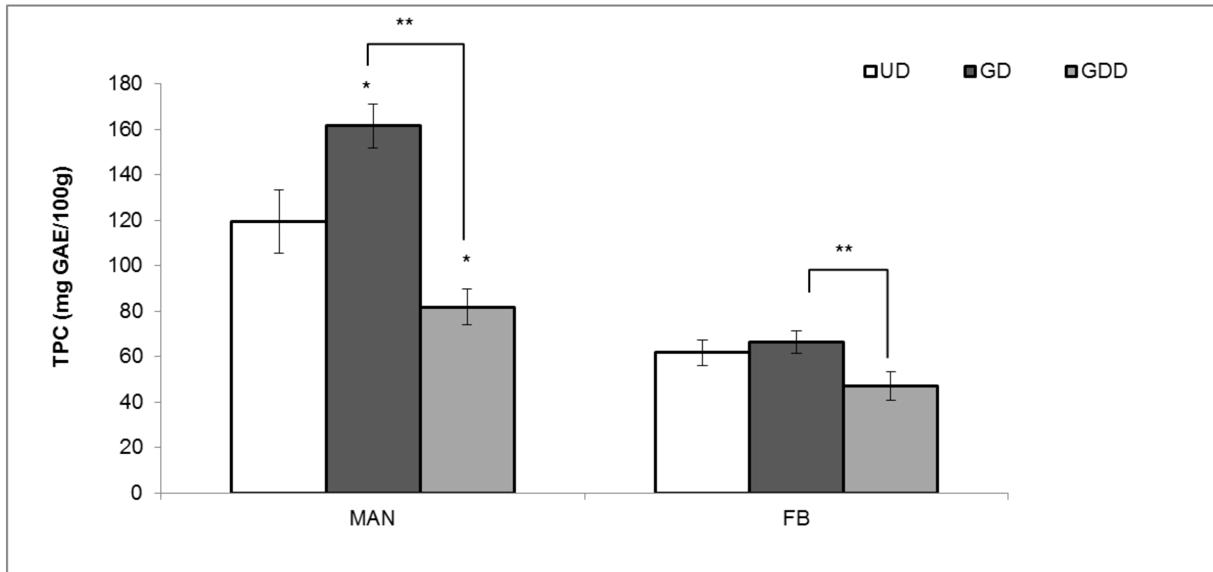

Figure 4.2a: TPC during SGDD of MAN and averages of FB honeys. Data is expressed as mean ± SEM. * Indicate significant difference compared to UD and ** indicates difference between GD and GDD, p≤0.05.

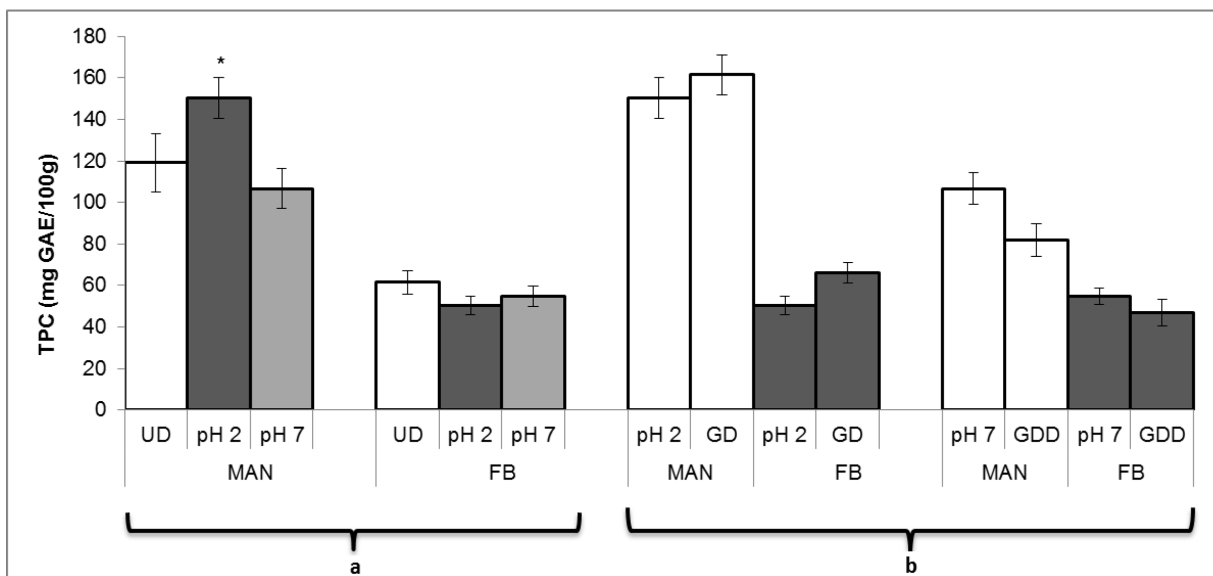

Figure 4.2b: a) Effect of pH on TPC in the absence of digestive enzymes compared to UD and b) the comparison between pH and each phase of digestion (pH2 vs. GD and pH7 vs. GDD) for MAN (n=1) and FB (n=5) honey. Data is expressed as mean ± SEM. * Indicates significant difference compared to UD p≤0.05.

A study by O'Sullivan *et al.*, (2013) found no significant change in TPC before and after *in vitro* digestion of 4 honeys which is similar to the effect observed for FB honey. In a study evaluating the bioaccessibility of phenolic compounds in soymilk, a significant TPC increase was observed following the gastric phase of digestion which decreased following duodenal digestion (Rodríguez – Roque *et al.*, 2013). The increase in polyphenol content observed following gastric digestion can be attributed to the acidic conditions and enzyme activities which can cause the hydrolysis of phenolic compounds that are bound to other food

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components (Rodríguez – Roque *et al*., 2013). In Figure 4.2b, it is shown that this effect in MAN is due to pH and not digestive enzyme activity. Baublis *et al*., (2000) and Liyana-Pathirana and Shahidi (2005) also reported that in the gastric phase of digestion, phenolic compounds are released from high molecular weight compounds such as carbohydrates and proteins (Saura-Calixto *et al*., 2007). In a bioaccessibility study of grapes an increase in TPC was observed with gastric digestion followed by a decrease with pancreatic digestion (Tagliazucchi *et al*., 2010). However interestingly after 2 h of pancreatic digestion an increase in TPC content was observed. With gastric digestion a significant increase in TPC for MAN was observed and this is probably due to the increased extraction of bound polyphenols, however, for honey little is known about the association of polyphenols with matrix components such as carbohydrate and protein.

In a study by Bermúdez-Soto *et al*., (2007) the stability of major polyphenols found in a commercial chokeberry (*Aronia melanocarpa*) juice during *in vitro* simulated gastric and pancreatic digestion were evaluated. Chlorogenic acid and caffeic acid derivatives found in chokeberry were not altered with gastric digestion when compared to the original juice. In contrast with pancreatic digestion, both neochlorogenic acid and chlorogenic acid levels were altered. Neochlorogenic acid levels decreased while chlorogenic acid levels increased. Evaluation of the effects of digestion on the pure, isolated phenolic compounds, found that chlorogenic acid was converted to neochlorogenic acid. Many of the phenolic compounds are not stable under these conditions and may degrade or undergo structural transformations producing different compounds (Bermúdez-Soto *et al*., 2007; Jamali *et al*., 2008).

A similar study by Chiang *et al*., (2013), evaluated the effects of digestion on the TPC of two types of European gooseberries the Tixia and Invicta varieties. Controls or undigested samples of this study contained heat-inactivated enzymes and the digested samples contained active enzymes. Both controls and treatments were subjected to *in vitro* GIT digestion and a significant increase in TPC was seen in digested samples compared to the undigested samples. These observations were different for both FB and MAN honeys as FB honey showed no change in TPC while MAN showed a decrease when UD was compared to GDD. A study done on millet grains also showed an increase in TPC throughout digestion (Chandrasekara and Shahidi, 2012). Different effects were observed for 23 juices (Wootton-Beard *et al*., 2011). A significant increase following gastric digestion was shown in all 23 juices while a further significant increase was observed in the duodenal phase of 19/23 juices while a decrease was seen for 4 juices. When the effect of SGDD was evaluated in 8 fruit juices, all showed a significant decrease when UD was compared to GDD (Cilla *et al*.,

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2011). A pomegranate juice study showed no significant difference in the phenolic acid content after gastric digestion however with a decrease seen in the pancreatic digestion (dialyzed and non-dialyzed samples) when compared to the TPC of the undigested juice (Pérez-Vicente *et al.*, 2002). A study by Bouayed *et al.*, (2011) showed no significant differences of TPC of all 4 variants of apples transitioning from gastric phase to intestinal phase of digestion. Furthermore when the TPC of gastric and intestinal digestion was compared to the TPC of methanol extracts, there were significant decreases in both gastric and intestinal digests illustrating the loss of phenolic acids. Differences in the effect of GDD on the TPC of several fruits and grains have been evaluated and this may be a function of matrix effects and the complexity of each sample and this includes the amount of fibre and protein as well as polyphenol composition and types. For both MAN and FB honey there is a significant decrease in TPC when transitioning from GD to GDD. This indicates that although honey is rich in polyphenols, these molecules are susceptible to the effects of GDD.

Factors such as pH and digestive enzymes affect the stability of the phenolic compounds. Related to the effect of pH as well as digestive enzymes, Figure 4.2b part a, illustrated a significant increase of TPC in a pH 2 fraction of MAN honey. This illustrates the influence of the acidic pH towards the release of phenolic acids (Rodríguez – Roque *et al.*, 2013) while in contrast no effect was observed for FB. A study of 5 types of millet grains showed no significant pH influences on TPC for 4 types of grains (kodo, pearl, foxtail and proso), however for one type of millet grain (finger), a pH effect was observed (Chandrasekara and Shahidi, 2012). Likewise for the honey samples evaluated in this study, the effect of pH is dependent on the type and composition of the sample.

4.4.1.2 TFC

The average TFC of FB honeys is compared to MAN honey (Figure 4.3a). For MAN, a significant increase in TFC in GD was observed when compared to UD and no change observed in GDD when compared to UD. When GD was compared to GDD, a statistical decrease was observed. For FB honey, the observed TFC trend followed the same trend observed for TPC, where the average differences were not significant throughout SGDD when UD was compared to both GD and GDD. Comparison between GD and GDD showed a statistical decrease of TFC.

In Figure 4.3b, a significant influence of pH was observed for MAN only, where an increase of TFC was observed at pH 2 and a decrease observed at pH 7 compared to UD. Increased TFC seen in pH 2 and the presence of pepsin in GD results in an increase in TFC. For FB

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honeys, the extraction of flavonoids was not influenced by both pH and digestive enzymes (Figure 3b part b).

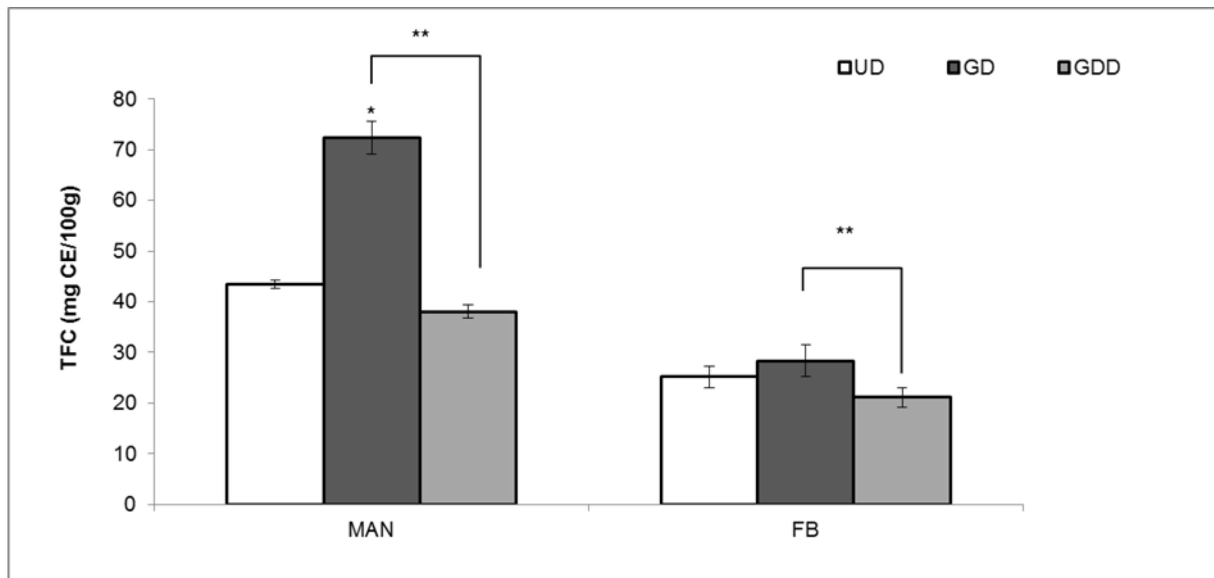

Figure 4.3a: TFC with SGDD of MAN and averages of FB honeys. Data is expressed as mean ± SEM. * Indicates significant difference when compared to UD and ** indicates difference between GD and GDD, p≤0.05.

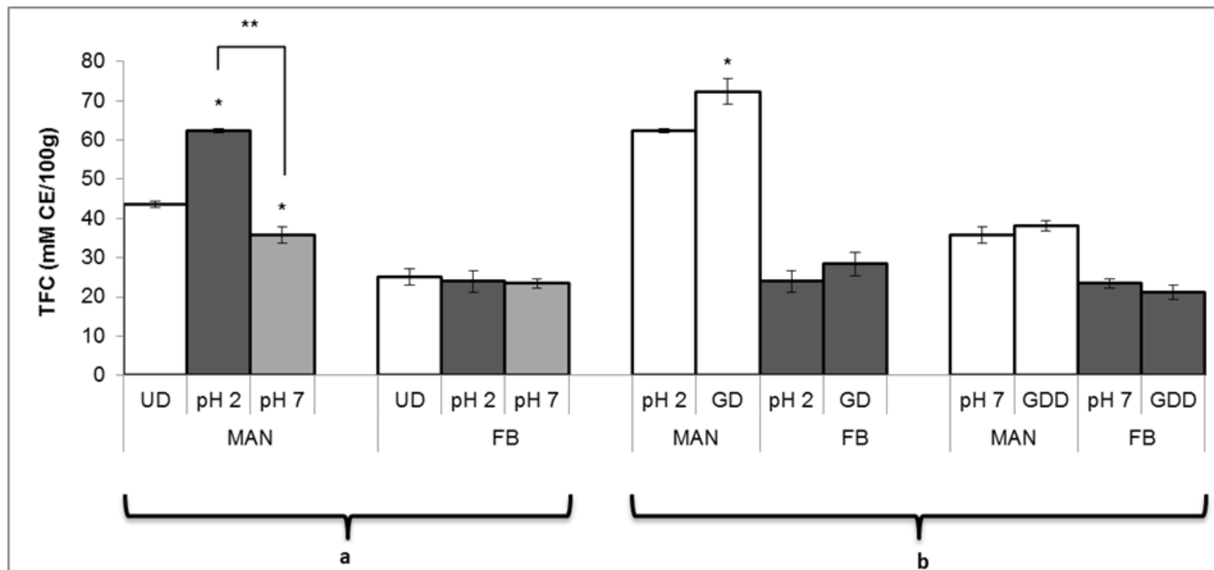

Figure 4.3b: a) Effect of pH on TFC, in the absence of digestive enzymes compared to UD and b) the comparison between pH and each phase of digestion (pH2 vs GD and pH7 vs GDD) for MAN (n=1) and FB (n=5) honey. Data is expressed as mean ± SEM, p≤0.05. * In part **a** indicates difference when compared to UD and ** indicates significant difference between pH 2 and pH 7. In part **b**, * indicates significant difference when GD is compared to pH 2.

Comparing these results to that of soymilk (Rodríguez – Roque *et al.*, 2013), an increase in extraction of the total isoflavones in gastric digestion was observed relative to the non-digested sample. Following duodenal digestion, no significant change was seen relative to the undigested sample. Comparisons between gastric and duodenal digestion showed significant losses of the total isoflavones. When grape flavonoids were compared before and

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after gastric digestion, an increase was observed (Tagliazucchi *et al*., 2010). In this study similar to TPC, an increase in flavonoid content in the pancreatic digestion was observed 2 h after incubation, however the anthocyanins level decreased. Evaluation of the pure flavonoids found in these samples revealed that catechin and quercetrin were stable under gastric conditions but transition from an acidic medium to the alkaline environment caused degradation, however not significant. Flavonoids (flavonols and flavan-3-ols) in chokeberry juice were stable in gastric digestion when compared to the original fraction (Bermúdez-Soto *et al*., 2007). However a decrease in these flavonoids was observed with pancreatic digestion when compared to the original fraction. Further evaluation of the pure flavonoid compounds (cyanidin 3-rutinoside and quercetin-3-rutinoside) showed stability in the gastric digestion when compared to the original fraction. In pancreatic digestion, flavonoids in chokeberry as well as pure commercial compounds decreased. A study done on millet grain by, Chandrasekara and Shahidi, (2012), showed significant increases of the flavonoids throughout gastric and intestinal phases of digestion. A similar study on the pomegranate juice, evaluated a specific family of flavonoids, the anthocyanins (Pérez-Vicente *et al*., 2002). With gastric digestion, anthocyanin levels were increased while with pancreatic digestion a decrease in both dialyzed and non-dialyzed fractions was found relative to the undigested sample.

In general, TFC levels increase with GD but depending on the type of cereal or fruit variable effects are obtained for GDD which is either increased as for millet or reduced as found for several types of fruit. Likewise for MAN, TFC is increased but with GDD is reduced when compared to the GD. TFC is unaltered for FB, but is reduced following GDD when compared to GD. For MAN increased TFC following GD was a function of pH and enzyme activity, indicating increased extraction or release of flavonoids from the food matrix. These factors did not affect the TFC of FB. No studies could be found that investigated the effects SGDD on the TFC of honey.

<u>4.4.2 Antioxidant activity</u>

<u>4.4.2.1 TEAC</u>

The average of TEAC of all FB honeys compared to MAN is presented in Figure 4.4a. MAN showed an unchanged antioxidant activity with GD and a significant reduction of activity following GDD when compared to UD. For FB honeys, no statistical differences were found throughout SGDD when compared to UD. However when GD was compared to GDD, a significant decrease in antioxidant activity was found.

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A pH effect was found for MAN with a significant reduction of antioxidant activity at pH 7 when compared to UD while no pH effect was seen for FB honeys (Figure 4.4b). Both honeys also showed no effect of digestive enzymes on the TEAC activity. The decrease in antioxidant activity was a function of change in pH (Figure 4.4b).

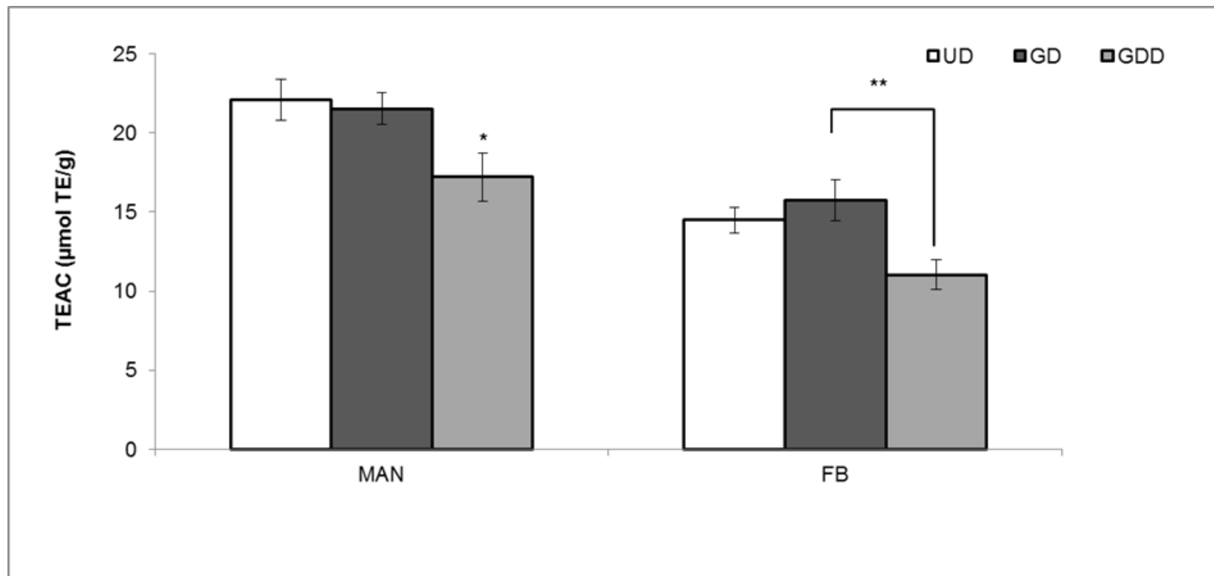

Figure 4.4a: Effect of SGDD on the antioxidant activity of MAN and FB honey determined by the TEAC assay. Data is expressed as mean ± SEM. * Indicates significant difference compared to UD and ** indicates differences between GD and GDD, p≤0.05.

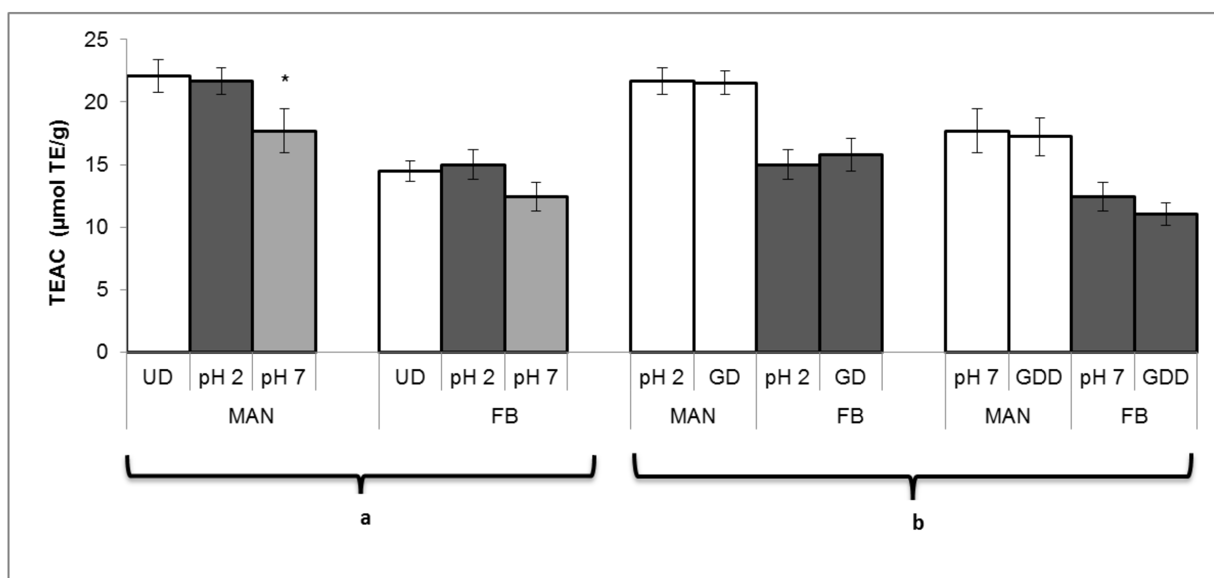

Figure 4.4b: a) Effect of pH on antioxidant activity determined by TEAC, in the absence of digestive enzymes compared to UD and b) the comparison between pH and each phase of digestion (pH 2 vs GD and pH 7 vs GDD) for MAN (n=1) and FB (n=5) honey. Data is expressed as mean ± SEM. * Indicates significant difference compared to UD, p≤0.05.

Comparing these results with studies used above, 23 vegetable juices showed an increase in activity for 12/23 juice when the undigested juice was compared to the duodenal phase of

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digestion (Wootton-Beard *et al*., 2011). However for beetroot juice and 2 Cawston Press juices (apple and rhubarb and apple with beetroot) showed significant reduction in antioxidant activity which resembles the trend for MAN honeys. However a different trend for other functional foods was observed. Such studies include the TEAC activity of millet grains. All five types of millet grains showed increasing antioxidant activity in a gastric phase and a further increase with intestinal digestion (Chandrasekara and Shahidi, 2012). Similar results were seen for polyphenols in grapes where an increase in antioxidant activity was also seen with GD and a further increase in pancreatic digestion (Tagliazucchi *et al*., 2010). These observations were further supported by the *in vitro* digestion of pure phenolic compounds (gallic acid, caffeic acid, catechin, quercetin and resveratrol), which displayed an increased antioxidant activity in the pancreatic/intestinal digestion compared to the gastric digestion (Tagliazucchi *et al*., 2010). Effect of digestion on 8 fruit juices also showed an increase in antioxidant activity when UD was compared to GDD (Cilla *et al*., 2011).

An alkaline pH showed a decrease in antioxidant activity of MAN (Figure 4.4b). This illustrates the influence of pH especially pH 7 which significantly decreases antioxidant activity which is also seen in GDD (Figure 4.4a). Comparing these results to that of millet grains, an increase in antioxidant activity was shown in pH fractions, thus illustrating the contribution of pH towards the antioxidant activity of millet grains (Chandrasekara and Shahidi, 2012) and this may be related to the type and complexity of the polyphenols found in samples analysed.

4.4.2.2 ORAC

The effect of SGDD on the antioxidant activity determined by the ORAC assay for MAN and FB averages is shown in Figure 4.5a. No statistical difference was observed throughout SGDD for both MAN and FB honeys, indicating that SGDD did not affect antioxidant activity measured with the ORAC assay. Additionally, there was no pH and enzymatic influence on antioxidant activity (Figure 4.5b).

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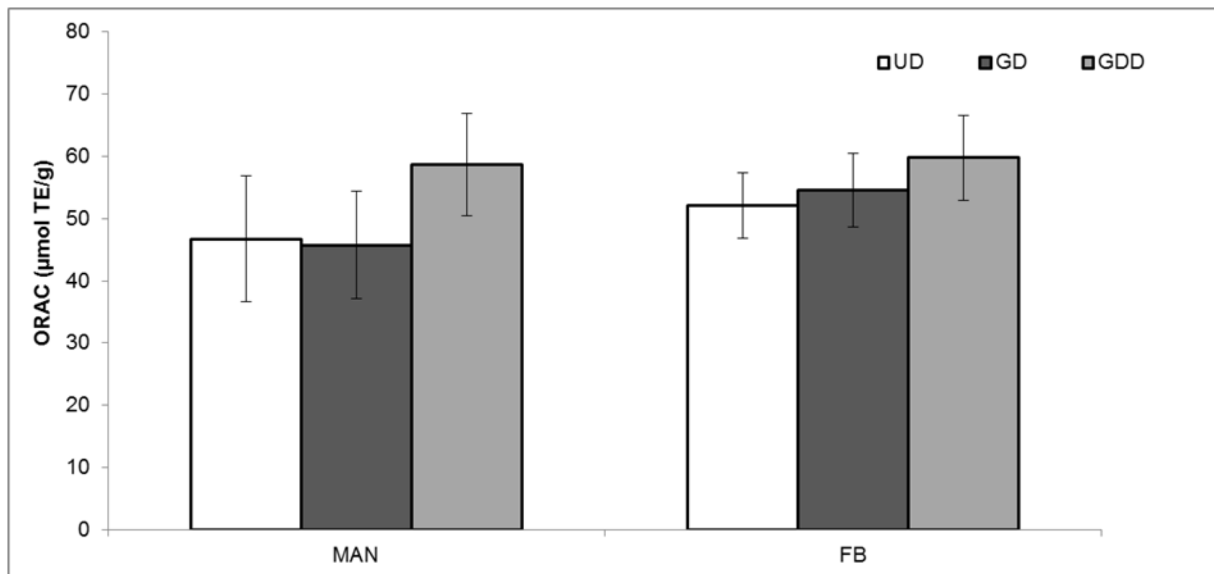

Figure 4.5a: Effect of SGDD on the antioxidant activity of MAN and FB honey averages determined by the ORAC assay. Data is expressed as mean ± SEM. No statistical difference was obtained, p≤0.05.

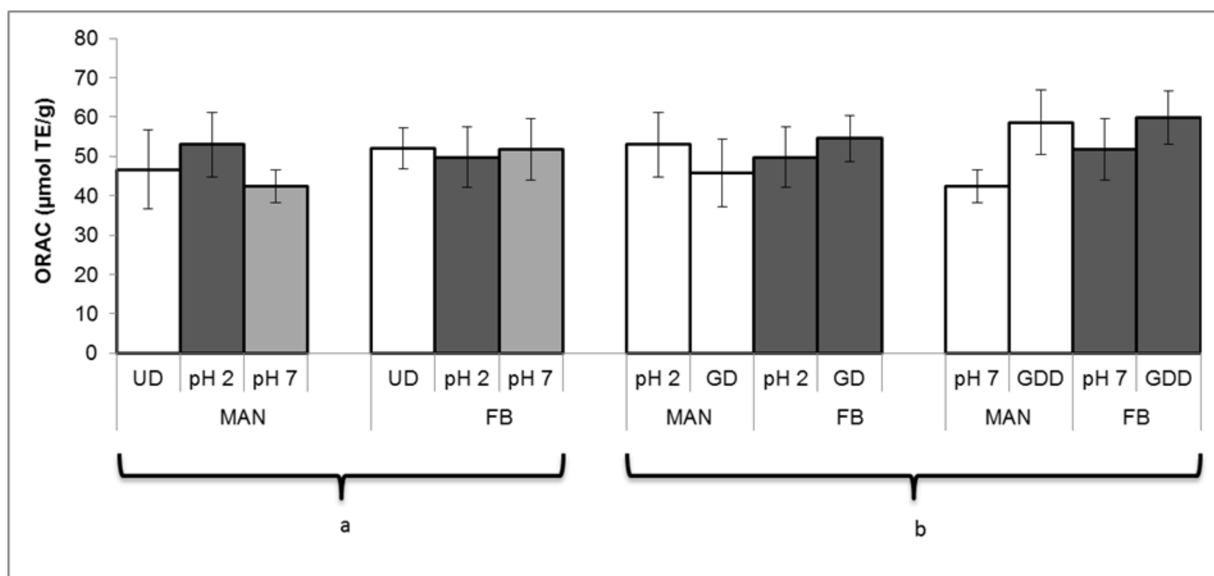

Figure 4.5b: a) Effect of pH on antioxidant activity determined by ORAC, in the absence of digestive enzymes compared to UD and b) the comparison between pH and each phase of digestion (pH 2 vs GD and pH 7 vs GDD) for MAN (n=1) and FB (n=5) honey. Data is expressed as mean ± SEM. For both part **a** and **b**, no statistical difference were obtained, p≤0.05.

Comparing these results to that of gooseberries (Chiang *et al.*, 2013), similar ORAC results were obtained with one type of gooseberry fruit (Tixia) where the antioxidant activity was not affected by *in vitro* simulated digestion. However for the other type known as Invicta, different results were obtained, where significant increases were shown for digested samples when compared to undigested samples. Similar increase in activity throughout digestion was also found for all 5 types of millet grains (Chandrasekara and Shahidi, 2012) as well as for 8 fruit juices (Cilla *et al.*, 2011).

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Variable pH effects were found for 5 types of millet grains that had been subjected to simulated digestion (Chandrasekara and Shahidi, 2012). For varieties, pearl and proso, levels were increased, for kodo and foxtail unchanged and for finger decreased. While following digestion the antioxidant activity of 8 fruit juices was increased when measured with the ORAC assay (Cilla *et al*., 2011). This implies the variable effects of pH and digestion is due to the concentration, type as well as the pH dependent extractability or sensitivity of the polyphenols found in cereals, fruit as well as honey.

Chemical assays are known to be affected by pH e.g. the FRAP assay is reported to work better with samples of lower pH (Huang *et al*., 2005) and the ORAC assay shows sensitivity to samples with pH values < 7 (Huang *et al*., 2014). Therefore, as mentioned in the materials and methods section, a blank, only ddH_2O was subjected to *in vitro* SGDD to rule out the possible effect of changes in pH and/or the digestive enzymes on the ORAC assay. As shown in Figure 4.6 there was no significant quenching of AAPH by control samples, indicating that any increase in antioxidant activity by digested samples is due to sample components released from food matrix and not digestive enzymes.

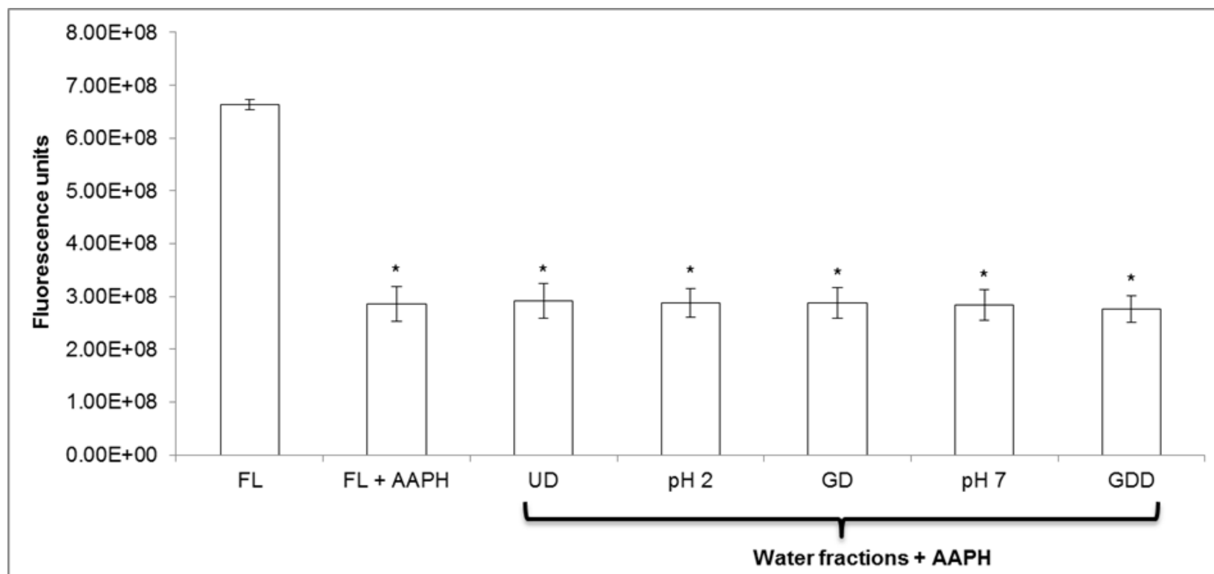

Figure 4.6: Effect of digestion solutions (no honey added) on the oxidative effects of AAPH on fluorescein compared to control of fluorescein only. Data is expressed as mean ± SEM. * Indicates significant difference when compared to FL, p≤0.05. No significance differences were found between AAPH only and digestion solutions/water digests containing AAPH.

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4.4.3 Cellular antioxidant activity

4.4.3.1 Caco-2 cell line

Chemical assays allow the rapid evaluation of antioxidant activity however to determine if these effects are physiologically relevant, cell culture models are used. In this study two different cellular models, the Caco-2 and SC-1 cell lines were used and the ability of each digest to protect against oxidative damage was evaluated. AAPH alone was calculated as causing 100% oxidative damage. Undigested MAN and FB significantly reduced the oxidative effects of AAPH in the Caco-2 cell line (Figure 4.7a) showing that both honey types have CAA. With GD, differences in measured CAA did not differ significantly from UD. Following GDD, compared to GD there was a loss of CAA for MAN although some CAA was retained when compared to the AAPH control. For FB, there was a complete loss of CAA and the measured % oxidative damage in Caco-2 cells was similar to the effect of AAPH. This in contrast to antioxidant activity measured with chemical assays where it was shown that antioxidant activity was essentially unchanged. The contribution of pH and digestive enzymes to these observed effects was then evaluated as shown in Figure 4.7b.

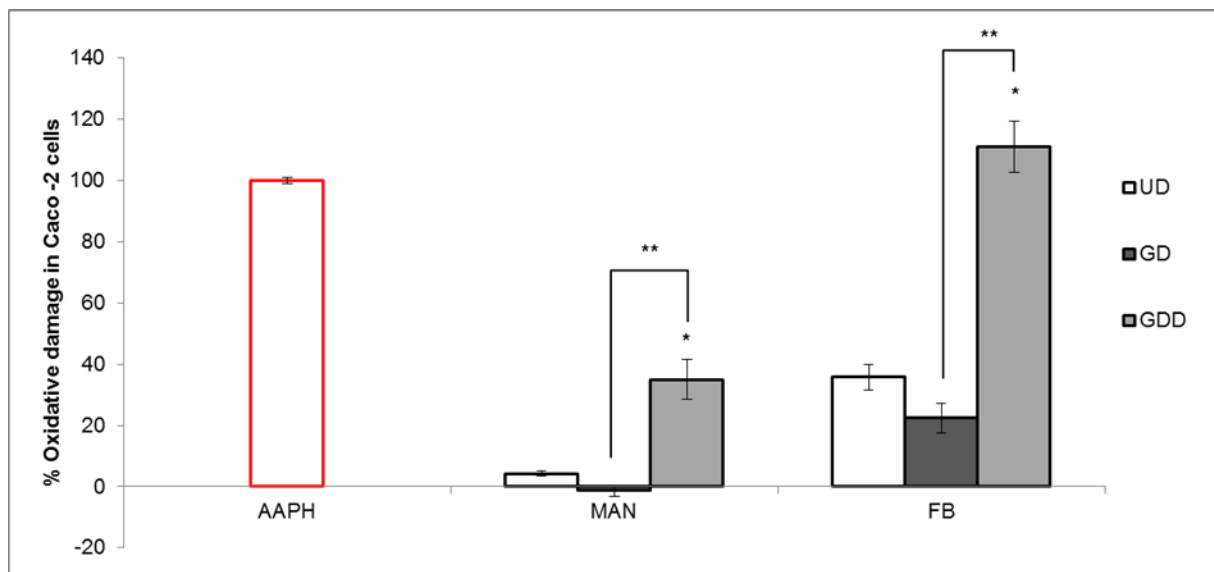

Figure 4.7a: Cellular antioxidant activity of MAN and averages of FB honeys of each phase of SGDD in Caco-2 cells compared to the 100% production by AAPH alone. Data is expressed as mean ± SEM. * Indicates significant difference compared to UD and ** indicates differences between GD and GDD, $p \leq 0.05$.

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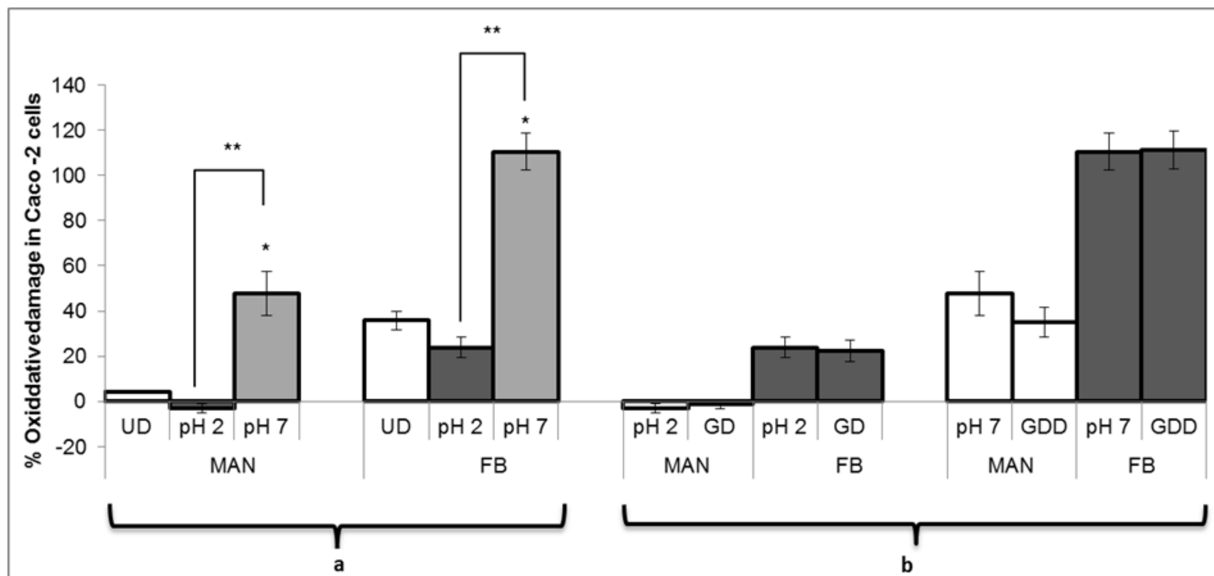

Figure 4.7b: a) Effect of pH on the cellular antioxidant activity in the absence of digestive enzymes compared to UD and b) the comparison between pH and each phase of digestion (pH2 vs GD and pH7 vs GDD) for MAN (n=1) and FB (n=5) honey. Data is expressed as mean ± SEM. * Indicates significant difference (p≤0.05) when compared to UD. ** Indicates differences between pH 2 and pH 7.

In Figure 4.7b, part a, the effect of pH alone is evaluated. There were no significant differences between UD vs. pH 2 but a subsequent increase of % oxidative damage at pH 7 for both MAN and FB honeys. This is similar to the effect observed in 4.7a. In Figure 4.7b, part b, the presence of the digestive enzymes did not alter the observed effects. There were no differences between pH 2 and GD and pH 7 and GDD for both honey types. This confirms that in an acidic environment polyphenols are stable and extracted more, whereas in an alkaline environment polyphenols are degraded or oxidised. In addition MAN compared to FB honey more effectively protects Caco-2 cells against oxidative damage.

4.4.3.2 SC-1 cell line

The effect observed in the Caco-2 cell line was confirmed in a second cell line, the SC-1, mouse fibroblast cell line. For SC-1 cells a similar trend was observed (Figure 4.8a). UD samples effectively protected SC-1 cell against oxidative damage, in an acidic environment CAA was retained and with GDD, CAA was lost. However the % oxidative damage measured in the SC-1 cell line was less than that measured using the Caco-2 cell line. Differences were also observed between the individual honey samples (Figure 3.4) and this may also account for the poor correlation found between DCFH-DA data generated for the Caco-2 and the SC-1 cell lines (Table 4.2). Similar to the Caco-2 cell line, MAN presented with the best CAA protection compared to FB. Therefore, CAA is not only a function of the phase of digestion but also the sensitivity of the cell line and type used for evaluation.

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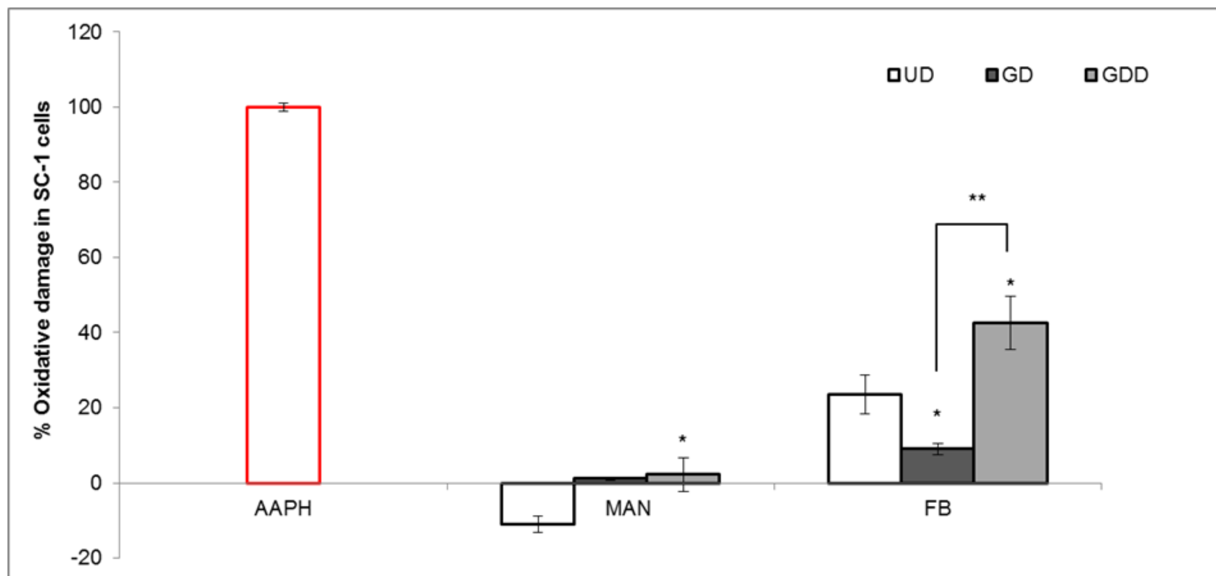

Figure 4.8a: Cellular antioxidant activity of MAN and averages of FB honeys following SGDD in SC-1 cells compared to the 100% production by AAPH alone. Data is expressed as mean ± SEM. * Indicates compared to UD and ** indicates difference when GD is compared to GDD

In the SC-1 line, a total inhibition in AAPH induced oxidative damage was observed for all phases of digestion although the observed effect was less than the UD sample. For the FB honey compared to UD, with GD there was an increase in CAA which was lost with GDD. Although differences were not significant the effect of pH showed a similar pattern (Figure 4.8a, section a). In addition, a significant increase in the % oxidative damage following GDD compared to pH of the FB honey was observed, indicating that in these honey samples, the presence of digestive enzymes increases oxidative effects.

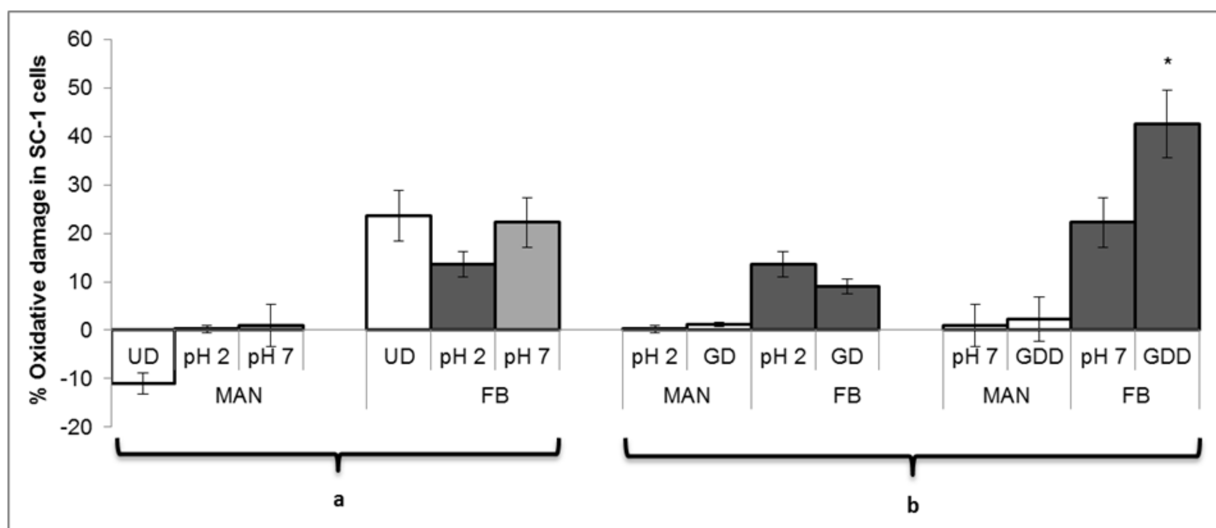

Figure 4.8b: a) Effect of pH on the cellular antioxidant activity in the absence of digestive enzymes compared to UD and b) the comparison between pH and each phase of digestion (pH 2 vs GD and pH 7 vs GDD) for MAN (n=1) and FB (n=5) honey. Data is expressed as mean ± SEM. * Indicates significant difference, $p \leq 0.05$. * In part **b** indicates difference when GDD is compared to pH 7.

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Few studies have been undertaken to determine CAA *in vitro* following digestion of honey and other foods. However, O'Sullivan *et al*., (2013) reported that honey digests compared to undigested samples caused a loss in Caco-2 cellular viability, measured with the MTT assay following 24 h exposure which could be due to increase oxidative damage in digested samples as seen in the CAA assay. The cause of reduced CAA in honey may be due to polyphenol oxidation or degradation. Factors that have been identified to promote the autoxidation of polyphenols during digestion are elevated pH (6.0 – 7.5) as found in the duodenum, digestive secretions such as bile, dissolved O_2 and dietary ROS (Neilson *et al*., 2007). In the GDD phase, $NaHCO_3$ is used to increase sample pH to 7, before the enzyme pancreatin is added. Therefore, the additions of $NaHCO_3$ or the enzyme pancreatin to samples were identified as possible agents of pro-oxidant activity, the former via formation of H_2O_2 by $NaHCO_3$. During this process phenoxide anions are formed which are then converted to phenoxyl radicals by oxygen and finally H_2O_2 (Odiatou *et al*., 2013). This effect was evaluated by measuring the H_2O_2 levels in digested honey samples with the FOX assay.

Honey has inherent H_2O_2 content (chapter 3, Table 3.1) formed by the oxidation of glucose by the enzyme glucose oxidase when honey is ripening (Irish *et al*., 2011). With GD and GDD, significant increases of H_2O_2 levels were seen for MAN honey when compared to UD (Figure 4.9a). For FB honeys, GD showed an unchanged H_2O_2 content and a significant increase of H_2O_2 in GDD relative to UD. When GD was compared to GDD, both MAN and FB honeys showed significant increases in H_2O_2 content.

The effect of pH and digestive enzymes on H_2O_2 formation was evaluated (Figure 4.9b). With SGDD, for MAN honey there was a loss of H_2O_2 at pH 7 while for FB honey H_2O_2 levels were unchanged. In the presence of digestive enzymes there was an increase in the levels of H_2O_2. In contrast with GD, H_2O_2 was unchanged while with GDD, levels were increased.

The phenolic acids present in Manuka honey are gallic acid, syringic acid, 2-methoxybenzoic acid, phenyllactic acid, methyl syringate, abscisic acid and 4-methoxybenzoic acid (Stephens *et al*., 2010). Gallic acid in cell culture forms H_2O_2 and the anti-proliferative effect of gallic acid are attributed to H_2O_2 formation (Lee *et al*., 2005). Neilson *et al*., (2007) proposed that the degradation of catechins resulted in the formation of H_2O_2 at neutral pH and in the presence of O_2. Akagawa *et al*., (2003) also proposed that polyphenols are oxidized in the absence of metal ions by O_2 to yield the semiquinone and O_2^- which is spontaneously converted to H_2O_2. At a neutral pH associated with GDD no increase in H_2O_2 levels was found. However, the presence of digestive enzymes in GD and GDD results in

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H_2O_2 formation which is the highest following GDD. This high level of H_2O_2 following GDD may account for the loss of antioxidant activity observed in the SC-1 and Caco-2 cells.

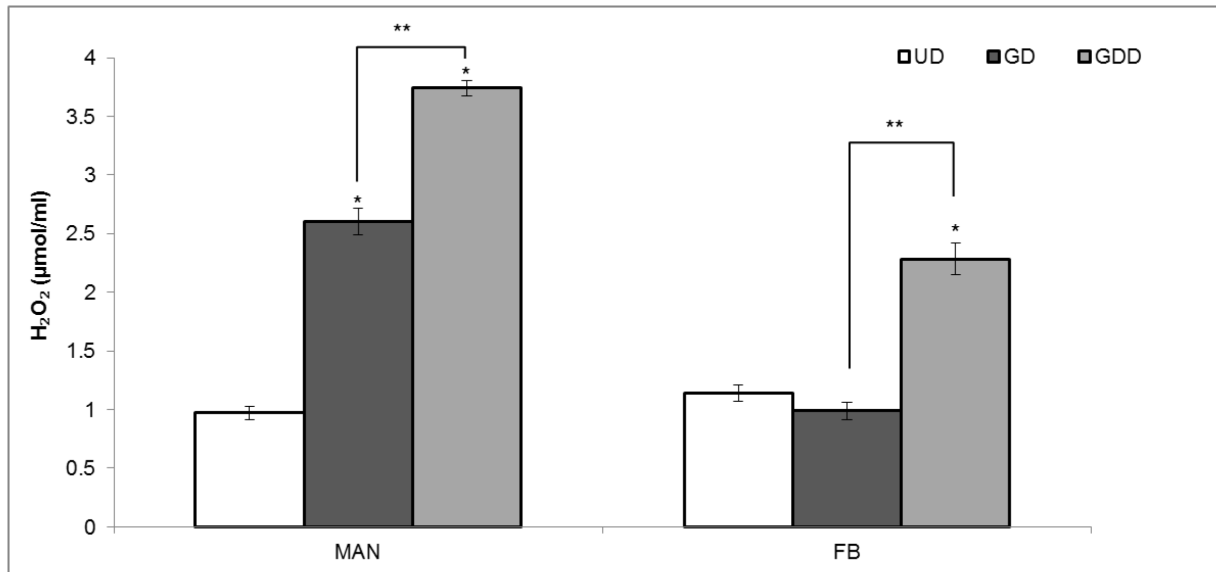

Figure 4.9a: Effect of SGDD on the H_2O_2 content of MAN and averages of FB honeys determined by the FOX assay. Data is expressed as mean ± SEM. * Indicates significant difference compared to UD and ** indicates differences between GD and GDD, p≤0.05.

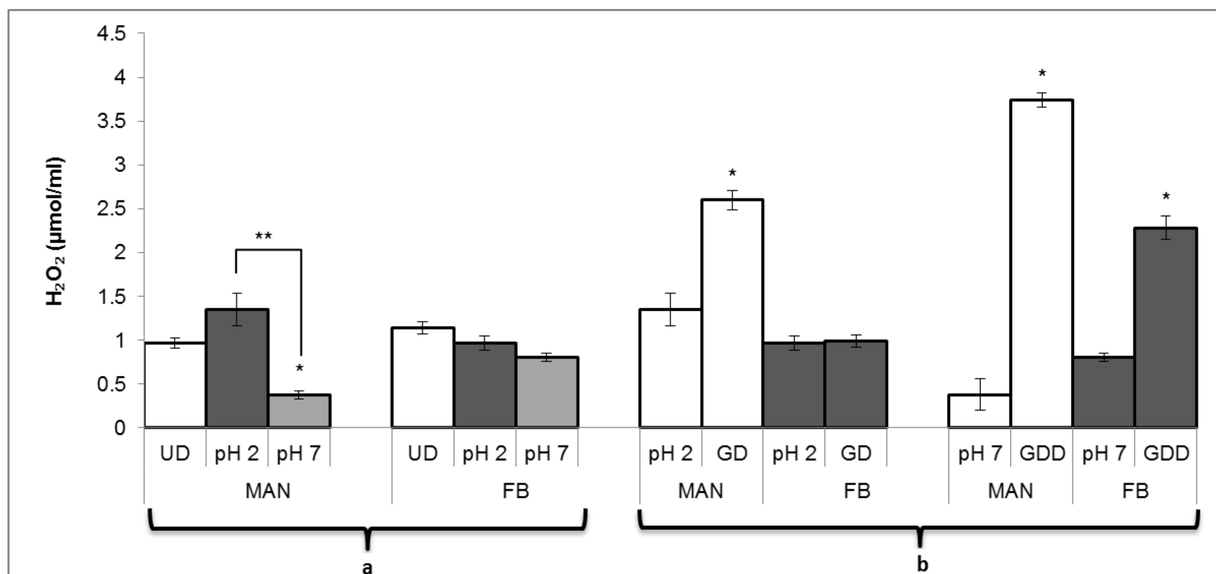

Figure 4.9b: a) Effect of pH on H_2O_2 content in the absence of digestive enzymes compared to UD and **b)** the comparison between pH and each phase of digestion (pH 2 vs GD and pH 7 vs GDD) for MAN (n=1) and FB (n=5) honey. Data is expressed as mean ± SEM. * In part **a** indicates difference when compared to UD and ** indicates significant difference between pH 2 and pH 7. In part **b**, * indicates significant difference when GD is compared to pH 2 and GDD compared to pH 7.

In an extensive review Manach *et al*., (2005) evaluated the bioavailability of polyphenols. Urinary excretion is 0.3 – 43% of the ingested polyphenol dosage. The best absorbed polyphenol was gallic acid followed by the isoflavones, catechins, flavanones and to a lesser degree the quercetrin glucosides. The auto-oxidation of these molecules or conjugated

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derivatives within the urinary system may contribute to the H_2O_2 found in the urine. As many polyphenols are not absorbed, the formation of H_2O_2 in the duodenum may have an important cellular regulatory or antibacterial function. This increase in H_2O_2 has been reported to be the basis for the antimutagenic effects of polyphenol compounds (Nakayama *et al*., 2002; Long *et al*., 2010; Odiatou *et al*., 2013). High levels of H_2O_2 are cytotoxic and can cause damage to the mucosa of the GIT. However, some polyphenols do not form H_2O_2 and retain antioxidant activity. The controlled shedding and cellular renewal by stem cells (Halliwell *et al*., 2000) as well as the presence of mucus limits damage that can lead to DNA damage, mutations and the development of cancer. A study by Long *et al*., (2010) evaluated H_2O_2 formation by polyphenols under different cell culture media. Rosmarinic acid, hydroxytyrosol and delphinidin chloride underwent oxidation and produced H_2O_2. However for polyphenols such as tyrosol, apigenin, resveratrol, curcumin and hesperatin, a non-significant H_2O_2 formation was seen. These authors further highlighted that the presence of adjacent phenolic –OH groups triggers the formation of H_2O_2 (Long *et al*., 2010; Halliwell, 2008).

The observed formation of H_2O_2 has little effect on the measurement of TPC and possible interference with the ORAC assay is minimal. In the ORAC assay AAPH acts as a peroxyl generator. The peroxyl radical abstracts a hydrogen atom from fluorescein, which is the fluorescent probe, and over time the fluorescence intensity starts to decrease (Ou *et al*., 2002). In the HORAC (hydroxyl radical averting capacity) assay, the principle is the same however 0.75 – 1.1 M H_2O_2 with the cobalt ion as catalyst is used to generate the hydroxyl radical (Ou *et al*., 2002). The amount of H_2O_2 generated with digestion is in comparison low and without a catalyst no hydroxyl radicals will be generated and therefore the effect on the ORAC assay is minimal. In contrast, cells *in vitro* are highly sensitive to H_2O_2, the presence of cellular metal ions such as well as metal ions in honey (Graham, 1992) iron can result in the generated H_2O_2 being converted to free radicals that can cause oxidative damage resulting in an increase in fluorescence measured with the DCFH-DA assay. The concentration and the type of polyphenol will determine the observed effect.

To summarise (Table 4.1) with GD of MAN, TPC and TFC is increased, while antioxidant activity is unchanged (TEAC and ORAC assays). In both cell lines, CAA is unchanged with GD. For MAN, with GDD, TPC and antioxidant activity measured with the TEAC assay is reduced. With the ORAC assay no effect is observed while with the DCFH-DA assay, CAA is reduced. For FB, following GDD compared to GD the polyphenol content and antioxidant activity was reduced. With the ORAC assay no change in antioxidant activity was observed

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while the CAA was reduced. For both this loss of CAA was due to the formation of H_2O_2 in the presence of digestive enzymes.

Table 4.1: Summary of the effect of SGDD on the antioxidant properties of honey

ASSAY	MAN		FB	
	GD	GDD	GD	GDD
Antioxidant content				
TPC	Increased	Decreased	-	-
TFC	Increased	-	-	-
Antioxidant activity				
TEAC	-	Decreased	-	-
ORAC	-	-	-	-
Cellular antioxidant activity				
DCFH-DA (Caco-2)	-	Decreased	-	Decreased
DCFH-DA (SC-1)	-	Decreased	Increased	Decreased

Correlations

For each phase of digestion, the measured FB antioxidant properties were correlated. Correlations between antioxidant assays are usually high between samples (Alvarez-Suarez *et al.*, 2010c; Serem and Bester, 2012).

For UD honey, strong correlations were found between TPC vs TFC, TEAC, ORAC and the DCFH DA (SC-1) assay (Table 4.2).

The correlations for UD compared to GD increased for TPC and TEAC as well as TPC and ORAC from 0.79 to 0.91 and 0.77 to 0.93, respectively. Between the TEAC and ORAC assays, correlations improve dramatically from 0.23 to 0.96 as shown in Table 4.2, indicating that both assays had improved antioxidant activity following GD due to the release of phenolic acids in an acidic environment (Rodríguez – Roque *et al.*, 2013), which generates a stable ideal environment for the optimal measurement of antioxidant activity.

For GDD, a strong correlation was seen between TPC and TEAC and between DCFH-DA Caco-2 and DCFH-DA SC-1, as presented in Table 4.2. Correlations values increased from r = 0.79 (UD) to r = 0.98 (GDD) for TPC and TEAC. A decrease in correlation was found for TPC vs. ORAC whereas the correlation for TEAC vs. ORAC remained the same. The lowered correlation can be accounted for by the instability of polyphenols with GDD resulting in reduced activity as seen with TEAC and the DCFH-DA.

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Table 4.2: Correlations of antioxidant content, chemical and cellular antioxidant activities of FB honeys for UD , GD and GDD

	TFC	TEAC	ORAC	DCFH-DA – Caco-2	DCFH-DA – SC-1
TPC	**-0.75** / 0.07/ -0.50	**0.79 / 0.91/ 0.98***	**0.77 / 0.93/** 0.22	-0.19 / 0.24/ -0.06	**-0.93** / -0.67/ -0.57
TFC	**1**	-0.42 / 0.36/ -0.43	-0.66 / 0.38/ 0.10	-0.18 / -0.69**/** 0.33	0.53 / -0.29/ 0.54
TEAC	-	**1**	0.23 / **0.96/** 0.29	-0.24 / -0.16/ -0.19	**-0.82 / -0.73/**- 0.66
ORAC	-	-	**1**	-0.23 / 0.01/ -0.61	**-0.70** /- 0.58/ -0.62
DCFH-DA Caco-2	-	-	-	**1**	0.52 / 0.33/ **0.82**
DCFH-DA SC-1	-	-	-	-	1

Bold indicates a significant correlation, *UD/GD/GDD underlined indicates a significant increase in correlation with GD and GDD compared with UD.

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4.5 Conclusion

In general antioxidant content for MAN in GD is increased while antioxidant activity is unaltered. With GDD, the polyphenol content is decreased of which translates into a reduction in antioxidant activity (TEAC) assay. CAA with GD is unaltered in the Caco-2 and SC-1 cell lines, however decreased in GDD. For FB honey with digestion, antioxidant content or activity is unaltered. With GD a slight increase in CAA is observed (SC-1 cell line) but the activity is lost following GDD, which is due to digestive enzyme and induced H_2O_2 formation.

An increase in correlations is seen more in GD than GDD, illustrating that the gastric environment extracts more polyphenols resulting in increased antioxidant activity, and it is in the stomach where polyphenols may have the greatest protective effect.

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Chapter 5: The effect of *in vitro* simulated digestion on the anti-inflammatory properties of Fynbos honey

5.1 Introduction

Inflammation is a physiological response against hostile agents and is normally associated with tissue injury or infection. It aids in the elimination of toxins or pathological agents as well as tissue repair. This process involves the appearance of macrophages, granulocytes and monocytes at early stages in inflamed sites followed by the appearance of lymphocytes at a later stage (Calder, 2006). Exogenous triggers such as the lipopolysaccharide (a bacterial endotoxin) directly elicits an immunological response in macrophages and monocytes which in turn produce and release inflammatory cytokines and mediators such as NO. However, if inflammation is not controlled or properly co-ordinated, this reaction could lead to an over production of mediators and cytokines which can induce oxidative damage thus leading to the development and manifestations of inflammatory diseases, cancer and neurodegenerative diseases (Calder, 2006). Besides cancer other GIT associated diseases resulting from inflammation include peptic ulcers and gastritis.

Honey has been shown to effectively reduce inflammation, oedema, remove necrotic tissue and promote granulation, angiogenesis and epithelialization (Molan, 2001). The beneficial effect against induced colitis in animal models (Mahgoub *et al*., 2001) implies that anti-inflammatory activity is retained following digestion.

Kassim *et al*., (2010a), reported that Malaysian honey extracts inhibited NO production in LPS stimulated RAW 264.7 cells. In addition these extracts also protected murine fibrosarcoma (L929) cells from the tumour necrosis factor alpha (TNF-α) cytotoxicity. Another *in vitro* study on the anti-inflammatory properties of honey was evaluated by Tonks *et al*., (2003). In this specific study, several honeys were found to be able to induce the production of pro-inflammatory cytokines (TNF-α, IL-6 and IL-1β) in both MonoMac-6 (MM6) and human monocytes which are involved in wound healing. This study was confirmed by Timm *et al*., (2008), as natural honeys significantly induced the production of a pro-inflammatory cytokine (IL-6) in the MM6 cell line. An *in vivo* study by Kassim *et al.,* (2010b), showed the effects of Gelam (Malaysian) honey extracts by reducing pain and oedema in the paws of rats that were injected with LPS. Furthermore these observations correlated with the inhibition of PGE_2 and NO. The potential anti-

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inflammatory effects of honeys have been attributed to the action of polyphenols especially flavonoids (Nasuti *et al*., 2006; Viuda-Martos *et al*., 2008; Kassim *et al*., 2010b). No studies could be found where the beneficial anti-inflammatory effects following digestion are investigated using *in vitro* cellular models.

Aim

To determine the anti-inflammatory effects of MAN and FB honey following SGDD.

The objectives of this chapter are to determine the effect of SGDD:

a. On the chemical NO scavenging activities of each honey digest.
b. On NO production in the RAW 264.7 cell line.
c. On the NO inhibiting/scavenging activity in the RAW 264.7 cell, NO model.

5.2 Materials

All materials for this chapter have been described either in chapter 3 and/or chapter 4.

5.3 Methods

5.3.1 *In vitro* simulated gastro-duodenal digestion

The simulated *in vitro* gastro-duodenal digestion done in this chapter is as described in sections 4.3.1 and Figure 4.1 of chapter 4.

5.3.2 Chemical NO scavenging activity

Each sample and digest was diluted to 10% (v/v) and 1% (v/v) and chemical NO scavenging activity was determined as described in sections 3.3.6.1 of chapter 3.

5.3.3 Cellular NO scavenging activity: RAW 264.7 cell model

The RAW cell line was cultured and maintained as described in section 3.3.6.2.1. Each sample and digest was diluted to 10% (v/v) and cellular anti-inflammatory effects were determined as described in section 3.3.6.2.2.

5.3.4 Calculation of the observed and expected values

In the RAW 264.7 cellular model, honey can induce NO production if the RAW 264.7 cells are also stimulated to produce NO. The total NO production (expected levels) can be calculated as follows. The actual levels measured following stimulation and the addition of the honey or

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digests are the observed levels. The observed divided by the expected NO levels can provide information on the actual scavenging activity and this is explained as follows:

Expected (E) = [NO] of {unstimulated RAW 264.7, (+) honey} + [NO] of {stimulated RAW 264.7, (-) honey}
Observed (O) = [NO] of {stimulated RAW 264.7, (+) honey}
If O<E honey is anti-inflammatory
If O>E effect is not anti-inflammatory
Anti-inflammatory effect can be high (H) (>60%), medium (M) (30 – 60%) and low (L) (<30%)

5.3.5 Data management and statistical analysis

The same statistical analysis performed in chapter 4 was also performed for this chapter. Additionally, a t-Test was used to identify honey digests that had a pro-inflammatory effect using the Student's t-Test function in Microsoft Excel 2013.

5.4 Results and discussion

5.4.1 NO scavenging activity (chemical assay)

In chapter 3, the antioxidant properties of 5 FB honey samples with similar antioxidant content were determined. Evaluation of these samples for NO scavenging activity revealed that these samples were very different and therefore the NO scavenging activity of each individual honey sample is evaluated and then summarised.

The effect of SGDD on the NO scavenging activity of individual FB honeys is shown in Figure 5.1a. All honeys, including MAN effectively scavenged NO. With GD, NO scavenging activity was only increased for FB6. With GDD, NO scavenging was unchanged for FB1, FB3 and FB5, increased for FB2 and reduced for FB6 and MAN.

In summary (Figure 5.1b), SGDD does not alter the NO scavenging activity of FB honey. When UD was compared to GDD for MAN, a significant reduction in NO scavenging ability was found (from 85.55% to 54.20%). Regardless of this decrease MAN was still at the same level with FB honeys (54.20% vs.45.14%).

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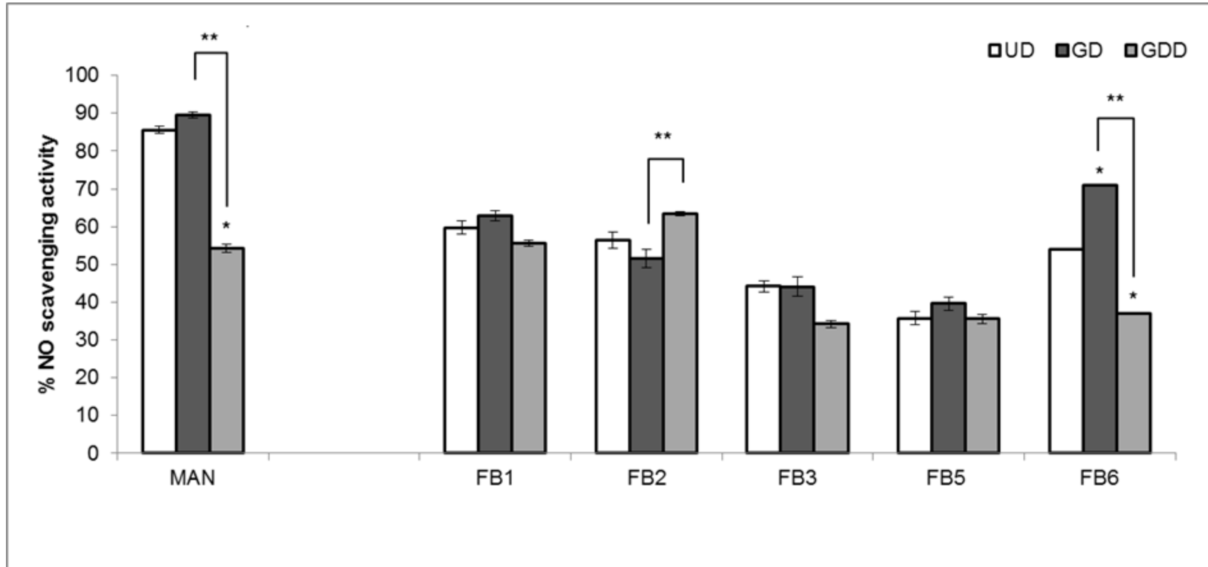

Figure 5.1a: NO scavenging activity of MAN and FB honeys at each phase of SGDD. Data is expressed as mean ± SEM. * Indicates significant difference compared to UD and ** Indicates difference between GD and GDD, p≤0.05.

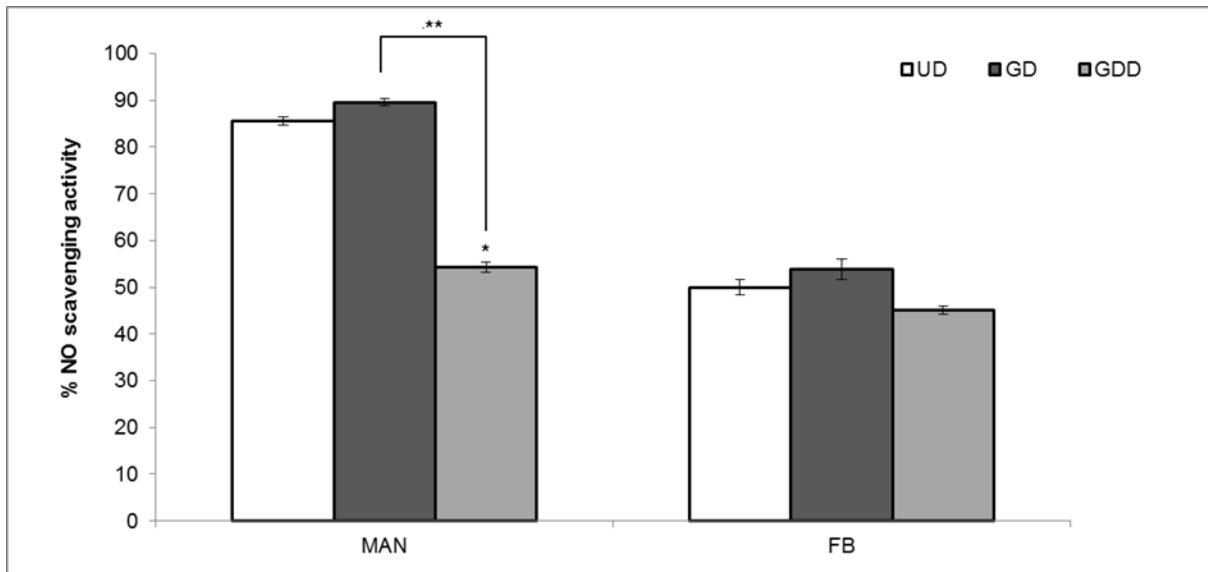

Figure 5.1b: Summary of NO scavenging activity with each phase of SGDD of MAN and FB honey. Data is expressed as mean ± SEM. *Indicate significant difference when compared to UD and ** indicates difference when GD is compared to GDD, p≤0.05.

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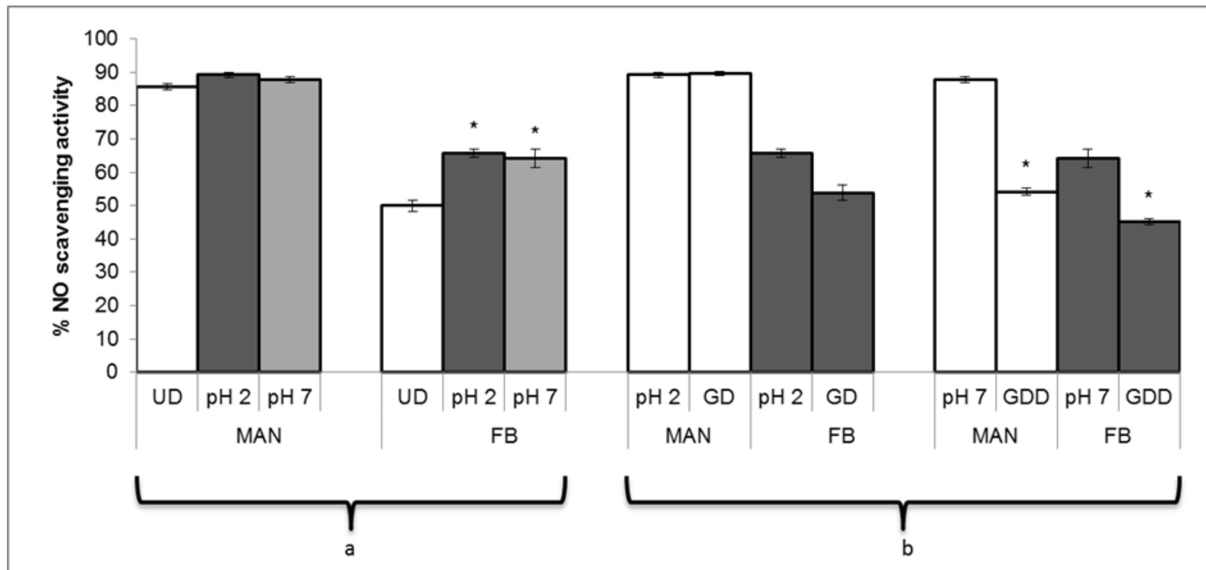

Figure 5.1c: a) Effect of pH on NO scavenging activity in the absence of digestive enzymes compared to UD and b) the comparison between pH and each phase of digestion (pH2 vs GD and pH7 vs GDD) for MAN (n=1) and FB (n=5) honey. Data is expressed as mean ± SEM. * In part **a** indicates significant compared to UD and ** in part **b**, indicates difference between pH and digests (pH 2 vs. GD) and (pH 7 vs. GDD).

The contribution of pH and digestive enzymes to this effect was evaluated (Figure 5.1c). pH had no effect on the NO scavenging activity of MAN. In contrast the NO scavenging activity of FB, GD and GDD was significantly greater than that of UD honey. The presence of pepsin did not alter the NO scavenging activity of MAN and FB while with pancreatin (GDD) the NO scavenging activity of both MAN and FB was significantly lower.

5.4.2 Cellular scavenging activity (RAW 264.7 cell model)

The RAW 264.7 murine macrophage cell line has been shown to be a useful model for inflammation as it synthesizes and releases inflammatory markers (McClain *et al*., 2002; Nakao *et al*., 2008). In addition, these cells are sensitive to the oxidant-antioxidant balance (Bor *et al*., 2006) and results are highly reproducible and therefore can serve as a model for NO inhibitory and antioxidant studies (So *et al*., 1999; Saha *et al*., 2004).

5.4.2.1 NO produced in non-LPS/ IFN-γ stimulated cells – Pro-inflammatory effect

As shown in chapter 3, Table 3.3, honey has been shown to have a pro-inflammatory effect and the effect of digestion on this effect was further evaluated.

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RAW 264.7 murine macrophage cells do not produce NO, however exposure to pro-inflammatory molecules such as LPS/IFN–γ induces NO production. The possible pro-inflammatory activity of each honey and digest was determined. The measured levels of NO was compared to RAW 264.7 murine macrophages stimulated with LPS/IFN–γ. UD honey samples did not induce significant levels of NO compared to RAW 264.7 cells in medium (cells only) Figure 5.2a. With GD and GDD, FB5 and FB6 did not induce significant NO formation. With GD NO levels were increased for MAN, FB1, FB2 and FB3 whereas with GDD, MAN, FB1, FB2 and FB3 showed an increase in NO production. Differences between GD and GDD were not significant. GD and GDD of FB1 and FB3, showed similar NO levels to that of cells stimulated with LPS/IFN–γ.

In summary, Figure 5.2b with both GD and GDD there was an increase in NO formation which was significant for MAN and for FB1, FB2 and FB3 where levels attained for FB3 was similar to that for LPS/IFN–γ stimulated RAW 264.7 cells. In summary both, MAN and FB honey stimulated NO formation in RAW 264.7 cells following GD and GDD digestion.

Simultaneously the viability of the RAW 264.7 macrophages (Figure 5.2c) was determined and following 24 h exposure to 10% honey cellular viability was unaltered compared to the control.

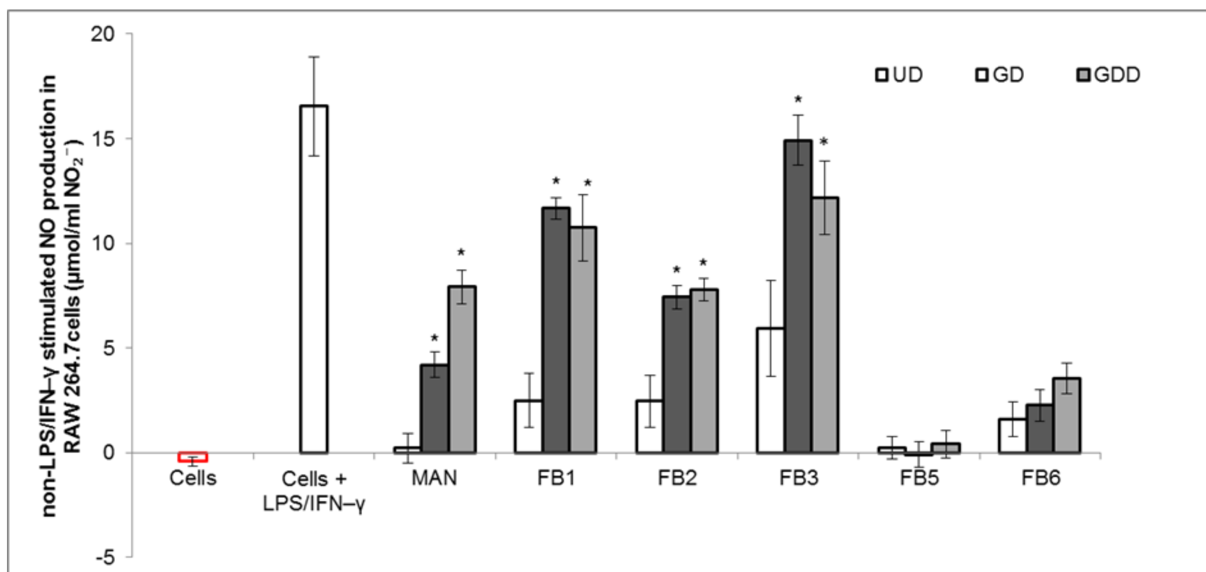

Figure 5.2a: RAW 264.7 cells induced NO formation by honey throughout SGDD (pro-inflammatory effect -no LPS and IFN–γ added). Cells (negative control) and cells exposed to LPS/IFN–γ (positive control). Data is expressed as mean ± SEM. * Indicates significant difference compared to cells only (negative control), p≤0.05.

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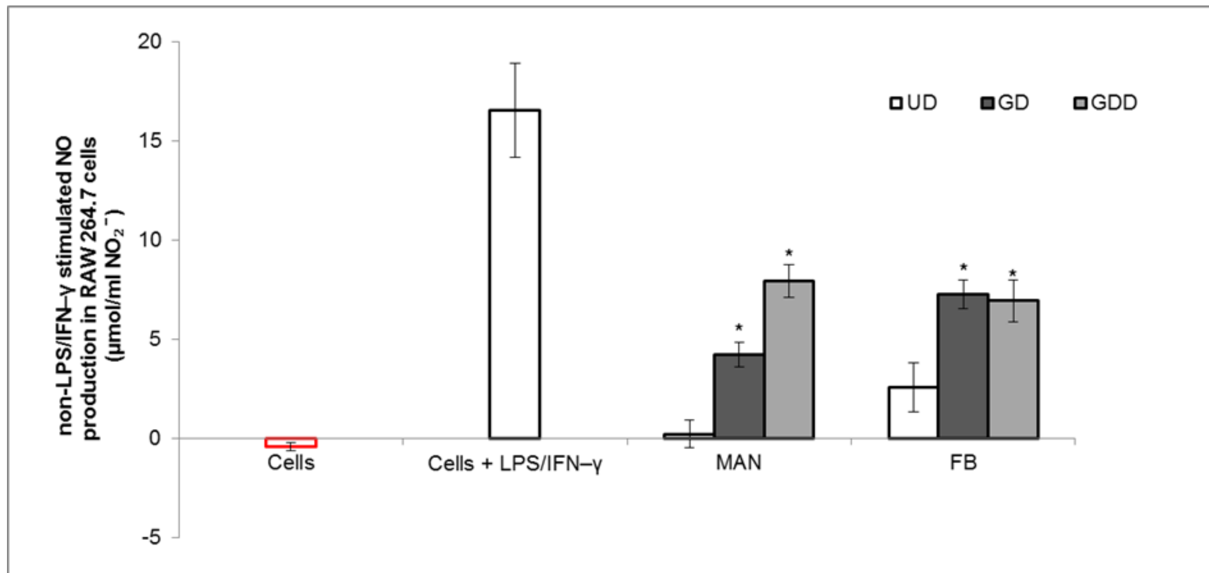

Figure 5.2b: Summary of NO production by MAN and averages of FB honeys in RAW 264.7 cells at each phase of SGDD. Cells (negative control) and cells exposed to LPS/IFN–γ (positive control). Data is expressed as mean ± SEM. * Indicates significant difference compared to cells only (negative control), $p \leq 0.05$

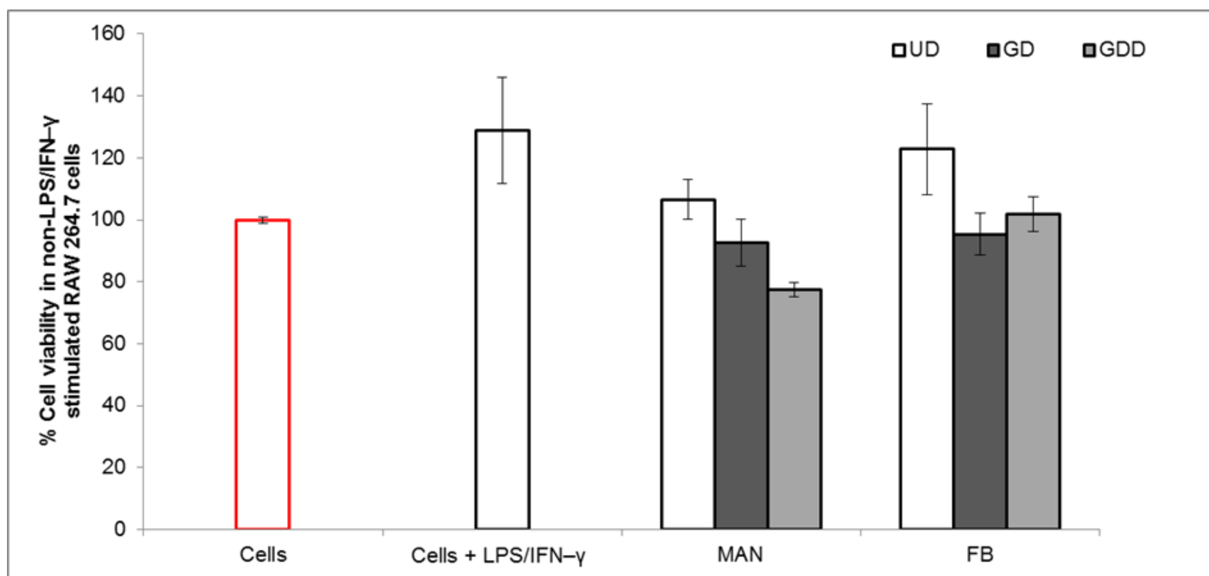

Figure 5.2c: % Cell viability in non-LPS/IFN–γ stimulated RAW 264.7 cells exposed to MAN and averages of FB honeys. Data is expressed as mean ± SEM. * Indicates significant difference, $p \leq 0.05$, compared to cells only (negative control). No statistical difference was found.

Honey has been shown to have an immunomodulator effect in wound healing by triggering the production of cytokines and ROS (Majtan, 2014). The release of NO levels in non- LPS/IFN–γ stimulated RAW 264.7 cells illustrates the pro-inflammatory effect of honey which is beneficial in acute wound healing. In the present study, following GD and GDD, NO levels were increased especially for FB5 and FB6 FB1 and FB3, where NO levels were similar to RAW 264.7 cells

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stimulated with LPS/IFN–γ. Similar pro-inflammatory effects were reported by Tonks *et al.*, (2003). The release of pro-inflammatory cytokines (TNF-α, IL-6 and IL-1β) from the MM6 cell line and human monocytes cells was induced by Manuka, jelly bush and pasture honeys. Timm *et al.*, (2008), further confirmed a significant production of a pro-inflammatory cytokine (IL-6) induced by natural honeys on the MM6 cell line. None of these studies have investigated the effect of digestion on the pro-inflammatory effects of honey.

When the influence of pH and digestive enzymes was investigated, Figure 5.2d, part a, showed a significant increase of NO production in both pH 2 and 7 fractions of MAN and FB honeys when compared to UD. This effect is unrelated to the pH sensitivity of Griess assay as for each data point an equivalent digestion control was used. Part b, shows that the presence of digestive enzymes did not affect NO release.

A cell viability test was conducted and no toxicity was seen (Figure 5.2e) when cell viability of all fractions was compared to the cells not stimulated with LPS/IFN–γ (cells only).

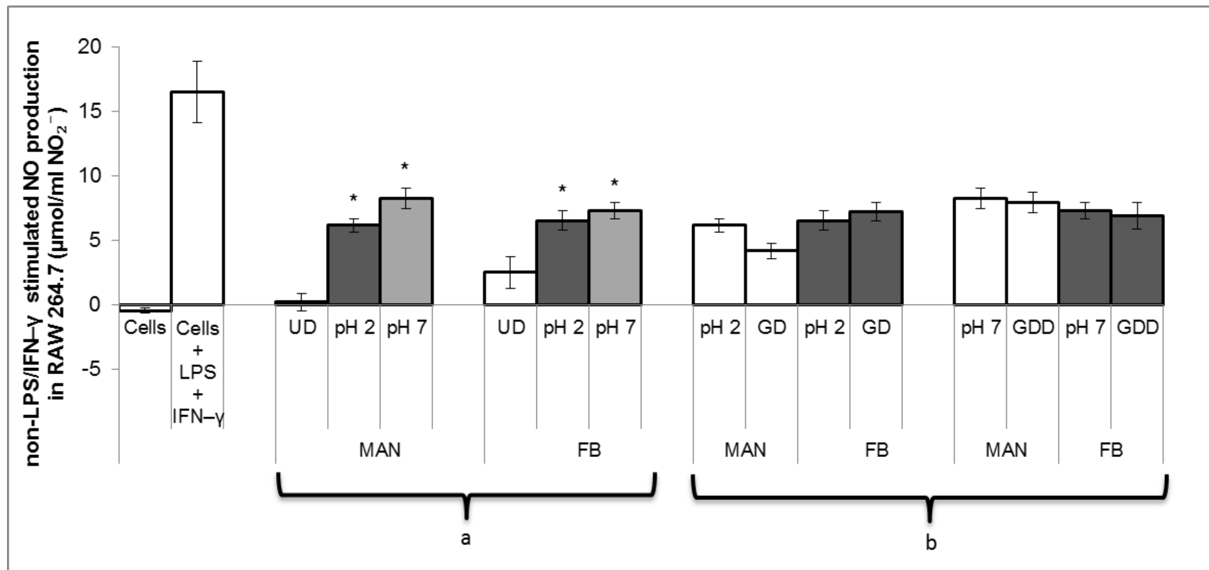

Figure 5.2d: a) Effect of pH on NO production in LPS/IFN–γ non-stimulated RAW 264.7 cells in the absence of digestive enzymes compared to UD and b) the comparison between pH and each phase of digestion (pH 2 vs GD and pH 7 vs GDD) for MAN (n=1) and FB (n=5) honey. Data is expressed as mean ± SEM. * Indicates significant difference when compared to UD, p≤0.05.

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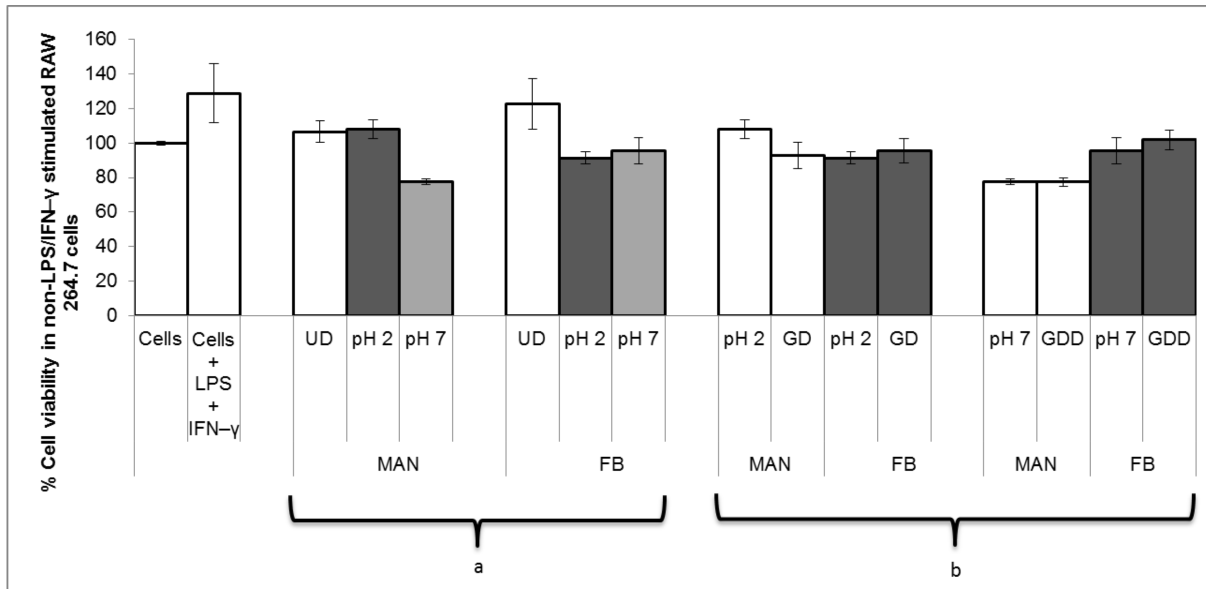

Figure 5.2e: a) % Cell viability in non-LPS/IFN–γ stimulated RAW 264.7 cells comparing undigested fractions to pH treated fractions and b) comparing pH to its relevant digestion phase (pH 2 vs. GD and pH 7 vs. GDD). Data is expressed as mean ± SEM. * Indicates significant difference, p≤0.05, compared to cells only (negative control). No statistical difference was found.

Honey has been found to contain NO metabolites (nitrates/ nitrites) as well as *N*-nitroso compounds. Levels are so low that any harmful effects are minimal (Al-Waili, 2003; Beretta *et al*., 2010). NO in the body can be produced enzymatically (NOS enzymes) and non-enzymatically (stomach). The non-enzymatic production of NO in the stomach involves the conversion of nitrites into nitrous acid (HNO_2) which then decomposes into NO and other nitrogen oxides (McKnight *et al*., 1997). Lundberg *et al*., (1994) reported a correlation between pH and the release of intra-gastric NO. The more acidic the pH, the greater is the release of intragastric NO (Weitzberg and Lundberg, 1998). Intra-gastric NO has therapeutic effects on the mucosal lining of the stomach. This includes the regulation or increasing of mucous production and mucosal blood flow (Brown *et al*., 1992; Whittle, 1993) as well as preservation of the gastric capillaries of the mucosa (Beretta *et al*., 2010). Intragastric NO production also protects against micro-organisms, necrotizing agents (Kitagawa *et al*., 1990) and non-steroidal anti-inflammatory drugs (NSAIDs) (Beretta *et al*., 2010). Studies have shown that gastric acid alone is not potent enough to kill micro-organisms, however with formation of HNO_2 and NO_2 (Benjamin *et al*., 1994; Dykhuizen *et al*., 1996) antibacterial activity is increased (Kono *et al*., 1994, Kaplan *et al*., 1996). A concentration of 20 – 100 ppb (0.67 – 3.33 µM), NO has been shown to inhibit certain microbial growth and an increase in vasodilation (Mancinelli and McKay, 1983). In the presence of HNO_2, ascorbic acid (also present in honey) via the ascorbate anion produces NO in aqueous solutions over a wide pH range (Bartsch *et al*., 1988).

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In the intestines, NO production is due to iNOS enzyme activity present in the epithelial cells of the mucosa. NO produced in this region has been found to play an important role in inflammation, however an excessive NO production is implicated in the pathogenesis of diseases such as inflammatory bowel disease and peptic ulcers (McCafferty *et al.*,1997). A study by McCafferty *et al.*, (1997) evaluated the role of iNOS in intestinal inflammation (colitis). Colitis was induced in both wild-type and iNOS-deficient mice and the effects were studied over a 7 day period. In both groups damage to the mucosa with the extent of damage being the greatest in the iNOS-deficient mice was observed. After 24 and 72 h the levels of iNOS RNA was increased while in the iNOS deficient mice, no iNOS RNA was detected. Furthermore inflammation in the wild-type mice resolved while in the iNOS mice there was an increase in the damage. Therefore it would appear that the induction of iNOS during inflammation may be essential to prevent or reduce tissue damage.

The requirement for intragastric NO production, is an acidic environment in direct contact with cells that form iNOS. In this study the gastric digest was added to the cell culture media which due to its buffering capacity would prevent intragastric NO production. As there is also increased NO production after GDD this indicates that NO formation is not due to pH but rather that several of the honey samples contain molecules that can induce NO formation.

Tonks *et al.*, (2003), quantified LPS levels in Manuka, jelly bush, pasture as well as artificial (syrup) honey solutions. The LPS levels in these honeys were extremely low, 5.6×10^{-5} – 6.9×10^{-4} µg/ml and 0.9×10^{-5} µg/ml for the artificial honey or syrup, negative control. Timm *et al.*, (2008) reported an endotoxin range of 69 – 125 ng/g LPS in Danish and Manuka honeys and Raynaud *et al.*, (2013) quantified a content of 94.2 ng/g LPS in thyme honey. Based on the data of Tonks *et al.,* (2003), a 1% honey solution would contain 5.6×10^{-7} – 6.9×10^{-6} µg/ml. For the stimulation of the RAW 264.7 cells, 100 ng/ml LPS is used and only together with 25 U/ml IFN-γ are NO levels increased. The values reported in honey are 0.00066 – 0.0069% of the amount of LPS required for the stimulation of NO formation.

Raynaud *et al.,* (2013) reported that thyme honey induced macrophage stimulation and this resulted in increased levels of PGE_2 and the overexpression and activation of activator protein - 1 (AP-1) and NF-κB transcription subunits. This pathway is also associated with the activation of iNOS and subsequently NO production. Gannabathula *et al.,* (2015) attributed this effect to the presence of apisimin and plant arabinogalactans (AGP). Both act synergistically to increase

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TNF-α production. Apisimin is a serine-valine rich peptide that was initially isolated from honeybee royal jelly. Evaluation of its sequence revealed that apisimin contains 19 pepsin cleavage sites using the ExPASy PeptideCutter tool (Gasteiger *et al*., 2005). Although there may be increased extraction of apisimin, gastric digestion would result proteolytic digestion and consequently a loss of activity.

In contrast, AGP (consisting of a protein core and branched polysaccharide chains) are resistant to digestive enzymes and form short chain fatty acids following degradation by intestinal microflora (Robinson *et al*., 2001). The observed increased NO formation i.e. pro-inflammatory effect may be due to the presence of varying amounts of AGP in MAN, FB1, FB2 and FB3. Steinhorn *et al.,* (2011) described the presence of arabinogalactan-proteins present in Manuka and kanuka honey from New Zealand. These authors did not describe the amino acid composition of the protein core of these arabinogalactan-proteins and therefore the effects of digestion cannot be predicted. However it seems that with digestion the smaller arabinoglycan moiety that form may have a greater stimulatory effect than observed before digestion.

5.4.2.2 NO produced in LPS/IFN-γ stimulated cells- Anti-inflammatory effect

The effects of each honey and digest on NO production were further evaluated in LPS/IFN-γ stimulated RAW 264.7 cells. LPS/IFN-γ induced NO formation, but following addition of the honey digests NO levels were reduced and this reduction in NO was significant for the UD fractions of MAN, FB1 and FB6 (Figure 5.3a). A significant reduction in NO was seen for GD of MAN, FB5 and FB6 as well as the GDD of FB5 and FB6 (Figure 5.3a). In summary (Figure 5.3b), UD and GD of MAN honey scavenged NO while with GDD, NO scavenging activity is lost. For FB honey significant NO scavenging activity was found for undigested, FB1 and FB6 as well as the GD and GDD digests of FB5 and FB6.

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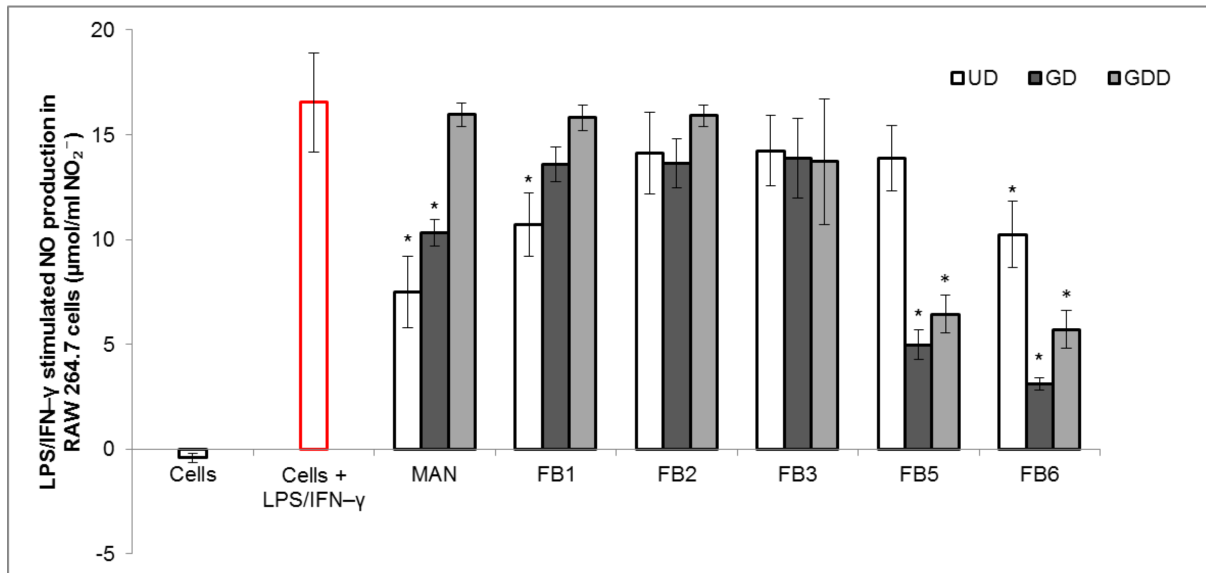

Figure 5.3a: NO production in LPS/IFN–γ stimulated RAW 264.7 cells at each phase of SGDD of MAN and FB honeys. Cells (negative control) and cells exposed to LPS/IFN–γ (positive control). Data is expressed as mean ± SEM. * Indicates significant difference compared to cells + LPS/IFN–γ, p≤0.05.

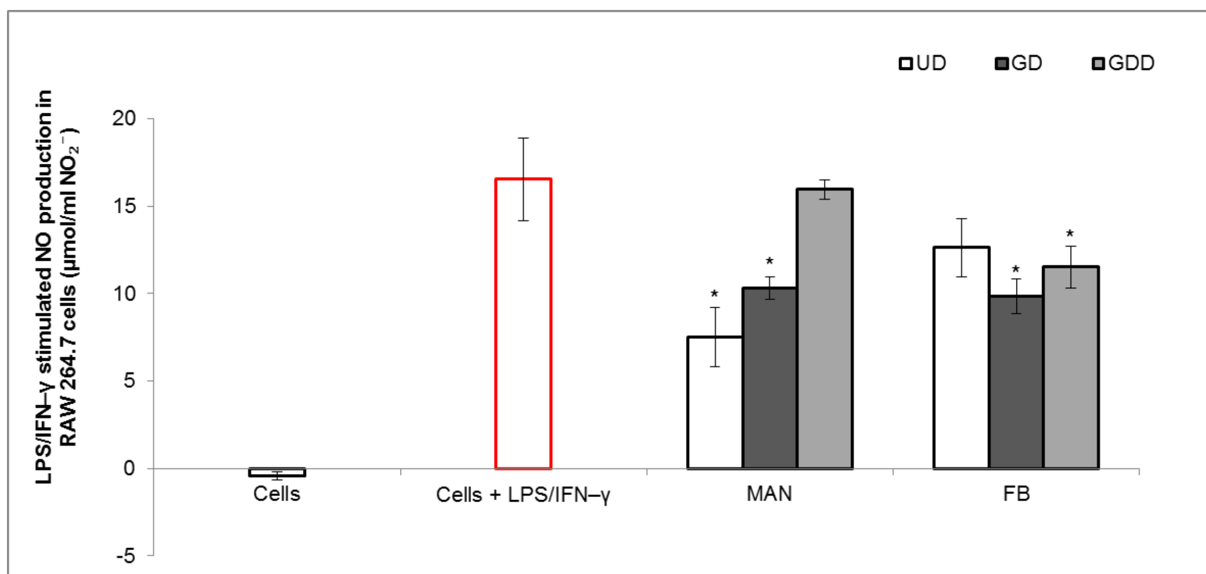

Figure 5.3b: Summary of the NO scavenging activity in LPS/IFN–γ stimulated RAW 264.7 cells at each SGDD phase for MAN and the average of FB honeys. Cells (negative control) and cells exposed to LPS/IFN–γ (positive control). Data is expressed as mean ± SEM. * Indicates significant difference compared to cells + LPS/IFN–γ, p≤0.05.

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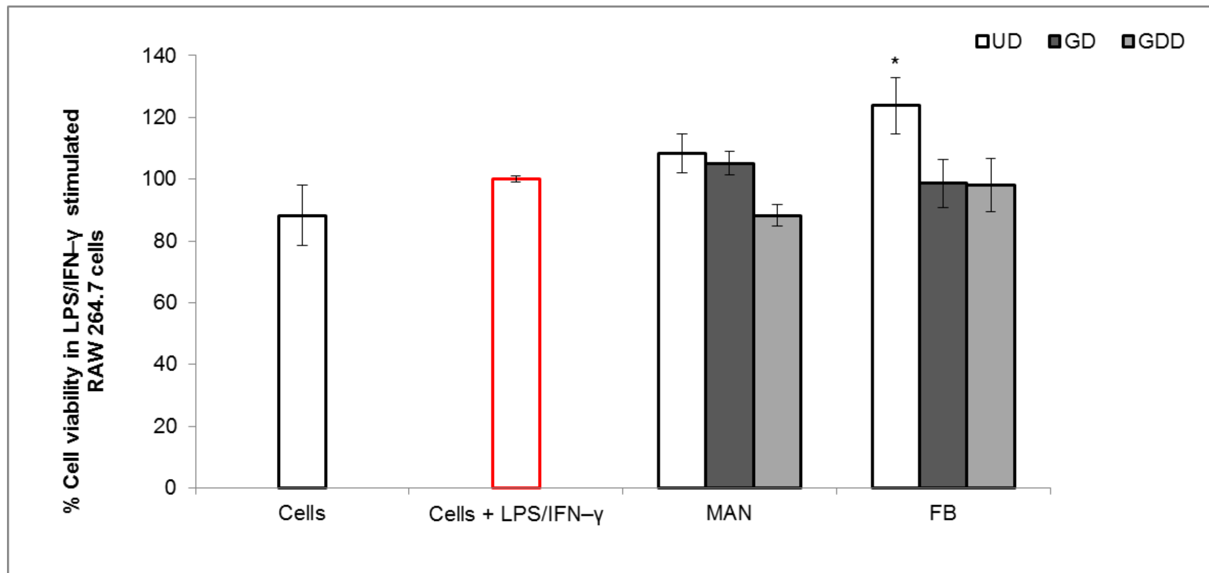

Figure 5.3c: Effects of MAN and FB honeys on % cell viability in LPS+ IFN- γ stimulated RAW 264.7 cells at each phase of SGDD. Data is expressed as mean ± SEM. * Indicates significant difference compared to cells + LPS/IFN–γ (positive control), p≤0.05.

A cell viability assay, linked to the results obtained in Figure 5.3b, showed no toxic effects on the RAW 264.7 cells for digests of both honey types. Instead a statistical increase in cell viability was seen in the FB, UD fraction when compared to the stimulated cells (cells + LPS/IFN–γ fraction). These results were similar to those obtained by Kassim *et al.*, (2010a) in the evaluation of NO inhibition by different alcohol honey extracts in LPS stimulated RAW 264.7 cells. Cell viability was not affected at all and no statistical significance was found.

The effect of pH on MAN honey (Figure 5.3d), showed a significant loss of NO scavenging activity at pH 7 when compared to UD. For FB honey, no pH effect was seen. When comparing digestion phase to its counterpart pH (GD vs. pH 2 and GDD vs. pH 7), there were no differences seen in MAN and FB honeys (Figure 5.3d, part b). A linked cell viability test, showed a significant increase in cell viability in UD for FB honeys when compared to the cell viability of stimulated cells (cells + LPS/IFN–γ) in Figure 5.3e, part a. The rest of the honey fractions, showed no statistical difference when compared to the cell viability in cells with LPS/IFN–γ.

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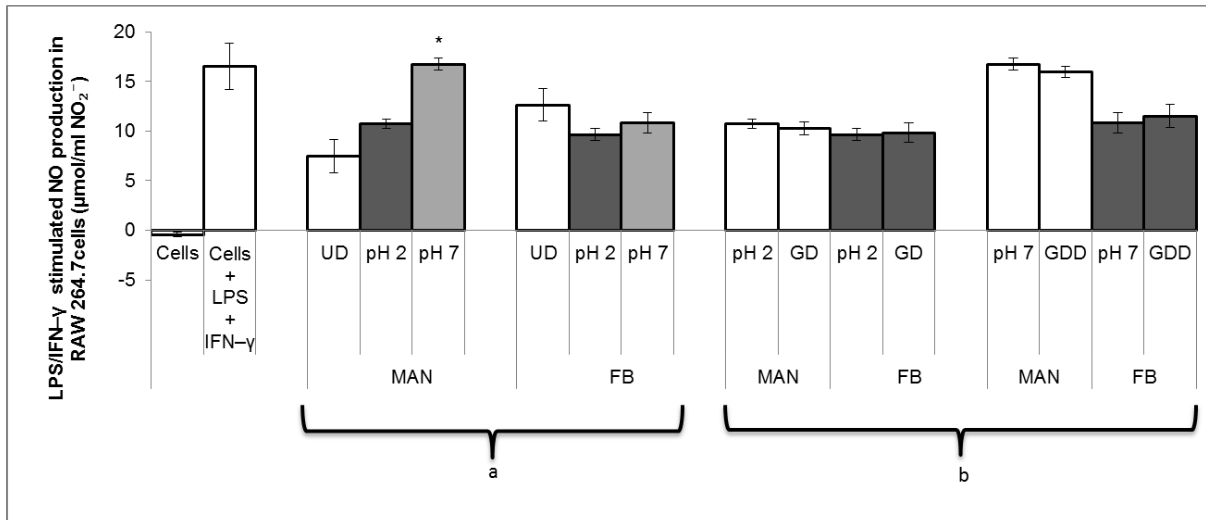

Figure 5.3d: a) Effect of pH on NO production in LPS/IFN–γ stimulated RAW 264.7 cells in the absence of digestive enzymes compared to UD and b) the comparison between pH and each phase of digestion (pH 2 vs GD and pH 7 vs GDD) for MAN (n=1) and FB (n=5) honey. Data is expressed as mean ± SEM. *In a indicates significant difference when compared to UD, p≤0.05.

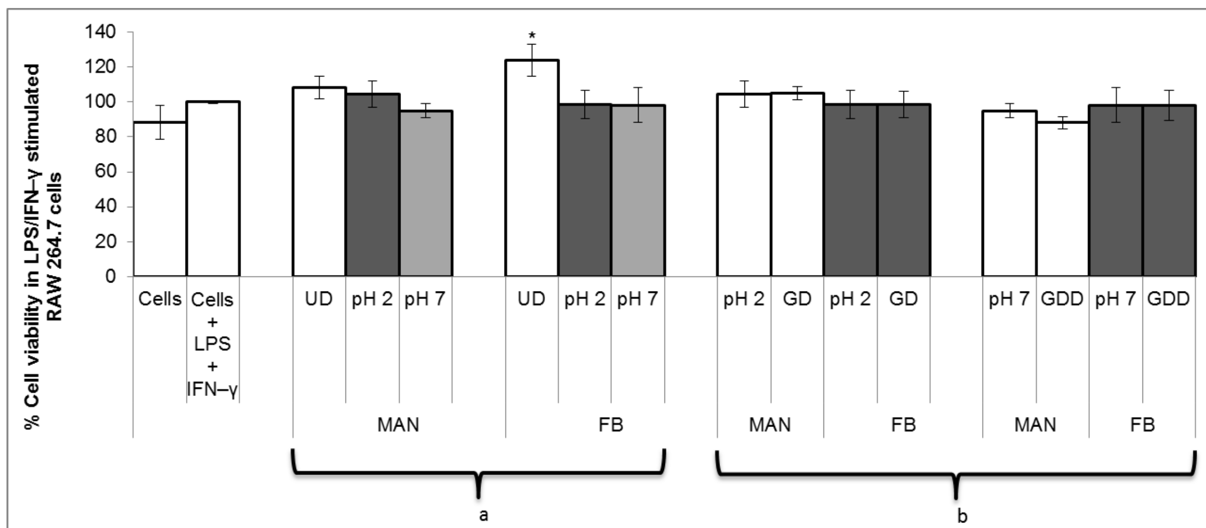

Figure 5.3e: a) % Cell viability of LPS/IFN–γ stimulated RAW 264.7 cells comparing undigested fractions to pH treated fractions and b) comparing pH to its relevant digestion phase (pH 2 vs. GD and pH 7 vs. GDD). Data is expressed as mean ± SEM. * Indicates significant difference compared to cells + LPS/IFN- γ (positive control), p≤0.05.

Kubes and McCafferty (2000), reported that, NO tends to have antioxidative properties during inflammation. In a study by Rubanyi *et al.*, (1991), cytoprotective effects of NO against the generated O_2^- radicals by human leukocytes were examined. It was found that the cytoprotection from O_2^- radicals were inhibited by the reaction with NO. Although NO can also react with the O_2^- radical to produce a cytotoxic oxidant known as the peroxynitrite ($ONOO^-$), it

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can also scavenge peroxyl radicals (Kröncke *et al*., 1997). In addition Kubes and McCafferty (2000), further reported that inflammation triggered by the O_2^- can be inhibited by NO donors or SOD, whereas inflammation triggered by non-oxidant dependent mediators cannot be inhibited by NO donors (Gaboury *et al*., 1993). Other studies have shown in both *in vitro* and *in vivo* models the antioxidant properties of NO donors in reducing injury and inflammation induced by H_2O_2 (Wink *et al*., 1993; Johnston *et al*., 1996).

The LPS endotoxin has been found to increase the permeability of the intestinal epithelia in animals (Deitch *et al*., 1991; Salzman *et al*., 1994) as well as in healthy humans (O'Dweyer *et al*., 1988) and patients with sepsis (Johnston *et al*., 1996). An *in vivo* study was undertaken with the aim of investigating the adverse effects of excess NO production on the functionality of the intestinal barrier in rats. After 12 h of LPS induced inflammation, plasma levels of NO was 450 umol/L. Excess prolonged NO production in this study further revealed, up-regulation of iNOS enzyme activity as well as an increase in the expression of the iNOS mRNA. Additionally the NO production induced the hyperpermeability of the intestinal epithelial lining, leading to bacterial translocations from the lumen of the gut to the lymph nodes in the mesentery region, resulting in bacterial infection. Excess NO production also led to the inhibition of mitochondrial respiration of the intestinal mucosa (Unno *et al*., 1997).

Jun *et al*., (2005) reported that isoflavones (genistein and biochanin A) scavenged NO in LPS stimulated RAW 264.7 cells. These two isoflavones were the most active among a pool of isoflavones extracted from the Kudzu roots (a leguminous plant). NO inhibitory effects by these flavonoids were achieved at concentrations higher than 50 µM. A similar study by Hämäläinen *et al*., (2007), evaluated the ability of 36 flavonoids to inhibit iNOS and NO production in LPS stimulated murine macrophage (J774) cells. The effect of 36 flavonoids representing the different flavonoid groups and related compounds were evaluated at a concentration of 10 and 100 µM. Compounds that induced cytotoxicity at a concentration of 100 µM were excluded from the study. These were gallic acid, dodecyl gallate and octyl gallate, rhamnetin, catechin, luteolin, apigenin and acacetin. Eight polyphenols exhibited more than 50% inhibition of NO production and these were quercetin, flavone, naringenin, diadzein, kaempferol, pelargonidin and isohamnetin. Furthermore these compounds were found to inhibit the expression of iNOS in the LPS/IFN–γ stimulated cells. These flavonoids also lowered the mRNA expression of iNOS as well as the transcription factor, NF-κB. Of these flavonoids, four out of eight of them also inhibited transcription factor, STAT-1, while the others, namely pelargonidin, isorhamnetin and

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naringenin (flavones) showed no inhibitory effects. Of these compounds, gallic acid, quercetin, luteolin, apigenin and kaempferol are found in honey and the observed NO inhibitory effects seen in MAN (UD & GD), FB5 and FB6 (GD & GDD) (Figure 5.3a) may be due to the presence of polyphenols. Mechanisms of NO reduction by polyphenols have been shown to either suppress the iNOS gene expression, or to block the NF-κB complex as well as to scavenge NO directly (Jun *et al*., 2005; Hämäläinen *et al*., 2007; Kassim *et al*., 2010b).

The effects of polyphenols have also been investigated *in vivo*. A study by Kassim *et al*., (2010b) evaluated the anti-inflammatory and anti-nociceptive properties of Gelam honey. Two alcohol extracts (methanol and ethyl acetate) were evaluated. These extracts were examined on the anti-inflammatory immune and non-immune animal models with a focus on two inflammatory mediators (NO and PGE_2). All animals in the groups were injected with either LPS or carrageenan into the footpad to induce oedema. The anti-inflammatory immune animal model received LPS while the non-immune/anti-nociceptive model received carrageenan. Each animal group was pre-treated with either, honey, honey ethyl acetate extract (HEAE), methanol extract of honey (HME), saline and indomethacin. The major phenolic compounds in the extracts were: ellagic acid, gallic acid, luteolin, quercetin, caffeic acid and chrysin. Paw oedema was monitored and measured and was significantly reduced by the both extracts (HEAE and HME) in the two models compared to animals treated with honey, saline and indomethacin. Furthermore, both extracts and honey were found to have anti-nociceptive activity as pain was significantly reduced as well as reducing NO and PGE_2 levels in both models

Honey can stimulate and/or inhibit NO production i.e. a pro-inflammatory or an anti-inflammatory effect. If the observed effect is only pro-inflammatory (formation of NO without stimulation and no inhibition of NO production), the amount of NO formed is the sum of the amount formed with and without LPS/IFN-γ stimulation. In contrast if only inhibition occurs no NO formation will occur without LPS/IFN-γ stimulation, NO production should be reduced with LPS/IFN-γ stimulation. Due to the possible presence of arabinoglycans that stimulate NO production and flavonoids that scavenge NO, the observed effect is the sum of the effects of these two types of molecules. Therefore honey types can be identified that will have a predominant pro-inflammatory or a predominant anti-inflammatory effect. This will determine the type of application with a honey with a predominant pro-inflammatory effect being used for the treatment of acute wounds while honey with an anti-inflammatory effect being used for chronic

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wounds. The pro-inflammatory (Table 5.1) and anti-inflammatory (Table 5.2) effects have been evaluated for each honey type.

To evaluate the pro-inflammatory effect, the individual NO or the nitrite content values produced by honey digests in non-LPS/IFN–γ stimulated cells was compared to the positive control of the experiment, that is cells + LPS/IFN–γ. This positive control yielded a nitrite content value of 16.54 µmol/ml NO_2^-. Honey digests that had values similar to 16.54 µmol/ml NO_2^- were considered to have pro-inflammatory effect which is ideal for acute wounds. As shown in Table 5.1, all UD samples of MAN and FB honeys produced statistically lower values of NO as compared to the control value (16.54 µmol/ml NO_2^-) and therefore do not have a pro-inflammatory effect. However with SGDD, FB3 showed pro-inflammatory effect with digestion. Similar effects were also seen for FB1 with the pro-inflammatory effect shown for pH samples only.

All MAN and FB honey digests showed potential NO scavenging abilities as the observed values were lesser than the expected values (Table 5.2). For MAN, pH 7 and GDD digests showed higher scavenging abilities with values greater than 60%. Among FB honeys this effect was seen for UD and GDD fractions of FB2, UD of FB3 and UD of FB5 with the highest NO scavenging value of 82.23%. Moderate scavenging was found for UD, pH 2 and GD digests of MAN. Among FB honeys, all FB1 digests, FB2 (pH 2, GD and pH 7), FB3 (pH 2, GD and pH 7 and GDD) and UD of FB6. UD digests of FB5 and FB6 showed higher and moderate scavenging abilities respectively. However through SGDD, these honeys presented with a reduced scavenging activity, less than >30% and therefore are lower scavengers during SGDD.

Therefore, MAN and FB honey samples show both pro-inflammatory and anti-inflammatory properties. These properties are attributed to the immunomodulatory effect of honey, which will stimulate the release of pro-inflammatory cytokines and ROS for the beginning stages of inflammation. If inflammation persists to be chronic, honey has been shown to inhibit and scavenge the production of these pro-inflammatory cytokines and ROS illustrating its anti-inflammatory effect (Majtan, 2014). Based on the information depicted in both Table 5.1 and 5.2, UD of FB2, FB3 and FB5 have potential anti-inflammatory activities. Throughout digestion, this effect is seen for pH 7 and GDD of MAN and GDD of FB2. The pro-inflammatory effect is best shown throughout SGDD for FB3 and both pH conditions of FB1. This is illustrative of the use of honey in the treatment of GIT disorders such as ulcers and gastritis.

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Table 5.1: Identification of pro-inflammatory MAN and FB honey samples

	UD	pH 2	GD	pH 7	GDD
MAN	0.21	6.17	4.19	8.30	7.93
FB1	2.48	**12.49 = P**	11.67	**13.54 = P**	10.73
FB2	2.46	6.11	7.43	5.77	7.79
FB3	5.93	**12.72 = P**	**14.92 = P**	**13.77 = P**	**12.17 = P**
FB5	0.23	-0.25	-0.11	-0.08	0.40
FB6	1.59	1.70	2.25	3.58	3.55

P indicates sample is pro-inflammatory and not statistically different from NO induced with LPS+IFN-γ

Table 5.2: Identification of potential anti-inflammatory MAN and FB honey samples

Honey Fractions/Digests		Expected ($\mu mol/ml\ NO_2^-$)	Observed ($\mu mol/ml\ NO_2^-$)	% scavenging	Type of interaction
MAN	**UD**	16.75	7.46	44.72	M
	pH 2	22.70	10.75	47.36	M
	GD	22.73	10.31	45.36	M
	pH 7	24.83	16.72	67.34	H
	GDD	24.46	15.95	65.21	H
FB1	**UD**	19.02	10.69	56.20	M
	pH 2	29.02	12.96	44.66	M
	GD	28.21	13.59	48.17	M
	pH 7	30.08	16.89	56.15	M
	GDD	27.27	15.83	58.05	M
FB2	**UD**	19.00	14.13	74.36	H
	pH 2	22.65	13.49	59.56	M
	GD	23.97	13.62	56.82	M
	pH 7	22.31	12.51	56.07	M
	GDD	24.33	15.92	65.43	H
FB3	**UD**	22.47	14.24	63.37	H
	pH 2	29.26	13.68	46.75	M
	GD	31.46	13.87	44.09	M
	pH 7	30.31	15.04	49.62	M
	GDD	28.71	13.72	47.79	M
FB5	**UD**	16.77	13.89	82.83	H
	pH 2	16.29	5.33	32.72	L
	GD	16.43	4.97	30.25	L

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Honey Fractions/Digests		Expected (μmol/ml NO_2^-)	Observed (μmol/ml NO_2^-)	% scavenging	Type of interaction
	pH 7	16.47	4.32	26.23	L
	GDD	16.94	6.44	38.02	L
FB6	UD	18.12	10.23	56.09	M
	pH 2	18.24	2.83	15.51	L
	GD	18.79	3.10	16.50	L
	pH 7	20.12	5.47	27.19	L
	GDD	20.09	5.70	28.37	L

Expected = NO produced in non-LPS stimulated cells + Positive control (16.54 μmol/ml NO_2^-)

Observed = LPS/IFN-γ stimulated NO production in RAW 264.7 cells

H = high scavenging effect >60%, M = moderate scavenging effect 30-60% and L = low scavenging effect <30%

A correlation between chemical and cellular NO scavenging activity in LPS/IFN–γ stimulated RAW 264.7 model was expected. However the only correlation found was between gastro-duodenal digested samples (r = 0.72), and this is because with FB honey throughout digestion, chemical NO scavenging activity was maintained. The only correlation in FB honeys that was found was between the GD digested samples. For chemical NO scavenging activity with digestion, there was no change. However in a cellular model with both GD and GDD digestion, FB honeys showed an increase in NO scavenging abilities. MAN had decreased chemical NO scavenging with GDD but in a cellular environment did not have the same effect, instead GDD digestion showed an increase in NO production, Figures 5.1b and 5.3b.

5.5 Conclusion

In conclusion, undigested honeys (FB and MAN) have chemical and cellular anti-inflammatory activity. In a cellular environment, several honey samples are found to produce NO i.e. have a pro-inflammatory effect while other honey types are found to have anti-inflammatory effects related to NO scavenging. In general for both MAN and FB, the anti-inflammatory effect is the predominant effect. Digestion does not result in a loss of anti-inflammatory activity and in some instances is increased. During all phases of digestion cell viability is retained.

A correlation between chemical and cellular NO scavenging activity is seen in the GDD digest of FB honeys. The maintained chemical NO scavenging activity of FB honeys is reflected by the increased scavenging or inhibition of NO in a cellular environment.

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Chapter 6: Concluding discussion

Various health beneficial biological activities of honey have been identified hence deemed as a functional food mostly attributed to the presence of phenolic compounds. Many of these therapeutic effects have been identified for European honeys and few are documented for African honeys. A study by Serem and Bester (2012) reviewed the physicochemical properties, antioxidant activity, biological and cellular protection of southern African honeys and several of these honey samples were from the Western Cape region of South Africa. All of the southern African honeys were found to have significant antioxidant activity and provided biological and cellular protection against oxidative damage. The anti-inflammatory properties of New Zealand and Australian honeys (Tonks *et al*., 2003) as well as the Malaysian honeys (Kassim *et al*., 2010a; Kassim *et al*., 2010b) have been reported. No information is available concerning the anti-inflammatory properties of African honeys. Honey has been identified as a nutraceutical product and the aim of this study was to determine the effect of digestion on the bioactivity and further to identify the contribution of pH and digestive enzymes on the bioactivity of these samples.

The Cape Floristic Kingdom is a unique biodiversity region with over 8920 flowering plant species and it is expected that honey from this region will have unique properties. Six Fynbos honeys (FB1 – FB6) were evaluated and compared to a medicinal Manuka (MAN) honey, UMF 15+. The physicochemical properties of honey were evaluated. Each honey sample was subjected to SGDD and the chemical and cellular antioxidant and anti-inflammatory activities of each fraction was determined. Five fractions were obtained at the end of SGDD. These fractions included the gastric digest (GD), gastro-duodenal digest (GDD), pH fractions (pH 2 and pH 7) as well as the undigested/raw honey sample (UD).

Physicochemical properties were performed so as to assess the quality of honeys used in the study. All honeys proved to be of good quality and non-adulterated. All honeys complied with the regulatory standards of the Codex Alimentarius.

MAN had a higher TPC than all FB honeys and the TPC of all FB honeys were similar except for FB4 which presented with a significantly lower TPC. The TFC of MAN was higher than the TFC of the FB honeys. The TFC of all FB honeys was similar. The antioxidant activity of MAN (TEAC) was higher than FB honeys. The antioxidant activities of FB honeys were similar except for FB4, which presented with a lower TEAC activity. Antioxidant activity was further evaluated

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with the ORAC assay that is considered to be a physiologically relevant assay as it measures the activity of an antioxidant to donate electrons/hydrogen molecules to an unstable radical thereby quenching the radical. FB5, FB6 and FB1 were similar and were greater than the activity of MAN.

The principle of the ORAC assay is similar to that of DCFH-DA assay as both assays use AAPH as a radical generator and the time-dependent effect of antioxidants is measured. CAA measures the ability of antioxidants to protect cells such as Caco-2 and SC-1 *in vitro* against the oxidative effects of AAPH, 100% damage. In the Caco-2 cell line, MAN effectively protected these cells against oxidative damage a reduction from 100% to 4.28%. Among FB honeys, FB2 showed the highest CAA and oxidative damage was reduced from 100% to 19.50%. Other FB honeys (FB1, FB4, FB5 and FB6) were similar and the lowest level of CAA was found for FB3 with measured oxidative damage value of 58.83%. All honey samples more effectively protected the SC-1 cell line against oxidative damage when compared to the Caco-2 cell line. MAN honey, showed no damage at all (-10.97%) and was similar to FB6 (9.20%) and FB1 (10.00%). FB3 and FB4 presented with the least CAA with measured oxidative damage values of 46.50% and 41.38% respectively. The CAA of FB2 and FB5 were found to be similar to the darker honeys (MAN, FB1 and FB6) as well as FB3 and FB4.

ORAC assay, identified that FB honeys, FB1, FB5 and FB6 had greater antioxidant activity than MAN. Additionally, the CAA of FB1 and FB6 was similar to MAN, illustrating that in a cellular environment the antioxidant activity of FB honey is comparable to MAN. In general the ORAC assay, due to its mechanism that is physiologically relevant to human biology (Prior *et al.*, 2005) it can be concluded that several FB honeys have physiologically relevant antioxidant properties comparable to MAN honey.

Antioxidants such as polyphenols can reduce NO formation thereby preventing the formation of NOS. With the SNP assay, at a physiological pH, NO spontaneously forms in aqueous solutions. The ability of each honey to quench NO formation was evaluated. Residual nitrite content of honey was low. The NO scavenging activity of MAN was 85.55% while the average of 47.91% was obtained by FB honey. The most effective NO scavenging honeys were FB1, FB2 and FB6.

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Due to the complexity of honey it was necessary to determine if honey stimulates NO formation (pro-inflammatory effect, RAW 264.7 cells, no LPS/ IFN–γ stimulation,) or inhibits NO formation (anti-inflammatory effect, RAW 264.7 cells, with LPS/ IFN–γ stimulation,). A pro-inflammatory effect was observed for FB4 and an anti-inflammatory effect was observed for MAN, FB1 and FB6. Of the FB honeys evaluated the antioxidant and anti-inflammatory properties of FB1 and FB6 was similar to MAN.

Polyphenols contribute to the therapeutic properties of honey. Furthermore these phenolic compounds have been shown to possess health beneficial effects against the development or manifestations of chronic diseases. Due to such effects, it is important to assess if antioxidant and anti-inflammatory bioactivity is retained with digestion.

The effect of digestion on each of the measured activities was determined and was compared to UD honey. With GD, the TPC and TFC for MAN honey increased while the average TPC and TFC of FB honey was unchanged. With GDD, the TPC of MAN decreased while the TFC remained unchanged. For FB honeys, both TPC and TFC remained unchanged. With GD antioxidant activity (TEAC and ORAC assay) was unchanged for MAN and FB honeys. With GDD, activity was reduced for MAN but not FB. With digestion for both honey types, antioxidant activity measured with the ORAC assay was unchanged.

In the Caco-2 cell line, with gastric digestion, CAA for both MAN and FB honeys was unchanged. Following GDD, both MAN and FB honeys showed a loss in CAA from 4.28% to 34.98% and 19.50% to 111.10% for MAN and FB honeys respectively. In the SC-1 cells, with gastric digestion, the CAA of MAN remained unchanged while CAA increased for FB honeys in GD. As in the Caco-2 cell line, the CAA of the GDD was reduced. In general MAN was more effective in protecting cells *in vitro* against oxidative damage following digestion than FB honeys.

Changes in measured antioxidant content and activity were due to pH changes and digestive enzyme activity. An acidic environment and the presence of pepsin increased the measured TPC and TFC of MAN due to increased extraction at low pH. No effect was observed for FB honeys. Antioxidant activity of only MAN measured with the TEAC assay was reduced following GDD. No effect was observed for FB honey. For both honey types no change in antioxidant activity was observed when measured with the ORAC assay.

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With GD, CAA measured in the Caco-2 and SC-1 cell lines was unchanged, while with CAA a loss in activity was observed and this was found to be associated with an increase in the H_2O_2 content of the GDD. This effect was a result of pH change and the presence of pancreatin which causes polyphenol autoxidation at pH (6.0 – 7.5). Furthermore, $NaHCO_3$ addition used to adjust pH together with pancreatin was identified as the cause of increased H_2O_2 levels and consequently loss of CAA.

The effect of digestion on the NO scavenging activity was evaluated. With GD, No scavenging activity of MAN, FB1, FB2, FB3 and FB5 was unchanged while that of FB6 was increased. Following GDD, MAN and FB6 showed a reduction in NO scavenging. MAN showed a higher scavenging activity than FB honeys in the UD fraction however in the GDD fraction, the activity decreased to the level of FB honeys. Both pH 2 and pH 7 increased the NO scavenging activity of FB honeys but not that of MAN. Addition of digestive enzyme pancreatin in GDD, lowered the NO scavenging activity of both MAN and FB honeys.

Induction of NO formation (pro-inflammatory effect) was low in UD honey samples but with GD NO levels were increased for MAN, FB1, FB2 and FB3. With GDD, a further increase in NO levels was observed for MAN, FB1, FB2 and FB3. The levels of NO induced by MAN was similar to that of FB honey and was a function of pH. The ability of the honey digests to scavenge NO was then evaluated. Gastric digests of MAN, FB5 and FB6 as well as the GDD of FB5 and FB6 significantly effectively scavenged NO.

In summary, for MAN, effective NO scavenging was observed for UD and GD while for FB this was found for GD and GDD. NO scavenging of MAN was decreased at pH7 but had no effect on other honey samples. No pH or digestive enzyme effects were observed for FB. Correlation between assays for NO scavenging activity was poor and was high for only GDD.

Interestingly two types of honey were identified namely, those with pro-inflammatory activity and those with anti-inflammatory activity. Samples, FB1 (pH 2 and 7) and FB3 (pH 2, GD, pH 7 and GDD) had strong pro-inflammatory activity. Honey samples with anti-inflammatory activity could be divided into three groups with low, moderate and high activity. Honey samples with high NO scavenging activity were the UD samples of FB2, FB3 and FB5 and with digestion MAN (GDD and pH 7) and FB2 (GDD).

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In conclusion although the TPC, TFC and antioxidant activity (TEAC assay) of MAN are greater than FB honey, using the ORAC assay and anti-inflammatory assays the activities of several FB honeys are comparable to MAN honey. This study identifies that FB honey, is an important local nutraceutical product. Interestingly some of the honey samples were even found to have pro-inflammatory effects which increased with digestion.

Limitations and recommendations

A major limitation of this study is the use of only one MAN sample. For better analysis, several MAN honey samples should be used. In addition the MAN sample used is a honey type that has been developed for medicinal purposes and may not be an ideal control for honey samples that have been purchased from local farm stalls. An UMF15+ indicates that this honey has a high MGO level which translates into excellent antibacterial activity. Interestingly this study shows that this honey also has high antioxidant and anti-inflammatory activity. This implies that as a wound healing product the effect of this honey is not limited to eradication of infection but also improves oxidative status and reduces inflammation.

It is essential that the polyphenols in these honey samples be identified using ultra performance liquid chromatography-electrospray ionisation mass spectrometry (UPLC-PDA-ESI/MS) analysis. Using this technique changes in the concentrations of specific polyphenols can be measured, however it is especially difficult to identify the degradation or oxidation products of a complex mixture especially following SGDD. During this process where degradation products form unknown derivatives can also form and these may have additional bioactivity. Therefore once the major polyphenols have been identified the effect of SGDD can be determined on the activity and structure of individual polyphenols that are present in honey. This includes the identification of specific degradation products that form following GDD.

How honey induces increased NO formation in the RAW 264.7 cells is unknown. Honey may contain bacteria, and the presence of LPS can cause increased NO formation. Levels of LPS can be quantified using a kinetic limulus amebocyte lysate (LAL) assay. Gannabathula *et al.,* (2015) further reported the presence of apisimin and plant derived AGPs. Both proteins were found to synergistically enhance the release of TNF-α. It appears from this study with digestion the levels of NO are increased in MAN, FB1, FB2 and FB3. Levels of AGPs in these samples can be measured by using the radial agar diffusion assay which makes use of the β-galactosyl Yariv reagent that binds and precipitates these proteins. Furthermore, presence of apisimin can

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be determined with Western blotting and quantified with the enzyme-linked immunosorbent assay (ELISA).

The mechanism by which increased NO is formed or scavenged is unknown and can be determined with Western blotting analysis by measuring the levels iNOS and associated transcription factors such as STAT-1 and NF-κB. Polyphenols that have an anti-inflammatory effect would then reduce or inhibit the iNOS expression and the levels of transcription factors. Furthermore, the iNOS mRNA expression could be quantified by the quantitative real-time polymerase chain reaction (RT-PCR).

Future studies can focus on the bioavailability of honey derived polyphenols and the antioxidant and anti-inflammatory effects of these honeys in animal models. Examples of animal models that can be used are the carrageenan-induced paw oedema or GIT models of inflammation (induced gastritis and colitis). Biochemical methods could then be applied to study the effect on blood components such as the effect on the antioxidant status of plasma, GSH levels and the activity in erythrocytes of the antioxidant enzymes catalase, superoxide dismutase and glutathione peroxidase. Histology can be used to evaluate the histology of the skin and mucosa of treated animals compared to untreated animals.

Before commercialisation as a nutracetical product a large number of honey samples from this region must be evaluated and the effect of geographical sub-region and seasonal variation (Bogdanov and Martin, 2002) must be determined. As FB honey is from a specific defined region, pollen analysis and/or fingerprinting (Bogdanov and Martin, 2002) will be essential to confirm the origin of this honey type. Techniques that are used are: Melissopalynology which analyse pollen and creates a spectra that can be used to identify floral regions and therefore geographical region (Bogdanov & Martin, 2002; Noor *et al*., 2014), high liquid chromatography-diode array detection-tandem mass spectrometry (HPLC-DAD-MS/MS) and chemometric analysis which uses markers and fingerprints e.g. from polyphenols to differentiate between various honeys (Zhou *et al*., 2014) and geographical origin analysis based on volatile compounds or physicochemical characteristics to identify honeys from various regions (Karabagias *et al*., 2014).

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Chapter 7: References

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Appendix

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.
- FWA 00002567, Approved dd 22 May 2002 and Expires 20 Oct 2016.
- IRB 0000 2235 IORG0001762 Approved dd 22/04/2014 and Expires 22/04/2017.

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Faculty of Health Sciences Research Ethics Committee

30/10/2014

Approval Certificate
New Application

Ethics Reference No: 424/2014

Title: Effect of in vitro simulated gastro-duodenal digestion on the antioxidant and anti-inflammatory activity of South African Fynbos honey.

Dear Miss Innocentia Magoshi

The **New Application** as supported by documents specified in your cover letter for your research received on the 1/10/2014, was approved by the Faculty of Health Sciences Research Ethics Committee on the 29/10/2014.

Please note the following about your ethics approval:
- Ethics Approval is valid for 2 years.
- Please remember to use your protocol number (**424/2014**) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, or monitor the conduct of your research.

Ethics approval is subject to the following:
- The ethics approval is conditional on the receipt of 6 monthly written Progress Reports, and
- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

Dr R Sommers; MBChB; MMed (Int); MPharMed.
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes 2004 (Department of Health).

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