AN EXPLORATORY INVESTIGATION INTO THE PHYSICOCHEMICAL, ANTIOXIDANT AND CELLULAR EFFECTS OF A SELECTION OF HONEY SAMPLES FROM THE SOUTHERN AFRICAN REGION

by

JUNE CHEPTOO SEREM

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Abstract

The unique floral biodiversity of Southern Africa would be reflected in the phenolic acid and flavonoid composition as well as the antioxidant activity of honeys from this region. In this exploratory investigation the total polyphenolic (TPC) and flavonoid (TFC) content, antioxidant activity as well as the celluar protective effects of a selection of honeys collected in this region was evaluated. Thirteen honey samples representative of the Western Cape (WCa, WCb and WCc), Eastern Cape (ECa, ECb and ECc), South East Mozambique (SEMa, SEMb and SEMc) and Agricultural: A-E (Eucalyptus) (A-E1 and A-E2), A-L (Litchi) and A-O (Orange) were collected.

These samples were subjected to physicochemical analysis, the antioxidant content (TPC and TFC) and both enzymatic (catalase activity) and non-enzymatic activity, using the 2,2-diphenyl-2-picrylhydrazyl (DPPH), trolox equivalent antioxidant capacity (TEAC) and oxygen radical antioxidant capacity (ORAC) assays was determined. From the DPPH, TEAC and ORAC data the Relative Antioxidant Capacity Index (RACI) was calculated.

To determine whether high antioxidant activity translates into significant cellular protection, biological and cellular assays were undertaken. Using the pBR322 plasmid assay and the erythrocyte haemolysis assay the ability of honeys to protect against 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH) oxidative damage was evaluated. Further evaluation was undertaken in the SC-1 fibroblast cell line and the physiologically more relevant Caco-2 cell line. Toxicity and antioxidant effects were evaluated in the SC-1 cell line

while antioxidant effects were only evaluated in the Caco-2 cell line. The long-term mitogenic and toxic effects were determined in the SC-1 cell line using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Neutral Red (NR) and Crystal Violet (CV) assays. Short term, total- and intracellular antioxidant effects were determined in both cell lines using the dichlorofluorescein diacetate assay (DCFH-DA) assay. For all cellular experiments honey at concentrations of 0.01% and 1% were used.

The physiochemical properties of the honeys evaluated fulfilled the regulatory standards compiled in the Codex Alimentarius (CODEX STAN12-1981 revision 2001). The results were as follows: **SEMb** had the highest TPC (167.96 mg GAE/100g) and TFC (51.60 mg CE/100g) while **A-E2** had the highest catalase (38.48 µmol H_2O_2/g) activity. RACI revealed that **WCb** had the highest antioxidant activity. **SEMc** showed the highest protection of plasmid DNA against oxidative-induced strand breaks while **SEMa** showed the highest protection of erythrocytes against AAPH-induced haemolysis. Although correlations were found between antioxidant content and antioxidant activity assays, no correlation was found these parameters and the biological assays.

For the long-term cytotoxicity assay, AAPH showed significant cytoxicity at 0.78mM, 1.56mM and 0.28mM when measured using the MTT, NR and CV assays, respectively. Some honeys 4/13 and 3/13 showed a mitogenic effect at a concentration of 0.01% and 1% respectively. Toxic effects, were observed for 1/13 and 8/13 at 0.01% and 1% honey respectively. Toxicity after 72 h exposure varied from 10-30% (CV assay). The same concetrations of honey was used to determine the short-term, 2h, antioxidant effects in both the SC-1 and Caco-2 cell lines. No oxidative effect was found for all honeys at these concentrations.

For the DCFH-DA assay using the SC-1 cell line at 1%, 12/13 and 7/13 honeys showed total and intracellular protection respectively. The highest extracellular protection was for **SEMa** (% Protection (%P) = 95) and **SEMb** (%P = 93). Intracellular protection was the highest for **SEMc** (%P = 21) and **A-L** (%P = 20). At 0.01%, 7/13 and 8/13 honeys exhibited total and intracellular protection, respectively. For both the highest protection was found for **SEMc** (%P = 43, total and %P = 30, intracellular). For the Caco-2 cell line at 1%, 11/13 and 4/13 showed total and intracellular protection, respectively. Of these the highest extracellular protection was for **SEMb** (% Protection (%P) = 90). Intracellular protection was the highest for **ECa** (%P = 28) and **WCc** (%P = 26). At 0.01%, 4/13 and 8/13 honeys showed total and intracellular protection respectively. The highest extracellular protection was found for **SEMc** (%P = 62) and intracellular protection was **ECc** (%P = 28). The SC-1 cell line was found to

be the most sensitive to the antioxidant effects of honey compared to the Caco-2 cell line. The honeys **SEMa**, **SEMb** and **SEMc** showed protection against oxidative damage in both cell lines.

In conclusion, the antioxidant activity of honeys from Southern Africa is of a high quality. The **WC, SEM and EC** honeys showed the highest antioxidant effects and could provide health benefits against diseases associated with oxidative stress as indicated by these results.

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Declaration

I, June Serem hereby declare that this research dissertation is my own work and has not been presented for any degree of another University;

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Table of Contents

List of Tables

List of Figures

List of Abbreviations and Chemical Formulae

%	Percentage
ºC	Degrees centigrade
x g/RCF	Relative centrifugal force
µg	Micrograms
µl	Microlitres
µg/µl	Microgram per microlitre
A450	Absorbance at 450 nm
AAPH	2,2'-azobis(2-amidinopropane) dihydrochloride
ABTS	(2,2'-Azo-bis (3-ethylbenzothiazoline-6-sulfuric acid) diamonium salt)
$ABTS^{\cdot}$	ABTS radical
ACF	Aberrant crypt foci's
A-E	Agricultural eucalyptus
A-L	Agricultural litchi
$AlCl_3$	Aluminium chloride
ANOVA	Analysis of variance
A-O	Agricultural orange
AOM	Azoxymethane
$Asc^{\cdot-}$	Ascorbate radical
$AsCH^{-}$	Ascorbate
BSA	Bovine serum albumin
Caco-2	Human colon adenocarcinoma cell line
CE	Catechin equivalents
cm^2	Centimeters squared
CQ_{10}	Co enzyme Q10
CUPRAC	Copper reduction assay
CV	Crystal violet
DCFH-DA	Dichlorofluorescein diacetate
ddH_2O	Double distilled water

DMEM	Dulbecco's modified essential medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DPPH	2, 2-diphenyl-2-picrylhydrazyl
EA.hy929	Endothelial cell line
EC	Eastern cape
EC 1.11.1.6	Catalase
EC 1.13.4/ GOD	Glucose oxidase
ECa	Eastern cape sample a
ECb	Eastern cape sample b
ECc	Eastern cape sample c
EC_{50}	Median effective concentration
EDTA	Ethylene diamine tetra acetic acid ($C_{10}H_{16}N_2O_8$)
EGCG	Epigallocatechin gallate
EMEM	Eagle's modifies essential medium
ET	Electron transfer
FCS	Foetal calf serum
FC reagent	Folin-Ciocalteu's reagent
FDA	Fluorescein diacetate
FRAP	Ferric reducing ability of plasma assay
Fru	Fructose
GAE	Gallic acid equivalents
Glc	Glucose
Glc:Fru	Ratio of glucose to fructose
g/L	Gram per litre
GIT	Gastrointestinal tract
GPx	Glutathione peroxidase
GSH	Glutathione
GST	Glutathione S-transferase
h	Hour/hours

HAT	Hydrogen atom transfer
H_2O	Water
H_2O_2	Hydrogen peroxide
HCL	Hydrochloric acid
HCT-116	Colorectal cell line
Hela	Cervical carcinoma cell line
Hep-G2	Liver carcinoma cell line
iNOS	Nitric oxide synthase
KCl	Potassium chloride
KH_2PO_4	Potassium dihydrogen phosphate
$K_3Fe[CN]_6$	Potassium ferricyanide
LDL	Low density lipoprotein
L	Litres
$L^{\cdot}$	Alkyl radical
$LO^{\cdot}$	Alkoxyl radical
$LOO^{\cdot}$	Lipid peroxide radical
LSD	Least significant difference
M	Molar
mAu	Milli-absorbance units
mM	Millimolar
µM	Micromolar
nM	Nanomolar
mg	milligrams
mg/ml	Milligram per millimetre
ml	Millimetre
Max	maximum
MCF-IDA	Normal human mammary cells
MDA-MB-23/MCF-7	Breast adenocarcinoma cells
MIC	Minimum inhibitory concentration
Min	Minute

MRP	Maillard reaction products
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAD(P)H	Nicotinamide adenine dinucleotide phosphate
NaCl	Sodium chloride
$NaHCO_3$	Sodium hydrogen carbonate
Na_2HPO_4	Sodium disodium hydrogen phosphate
$NaHPO_4$	Sodium hydrogen phosphate
$NaNO_2$	Sodium nitrite
NaOH	Sodium hydroxide
NO	Nitric oxide
$NO^{\cdot}$	Nitric oxide ion
NR	Neutral red
O_2	Oxygen
$O_2^{\cdot -}$	Superoxide ion
OH^-	Hydroxyl radical
ORAC	Oxygen radical absorbent capacity
PAOXI	Phenol antioxidant index
P-BF	Protein concentration determined using Bradford method
PBS	Phosphate buffered saline
pBR322	Plasmid DNA
pH	Logarithmic scale for the measurement of the acidity or alkalinity of an aqueous solution
P-OD	Protein concentration determined using Optical Density
P-value	Probability value
r	Correlation
r^2	Square correlation
$R^{\cdot\cdot}$	Unsaturated lipid
$R^{\cdot}$	Linoleic radical
RH	Linoleic acid
RAC	Recycling antioxidant cycle

RACI	Relative antioxidant capacity index
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
ROO$^{\cdot}$	Peroxyl radical
ROOH	Hydroperoxide
rpm	Revolutions per minute
RT4	Murine bladder cancer cell line
SC-1	Mouse fibroblast cells
SD	Standard deviation
Se	Selenium
SEM	Standard error of mean
SEM	South Eastern Mozambique
SEMa	South Eastern Mozambique sample a
SEMb	South Eastern Mozambique sample b
SEMc	South Eastern Mozambique sample c
SOD	Superoxide dismutase
TE	Trolox equivalent
TEAC	Trolox equivalent antioxidant capacity
TFC	Total flavonoid content
TNF	Tumour necrosis factor
TPC	Total polyphenol content
TRAIL	TNF-related apoptosis inducing ligand
TRAP	Total peroxyl radical-trapping antioxidant parameter
T24/253J	Human bladder cancer cell line
Vit A	Vitamin A/Retinol
Vit B3	Vitamin B3/Niacin
Vit C	Vitamin C/Ascorbic acid
Vit E/TOC	Tocopherol
v/v	Volume to volume

vs	Versus
WC	Western Cape
WCa	Western Cape sample a
WCb	Western Cape sample b
WCc	Western Cape sample c
w/v	Weight to volume

CHAPTER 1: INTRODUCTION

'The fruit of the Bees is desired of all, and is equally sweet to kings and beggars and it is not only pleasing but profitable and healthful, it sweetens their mouthes, cures their wounds and convaies remedies to inward ulcers.'

This quote by St Ambrose (Honey-health, 2005), conveys in one sentence the versatility of honey. Honey is a common food source worldwide and is the only natural form of concentrated sugar available (Meda *et al.,* 2005), which has been used since the earliest of times. First writings on honey were dated to Sumeria 2100-2000 BC as a drug and ointment against ulcers, and also by Aristotle 384-322 BC as a salve for sore eyes and wounds (Mandal and Mandal, 2011). Later on Celcus, a Greek physicician in Rome ca. 25 AD suggested honey as a remedy against diarrhea (Bogandov 2008), in the Materia Medica, 1st century AD by Dioscorides as a good ingredient for preparation of epileptic medicines (Eadie, 2004), and in the Papyrus Ebers as a remedy for passing too much urine (diabetes mellitus) (Korczowski, 1985). Remedies of honey also feature in the holy books: in the Bible; King Solomon tells his son to eat honey as it is good, and in the Holy Hadith; Prophet Mohamed recommended honey to his one of his followers as a remedy against diarrhea (Bogandov 2008).

Scientific research has confirmed these beneficial effects and has identified honey as an important functional food. A functional food is a food that produces a beneficial effect in one or more physiological functions, resulting in increasing well-being and/or decreasing the risk of suffering from a particular medical condition (Gomez Caravaca *et al.,* 2006) and is used in western, alternative and traditional medicine. Honey is made by honey bees, Apis *mellifera* (Blasa *et al.,* 2006), using nectar from plants or honey dew, the latter being a sugar containing substance excreted by some plant sucking insects. It is made up of many components: 70% saccharides, the main sugars being fructose and glucose, 10% water, organic acids, mineral salts, vitamins, proteins and phenolic compounds. The biological properties that make it ideal as a medicine are: antibacterial, bacteriostatic, anti-inflammatory, wound and sunburn healing effects, antioxidant activity, radical scavenging activity and antimicrobial activity (Beretta *et al.,* 2005; Blasa *et al.,* 2007; Gómez-Caravaca *et al.,* 2006; Meda *et al.,* 2005; Ouchemoukh *et al.,* 2007). Many of these effects have been attributed to the polyphenols found in honey. Many studies have been done on the antioxidant activity of European honey and the major flavonoids found include: chrysin, pinocembrin, quercetin, kaempferol, luteolin, and myricetin. Phenolic acids found include caffeic, ferrulic, benzoic and gallic acid (Estevinho *et al.,* 2008; Kaškonienė *et al.,* 2009;

Pyrzynska and Biesaga, 2009; Truchado *et al.,* 2009). When it comes to honeys from Africa only a few studies have been done to identify the polyphenols responsible for antioxidant activity. These studies include honey from Sudan (Makawi *et al.,* 2009), that identified quercetin, kaempferol, apigenin, hesperetin and isorhamnetin as their major flavonoids and honey from Tunisia that identified myricetin and quercetin as the major flavonoids (Martos *et al.,* 1997). Surprisingly very little if any research has been done on the antioxidant properties of honey from the Southern Africa region especially considering the biodiversity of this region where for example in the Western Cape region of South Africa where there are 8920 species of indigenous flowering plants (Cowling, 1983; Goldblatt and Manning, 2002). To address this gap in knowledge of Southern African honeys, the purpose of this study is to undertake an exploratory study into the physicochemical properties, antioxidant activity and biological/cellular effects of a selection of honey samples from this region.

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

Oxidative stress is associated with aging and the development of chronic diseases such as cardiovascular diseases, cancers and diabetes (Milner, 2000; Viuda-Martos *et al.,* 2008). Oxidative stress is caused by free radicals and leads to oxidative damage in biomolecules e.g. DNA, proteins and lipids. It is generally believed that plant and plant-derived foods (e.g. honey) derived phenolics are high in antioxidant content and can either prevent, reduce and/or in some instances treat these diseases. The main areas of antioxidant research are nutritional, medicinal and other applications e.g. cosmetic. Nutritional antioxidant research involves the identification of plant and plant products (phytochemicals) with high antioxidant activities that are common to the diet of a particular population and to study the effects of these phytochemicals on the health of this population. These compounds are known as functional i.e. a food that promotes health, improves general well being and reduces the risk of developing certain diseases (Milner, 2000; Viuda-Martos *et al.,* 2008). Medicinal antioxidant research involves the identification, isolation and characterization of specific phytochemicals with unique structure and biochemical properties which can be used as a possible drug to promote wound healing, as chemotherapeutic agents for the treatment of cancer or as new nutraceutical products that can prevent disease. A central theme is that these compounds effectively reduce oxidative stress thereby having health benefit effects.

2.2 Reactive oxygen species

Reactive oxygen species (ROS) are molecules and free radicals derived from molecular oxygen. Molecular oxygen has two unpaired electrons in the outer shell both with the same spin and are very unstable. Highly reactive singlet oxygen forms if one of the unpaired electrons gains energy and changes its spin. These electrons are highly reactive and as a result are powerful oxidants and may react with other electron pairs especially those found in double bonds. The removal of one electron through a process known as reduction, results in relatively stable intermediates such as the superoxide anion ($O_2^{-\bullet}$) which is the precursor of most ROS (Turrens, 2003). Dismutation of $O_2^{-\bullet}$ either catalysed by superoxide dismutase or spontaneously results in the formation of H_2O_2 which may be reduced to water, either spontaneously or through the action of catalase. H_2O_2 can via the Fenton reaction, with iron as a catalyst form the hydroxyl radical $OH^{\bullet}$. $O_2^{-\bullet}$ may react with other radicals such as nitric oxide (NO) to form peroxynitrite ($NO^{\bullet}$) and like $OH^{\bullet}$ is also a powerful oxidant (Buetnner and Jurkiewicz, 1996; Turrens, 2003).

The types of ROS can be classified according to their longevity (Simon *et al.,* 2000): these are short lived diffusible entities, namely hydroxyl ($OH^{\bullet}$), alkoxyl ($RO^{\bullet}$) and peroxyl ($ROO^{\bullet}$) radicals, medium lifetime entities namely nitroxyl ($NO^{\bullet}$) and superoxide ($O_2^{\bullet}$) radicals and non radicals H_2O_2, organic hydroperoxides (ROOH), and hypochlorous acid (HOCl).

There exists in all cellular systems a steady-state whereby there is a balance between the oxidants that play a role in cell signaling and the rapid removal of these molecules by several antioxidant enzymes. Uncontrolled increase in these molecules due to exposure to pollutants or the effect of disease leads to free radical mediated chain reactions, the driving force being to gain a stable configuration. During this process the molecule with which the free radical reacts itself becomes a free radical and this will continue until two radical forms a stable, inert covalent bond. This cellular process of autoxidation involves in the following reactions. The first reaction involves the reaction with a constituent of the cell membrane, for example linoleic acid (RH), and an initiator, for example H_2O_2 which in the presence of iron (Fenton reaction) forms $OH^{\bullet}$. This results in the formation of the linoleic radical, $R^{\bullet}$, the radical group being on the carbon next to a double bond and H_2O (LH), as summarized below (Buetnner and Jurkiewicz, 1996; Wardman and Candeias, 1996):

$$\mathbf{RH + Initiator\ (L)\ (+\ catalyst) \rightarrow R^{\bullet} + LH}$$

The unstable $R^{\bullet}$ radical then reacts with atmospheric oxygen to form a peroxyl radical $ROO^{\bullet}$.

$$\mathbf{R^{\bullet} + O_2 \rightarrow ROO^{\bullet}}$$

The peroxyl radical of linoleic acid then reacts with another lipid molecule and forms a hydroperoxide, ROOH which then can react with another molecule and subsequently the chain reaction continues.

$$\mathbf{ROO^{\bullet} + R'H \rightarrow R + R'^{\bullet}}$$

Where $R'^{\cdot}$ is any other unsaturated lipid. This process will continue until two radicals combine to form a stable product.

2.3 Oxidative stress

Oxidative stress is defined as the oxidative modification of cellular macromolecules and includes: lipids, proteins, carbohydrates, and nucleic acids which can lead to altered cellular function (Vertuani *et al.,* 2004) leading to the development of chronic diseases such as cardiovascular and Alzheimer's disease and, cancer (Noguchi *et al.,* 2000; Simon *et al.,* 2000).

2.4 Antioxidants

Antioxidants are heterogeneous molecules found in a wide variety of plants, fruits and other sources such as honey. These molecules are organic substances (e.g. vitamin E, β-carotene or Epigallocatechin gallate (EGCG)) that are capable of counteracting the damaging effects of oxidation in animal tissues by acting as radical scavengers that breakdown the radical chain reaction (Huang *et al.,* 2005). Perez *et al.,* (2006), defines antioxidants as any substance that when present at low concentrations compared with those of an oxidizable substrate significantly delays or prevents oxidation of that substrate including various types of molecules *in vivo*.

2.4.1 Types of antioxidants

Antioxidants can be divided into several groups e.g. (Vertuani *et al.,* 2004), according to their nature; enzymatic or non-enzymatic (Table 2.1) or according to their structure; flavonoids or phenolic acids. These groups can further be subdivided either according to their mode of action, chemical origin (natural or synthetic), site of action (hydrophilic/lipophilic compartment) or their mechanism of action (preventative or chain breaking).

Table 2.1: Enzymatic and non-enzymatic antioxidants

Enzymatic	Non-enzymatic
Superoxide dismutase	Antioxidant co-factors (Se, CQ_{10})
Catalase	Oxidative enzyme inhibitors (aspirin, ibuprofen)
Glutathione peroxidase	Transition metal chelators (EDTA)
Thioredoxin	Radical scavengers (Vitamin C and E, EGCG)

2.4.1.1 Enzymatic antioxidants

Examples of enzymatic antioxidants are superoxide dismutase (SOD), catalase and glutathione peroxidase (Table 2.2). These enzymes are found either in the blood or the

cytoplasm and the main function of these enzymes is to neutralize the free radicals such as superoxide anion ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2) (Vertuani *et al.,* 2004).

Table 2.2: Reactions and sources of enzymatic antioxidants

Reaction			Cellular localization
$O_2^{\bullet -}$	Superoxide dismutase (SOD) →	$O_2 + H_2O_2$	SOD1=cytoplasm, SOD2=mitochondria SOD3=extracellular
H_2O_2	Catalase →	$H_2O + O_2$	Perioxsomes
H_2O_2	Glutathione peroxidase (GPx) →	$H_2O + O_2$	GPx1 and GPx4=cytoplasm, GPx2= extracellular, GIT, GPx3=extracellular plasma

2.4.1.2 Non enzymatic/chemical antioxidants

Non enzymatic activity is due to molecules that inhibit oxidative enzymes, serve as co-factors or are free radical scavengers. Antioxidants effects within the cell are complex and usually involve several vitamins, co-factors and antioxidant metals such as selenium. These effects occur either in the cell membrane, cytoplasm or mitochondria. These molecules react in two ways; directly by an intrinsic free radical scavenging mechanism or indirectly participating to the regulation and expression of enzymes (Vertuani *et al.,* 2004). The effects of Vitamin E, Vitamin C, selenium and coenzyme Q_{10} will be discussed in greater detail.

Vitamins exert their antioxidant activity directly by intrinsic free radical scavenging mechanism and/or indirectly as cofactors for enzymes (Vertuani *et al.,* 2004). Most vitamins are free radical scavengers, and play a role in the recycling antioxidant cycle (RAC). The function of these vitamins is summarized in Table 2.3.

Vitamin E (TOC)

Vitamin E (tocopherols and tocotrienols) (TOC) are found in biological membranes where TOC have both antioxidant and non-antioxidant function. The non-antioxidant functions of TOC include: stabilization of membrane structures and modulation of membrane fluidity and membrane permeability to small ions and molecules (Vertuani *et al.,* 2004). Its antioxidant function is to prevent the chain propagation step in lipid (L) autoxidation as shown in Figure 2.1. These antioxidants play an important role in the protection of a cell against oxidative damage as this is usually the first site of oxidative damage. TOC is oxidized to $TOC^{\bullet}$ by

radicals, however it is then recycled back to TOC by ascorbate (whose radical has lesser reactivity when compared to TOC$^{\bullet}$), and/or ubiquinone and/or glutathione.

Table 2.3: Summary of vitamins and their radical scavenging activity (Vertuani *et al.*, 2004).

Vitamin	Function
Retinol (Vit A)	Scavenge ROS by direct mechanism Retinoic acid inhibits NO production through inhibition of iNOS
Tocopherol (Vit E) (lipophilic regions)	Scavenge ROS by direct mechanism Upregulates antioxidant enzymes
Ascorbic acid (Vit C) (hydrophilic regions)	Scavenge ROS by direct mechanism Recycles α-tocopherol, prevents lipid oxidation
Niacin (Vit B3)	Inhibits lipid peroxidation induced by photosensitization Quenches ROS Increases GSH and GST Modulates Inos expression Superior to Vit E and C, and plays a role in the RAC
Riboflavin	Plays a role in the RAC Restores reducing capability of molecules e.g. dihydrolipoate Activates co-factors for glutathione reductase

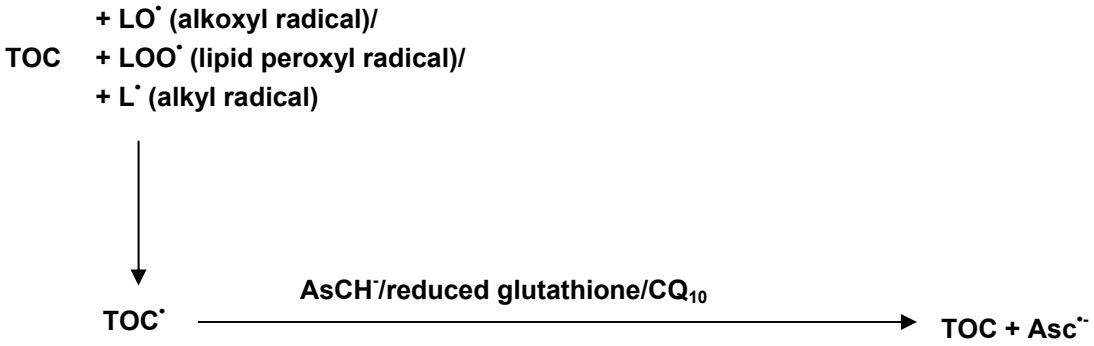


Figure 2.1: Antioxidant activity of Vitamin E and C. Adapted from (Vertuani *et al.,* 2004).

Vitamin C (ascorbate)

Ascorbate is found in high concentrations in fruits and vegetables and is an effective reducing agent.

$$AsCH^{-} + X^{\bullet} \xrightarrow{\text{electron oxidation}} Asc^{\bullet -} + XH \text{ (where X is an oxidizing free radical)}$$

The ascorbate radical is also a relatively unreactive free radical, and therefore can repair other antioxidant radicals. A disadvantage of ascorbate is that in the presence of catalytic metals, radical oxidations are initiated and the process is known as the cross-over effect.

[Low concentrations] $AsCH^-$ + metals (Fe^{3+}, Cu^{2+}) ⟶ less oxidative damage
[High concentrations] $AsCH^-$ + metals (Fe^{3+}, Cu^{2+}) ⟶ more oxidative damage

In summary, at low concentrations of ascorbate in the presence of metals ascorbate acts as an antioxidant, but at high concentrations, in the presence of metal ascorbate will act as a pro-oxidant (Buetnner and Jurkiewicz, 1996). When evaluating the antioxidant activity of plant or fruit extracts which usually have high ascorbic acid content in cell culture, the presence of Fe^{3+} in the cell culture medium can cause this cross over effect causing cell death or have no effect rather than an antioxidative effect.

Selenium and Co enzyme Q10

Selenium is an antioxidant found in extracellular space, the cytosol and in cell membranes. Its function is to preserve structural integrity of the above mentioned structures. In the cell it is the second level of protection against oxidative damage (Vertuani *et al.,* 2004). CQ_{10} also known as ubiquinol is a biological compound widely distributed in plants, animals and most microorganisms and it is present in biomembrane tissues. CQ_{10} acts both as an electron carrier and a proton translocator. Ubiquinol (reduced ubiquinone), acts as an antioxidant against free radical-mediated oxidations in membranes and lipoproteins (Vertuani *et al.,* 2004).

2.4.1.3 Polyphenolic antioxidants

Polyphenols are diversified group of phytochemicals derived from phenylalanine and tyrosine. They are considered as secondary metabolites that are synthesized during normal plant development (Naczk and Shahidi, 2004). Polyphenols are divided into flavonoids and phenolic acids. These molecules occur widely in plant kingdom and occur as secondary metabolites and include simple phenols, phenolic acids (both benzoic and cinnamic acid derivatives), coumarins, flavonoids, stilbenes, hydrolysable and condensed tannins, lignans, and lignins (Blasa *et al.,* 2007; Gomez Caravaca *et al.,* 2006; Naczk and Shahidi, 2004). Sources include fruits, vegetables, nuts, seeds, stems, flowers, tea, wine, chocolate, propolis, honey and medicinal plants. The functions of polyphenols in plants vary. Insoluble phenolic compounds are found in cell walls, whereas the soluble phenolic compounds are found within the plant cell vacuoles. These phenolics in plants have three major functions: contribute to the mechanical strength of the cell wall, regulate plant growth and morphogenesis and finally, protect the plant during stress and pathogen distress (Naczk *et al.,* 2006).

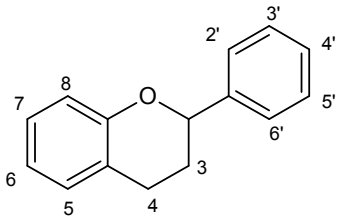
Flavonoids a subgroup of the phenolics can be divided into several classes and these classes as well as their dietary sources are presented in Table 2.4 and the structure thereof is presented in Figure 2.2 (Rice-Evans *et al.,* 1995).

Polyphenols have been shown to be effective free radical scavengers and have more antioxidant properties *in vitro* than tocopherols and ascorbate. These compounds are also reducing agents and scavenge the harmful ROS: $O_2^{\bullet-}$, H_2O_2, OH^-, and O_2^-. Their antioxidant activity arises from their high reactivity as hydrogen electron donors, their ability to stabilize and delocalize the unpaired electron (chain breaking function) as well as their ability to chelate transition metal ions (termination of the Fenton reaction) (Vertuani *et al.,* 2004).

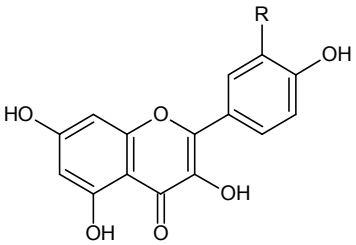
Of the flavonoids, catechin, quercetin and taxifolin have been found to have higher antioxidant activity. Although their basic structure is different, their hydroxylation pattern – 3,5,7,3',4'–OH is similar and this is associated with high antioxidant capacity (Vertuani *et al.,* 2004). Nevertheless, in the presence of Cu^{2+} and O_2, flavonoids and dihydroxycinnamic acids cause impairment of DNA functions via the production of radicals (Vertuani *et al.,* 2004).

Table 2.4: Flavonoid classes and their dietary sources

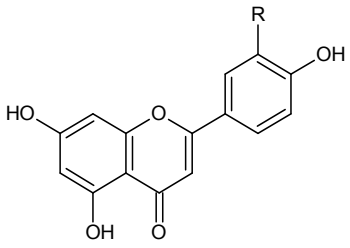
Class	Dietary sources
Flavanol	
Epicatechin Catechin Epigallocatechin Epicatechin gallate Epigallocatechin gallate	Green and black tea Red wine
Flavone	
Chrysin Apigenin	Fruit skin Celery, parsley
Flavanone	
Naringin Taxifolin	Peel of citrus fruits Citrus fruits
Flavonol	
Kaempferol Quercetin Myricetin	Endive, leek, broccoli, radish,grapefruit, black tea Onion, lettuce, broccoli, cranberry, apple skin, berries, olive, Cranberry, red wine, grapes
Anthocyanidins	
Malvidin Cyanidin Apigenin	Red grapes, red wine Cherry, raspberry, strawberry, grapes Coloured fruits and peels



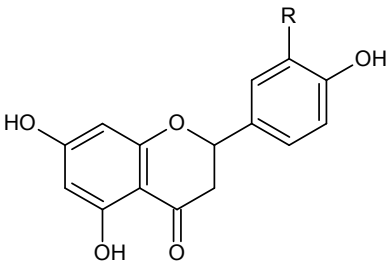
Flavonoid basic structure



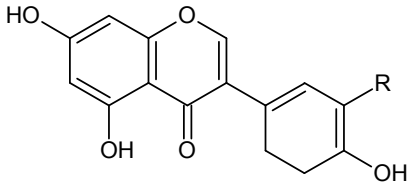
Flavonols (Quercetin, R=OH)



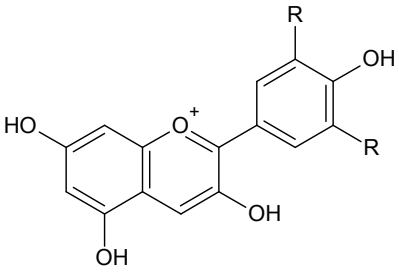
Flavones (Apigenin, R=H)



Flavanones (Naringenin, R=H)



Isoflavones (Genistein, R=H)

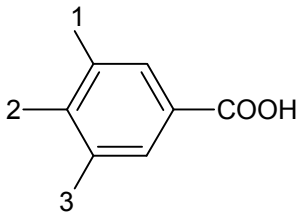


Anthocyanidins (Pelargonidin, R=H)

Figure 2.2: Basic structure of flavonoids and examples of each group

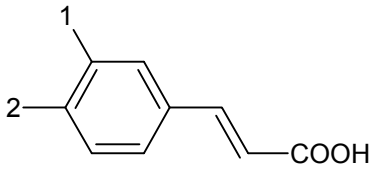
Phenolic acids are classed either as benzoic acid or cinnamic acid derivatives, according to their structure as shown in Figure 2.3.

Benzoic acid derivatives



Phenolic acid	Position		
	C1	C2	C3
Gallic acid	OH	OH	OH
Protocatechuic acid	H	OH	OH
Syringic acid	OCH_3	OH	OCH_3
3-Hydroxybenzoic acid	H	H	OH
Vanillic acid	H	OH	OCH_3

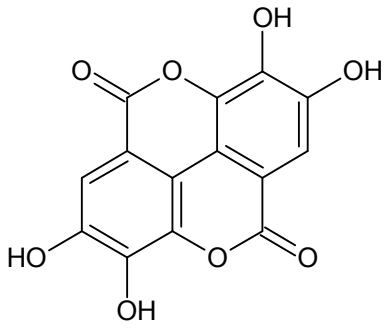
Cinnamic acid derivatives



Phenolic acid	Position	
	C2	C3
Caffeic acid	OH	OH
p-Coumaric acid	H	OH
Cinnamic acid	H	H
Ferulic acid	OCH_3	OH

Others

Ellagic acid



Chlorogenic acid

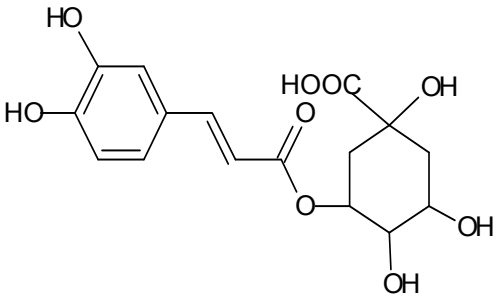


Figure 2.3: Basic structure of phenolic acids (Fukumoto and Mazza, 2000)

2.5 Chronic disease and antioxidants

The development of chronic diseases has a direct relationship with the production of ROS. ROS are generated in the body due to many factors and these include pollution, drugs and ionizing radiation (Blasa *et al.,* 2006). Diseases associated with excessive intracellular oxidative stress include: cardio- and cerebrovascular disease, cancers, as well as the neurogenerative diseases, Parkinson's, and Alzheimer's disease (Silva *et al.,* 2002). In these pathological conditions the ROS defense system does not function effectively due to overproduction of ROS. Oxidative stress leads to degradation of cellular components (DNA,

carbohydrates, proteins, polyunsaturated lipids), which in turn result in irreversible cellular dysfunction causing cell death. Antioxidants can effectively prevent or repair this oxidative damage. The mechanism of how antioxidants prevent or repair damage is summarized in Figure 2.4.

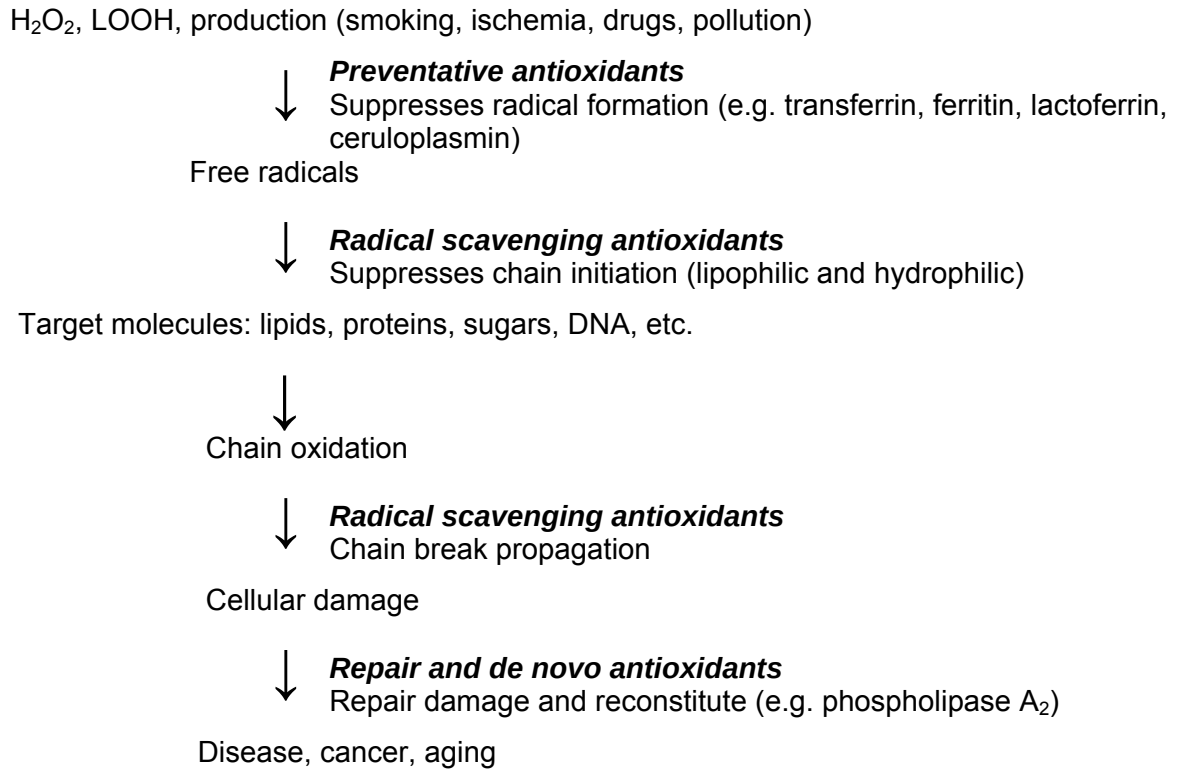


Figure 2.4: Schematic diagram showing the defense systems against oxidative damage induced by active oxygen and free radicals. Adapted from (Bourne and Rice-Evans, 1999; Cohn *et al.,* 1992)

The body deals with ROS using indigenous antioxidants (produced in body) e.g. catalase, reduced glutathione and endogenous antioxidants (from diet) e.g. vitamins, polyphenols in its defense system. This maintains the balance between antioxidant and pro-oxidant activities, which influences the amount of oxidants in the body (Blasa *et al.,* 2006; Dastmalchi *et al.,* 2007; Elisia and Kitts, 2008).

Clinical studies have shown that there is an inverse relationship between the consumption of fruits and vegetables and the risk of developing chronic diseases (Dastmalchi *et al.,* 2007; Silva *et al.,* 2002). Phytochemicals in the fruits and vegetables are responsible for the inhibition of these diseases due to their anti-hepatotoxic, anti-inflammatory, anti-atherogenic, anti-allergic, anti-osteoporotic and anti-cancer effect (Dastmalchi *et al.,* 2007; Silva *et al.,* 2002). Antioxidants present in the diet prevent oxidative damage and the eventual development of disease. This shows the link between phytochemicals and their effects as

radical scavengers of ROS. As a result, inhibition of free radical-induced oxidative damage by supplementation of antioxidants has become an attractive therapeutic strategy for reducing the risk of these diseases (Wei *et al.,* 2006).

The role of antioxidants is complex and each mediates their effect at different levels (Figure 2.4). The enzymes and proteins which reduce hydroperoxides (H_2O_2) and sequester metal ions, respectively, act as preventive antioxidants by suppressing the formation of free radicals. The radical scavenging antioxidants trap free radicals before they attack target molecules by inhibiting chain initiation and/or breaking the chain propagation by scavenging chain carrying peroxyl radicals. Enzymes such as protease and phospolipases repair the damage on proteins, lipids and DNA and the *de novo* enzymes reconstitute the membrane. These antioxidants act independently, cooperatively or synergistically (Bourne & Rice-Evans, 1999; Cohn *et al.,* 1992).

Besides the presence of enzymatic antioxidants such as catalase and reduced glutathione, blood is protected against free radical attack by various radical scavenging antioxidants. These are classified as lipophilic (vitamin E, ubiquinol and carotenoids), hydrophilic (vitamin C, uric acid, albumin and bilirubin) and the thiols which are both lipophilic and hydrophilic (e.g cysteine, methionine, lipoic acid, glutathione) (Held *et al.,* 1996). Hydrophilic antioxidants can scavenge radicals in the aqueous phase but cannot scavenge radicals in the lipophilic compartment. Once aqueous radicals attack lipids, the oxidation proceeds within the lipid layer, or when the oxidation is induced by the lipophilic radicals, the H_2O soluble antioxidants can no longer suppress the oxidation efficiently. H_2O soluble antioxidants function as preventative antioxidants (Cohn *et al.,* 1992). Whereas, Vitamin E (α-tocopherol) an important constituent of cellular membranes of all tissues, act primarily as a lipophilic radical scavenging antioxidant and suppresses the chain initiation and/or chain propagation by donating its phenolic hydrogen to the oxygen radicals. Though primarily lipophilic it also scavenges aqueous radicals by attacking from outside to suppress chain initiation (Cohn *et al.,* 1992). Thiols as mentioned above are both hydrophilic and lipophilic and act as redox sensitive switches, providing a common trigger for ROS and RNS mediated events (Held *et al.,* 1996; Vertuani *et al.,* 2004). These radical scavenging antioxidants are important for the repair of age/degenerative disease-related protein modifications.

Factors affecting bioavailability of antioxidants

Although the ability of antioxidants to prevent oxidative damage is well described, several other factors can affect the antioxidant activity positively or negatively. These factors include:

processing, digestion and absorption across mucosa of the gastrointestinal tract (GIT). Extensive heat processing of food causes the destruction of ascorbic acid while other antioxidants remain intact (Bourne and Rice-Evans, 1999). Likewise, some polyphenolics can be destroyed by the low pH and the effects of the digestive enzymes within the GIT. Alternatively, digestive enzymes may cause the release of polyphenolics from the food matrix. Some antioxidants such as ascorbic acid are absorbed across the GIT mucosa while others are not bioavailable. The lack of bioavailability is a major criticism of antioxidant studies. Those that are not absorbed still fulfill an important function by directly protecting the GIT mucosal membrane against oxidative damage such as that generated by the microflora of the GIT (Bourne and Rice-Evans, 1999; Rice-Evans *et al.,* 1995).

In contrast to the GIT, the skin is a natural barrier to the environment and the effect of antioxidants on the intact skin surface is limited. However when the skin surface is damaged, products or wound dressings that contain antioxidants, promote wound healing (Schäfer and Werner, 2008). Due to the lack of heat processing and the effects of digestion and pH both enzymatic and non-enzymatic antioxidant components can promote wound healing.

2.6 Honey

The presence of many constituents with varied properties that are both nutritional and medicinal has resulted in the classification of honey as a functional food (Gómez-Caravaca *et al.,* 2006). Unlike other foods that lay claim to one or two sources of antioxidants e.g. onions (quercetin), broccoli (kaempferol), oranges (hesperidin) (Rice-Evans *et al.,* 1997) honey contains a mixture of many types of antioxidants e.g carotenes, phenolic compounds, Maillard reaction products (MRP's), ascorbic acid and catalase activity, making it an exceptional functional food.

Honey, produced by honey bees, *Apis Mellifera*, is made from two sources: nectar of plants (nectar honey) and secretions of living parts of plants, or excretions of plant sucking insects (honey dew honey). Its composition is influenced by plant species, climate, environmental conditions and contribution of the bee keeper. Honeys consist of 70% saccharides, mainly fructose and glucose. The proportion of fructose to glucose in honeys is indicative of honeys granulation and is dependent on nectar source (Finola *et al.,* 2007). Ten percent is water, organic acids, mineral salts, vitamins, proteins, phenolic compounds. One percent of honey is free amino acids with proline being the predominant amino acid (Ouchemoukh et al. 2007). Other components are the products from the Maillard reaction (Bertoncelj *et al.,* 2007; Blasa *et al.,* 2006) which form when sugars condense with free amino acids, leading to the

formation of a variety of brown pigments, the Maillard reaction products (MRPs). These products are non-nutrient antioxidants (Turkmen *et al.,* 2006).

The polyphenol compounds are of particular interest as these mostly contribute to the antioxidant activity of honey. Flavonoids found in honey include chrysin, pinocembrin, pinobanksin, quercetin, kaempferol, luteolin, galangin, apigenin, hesperetin, and myricetin. Phenolic acids include caffeic, coumaric, ferrulic, ellagic, chlorogenic acid (Blasa *et al.,* 2007). Beside their medicinal properties, the antioxidant content of honey makes it ideal for many other uses, such as a preservative as it retards deterioration, rancidity and discoloration of food (Meda *et al.,* 2005). The structure of polyphenols found in honey is presented in Figure 2.5 (Estevinho *et al.,* 2008; Kaškonienė *et al.,* 2009; Pyrzynska and Biesaga, 2009; Truchado *et al.,* 2009).

2.6.1 Medicinal/therapeutic effects of honey

Honey has been used in ethno medicine since early times in the treatment of burns, GIT disorders, asthma, infected wounds and skin ulcers. Scientific studies have confirmed the anti-inflammatory, anti-microbial, anti-allergic, anti-oxidant, vascular and cytotoxic anti-tumour activity of honey (Beretta *et al.,* 2005; Blasa *et al.,* 2007; Ouchemoukh *et al.,* 2007).

2.6.1.1 Honey in wound healing

Skin acts as a natural barrier against the environment and when insults and injuries occur a multi-step process begins, which includes inflammation. With the inflammatory response inflammatory factors kinins, prostaglandins and histamine are released and through a chemotaxic process macrophages and neutrophils enter the wound site. This results in the release of proteolytic and pro-inflammatory enzymes, and ROS. ROS at low concentrations act as a defense against invading pathogens, mediates intracellular signaling, and are efficient for wound angiogenesis. However, at high concentrations ROS are harmful and result in the formation of radical such as H_2O_2, as shown in Figure 2.6 (Schäfer and Werner, 2008).

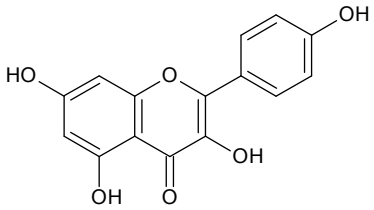
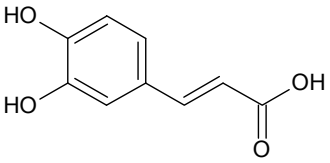
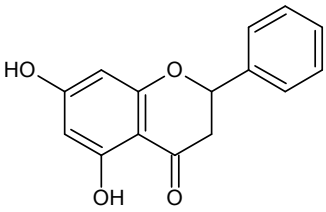
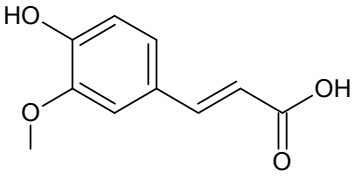
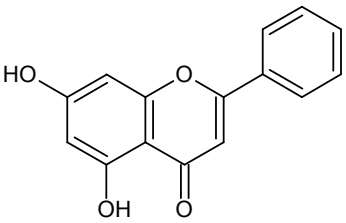
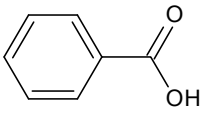
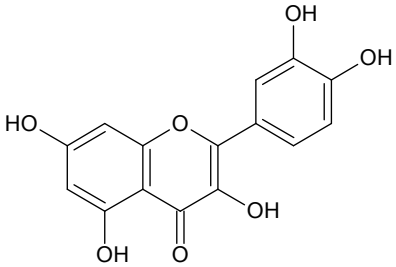
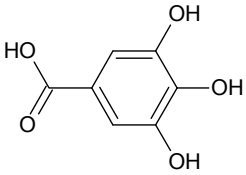
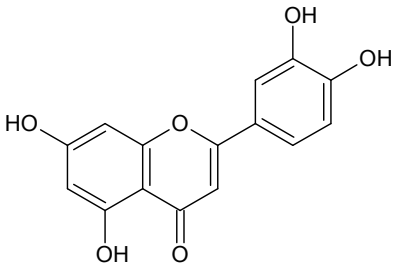
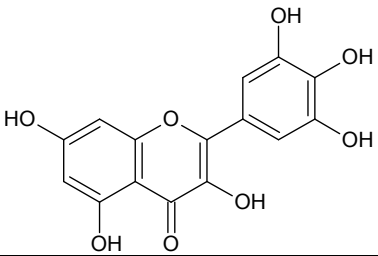
Flavonoids	Phenolic acids
Kaempferol 	**Caffeic acid** 
Pinocembrin 	**Ferrulic acid** 
Chrysin 	**Benzoic acid** 
Quercetin 	**Gallic acid** 
Luteolin 	
Myrecitin 	

Figure 2.5: Most common flavonoids and phenolic acids found in European honeys. (Estevinho *et al.,* 2008; Kaškonienė *et al.,* 2009; Pyrzynska and Biesaga, 2009; Truchado *et al.,* 2009)

Honey used in wound dressing either produces radicals (H_2O_2) at low concentrations, to fight invading pathogens, or due the presence of the enzyme catalase converts excess H_2O_2 to water and oxygen, preventing the formation of free radicals. Secondly, the availability of polyphenolic antioxidants in honey detoxify lipid peroxides and interfers with the Fenton reaction, as shown in Figure 2.6. Furthermore, the acidic pH of honey serves an antiseptic function. All these benefits of honey make it ideal for wound repair and therefore ideal for treatment of burns and specifically wound dressings.

Honey has been described to have antibacterial effects and this also promotes wound healing. Molan, (1999), reported on infected wounds treated with honey and observed complete inhibition of a collection of strains of Methicillin-resistant *Staphylococcus aureus* (MRSA) and sterile wounds within 3 - 6 days when 1 - 4% (v/v) honey was used. When a 2 - 4% (v/v) honey was used, there was complete inhibition of 58 strains of coagulase positive *staph aureus* and wounds were sterile within 7 days. When a 5.5 - 8.7% v/v honey used, there was complete inhibition of 20 strains of pseudomonas and wounds were sterile within 7-10 days. The high osmolarity of honey leads to inhibition of microbial growth through sugar molecules 'tying up' water molecules so that bacteria have insufficient water to support their growth. Even when honey is diluted down to an extent that osmolarity is reduced to a level that ceases to inhibit bacterial growth, honeys additional antibacterial components still ensure sterility. This is due to the presence of H_2O_2 as well as the presence of polyphenolic compounds. Honey contains both catalase (EC 1.11.1.6), which is a peroxidase that converts H_2O_2 to H_2O and O_2 and glucose oxidase (GOD, β-D- glucose:oxygen-1-oxidoreductase EC 1.13.4) which is an oxido-reductase that catalyses the oxidation of glucose to H_2O_2 and D-glucono-δ-lactone. The rate at which catalase, calatyses the conversion of H_2O_2 produced by glucose oxidase to H_2O and O_2 will be a function of factors such as substrate concentration, density due to high sugar content and pH. As H_2O_2 accumulates due to wound derived inflammation the forward reaction catalysed by catalase with the production of H_2O and O_2 is favoured without subsequent tissue damage that can occur at high H_2O_2 concentrations which includes lipid peroxidation, DNA and protein modification that can lead to cellular dysfunction or death (Molan, 1999).

Other effects of honey are its deodorizing effect (Molan, 1999) whereby malodours that are caused by the bacterial metabolism of amino acids into ammonia, amines, and sulphur compounds. Honey has a high glucose content which is used by infecting bacteria in preference to amino acids resulting in the formation of lactic acids as opposed to the malodorous products. Honey also has a debriding action which removes debris from wounds. This occurs via an osmotic action which causes an outflow of lymph and wound

fluid which causes the debris from the wound bed to lift. A further benefit is that the wound remains moist which prevents the tearing away of newly formed tissue when the dressing is removed (Molan, 2001). Furthermore, honey has been found to stimulate the growth of new tissue growth and this includes the formation of new healthy granulation tissue and epithelium. Low concentrations of H_2O_2 stimulate angiogenesis and the growth of fibroblasts which results in increased collagen production. Angiogenesis then provides more oxygen and nutrients to the wound site and tissue regeneration can occur.

2.6.1.2 Honey and the gastrointestinal tract

Nasuti *et al.,* (2006) evaluated the effects of two formulations that contain 86% or 96% honey in indomethacin treated rats, the results indicated a significant decrease in the ulcer index, microvascular permeability and myeloperoxidase activity (a marker of leukocyte infiltration). Honey has also been investigated as a possible alternative therapy that can contribute to the eradication of *Helicobacter pylori.* Manyi-Loh *et al.,* (2010) investigated the ability of extracts of three South African honeys to reduce the minimum inhibitory concentration (MIC) of *Helicobacter pylori.* These authors found that at a 75% (v/v) was the most active and chloroform extracts of these honeys were the most active in inhibiting activity, in comparison to methanol extracts. Further evaluation of the active ingredient that mediates this effect must still be identified. Furthermore factors such as pH and effect of proteolytic enzymes on activity of honey must still be investigated, although Nasuti *et al.,* (2006) observed beneficial effects in rats at the low pH of the stomach.

2.6.1.3 Honey and its effect on inflammation

Besides the anti-inflammatory effect in the stomach reported by Nasuti *et al.,* (2006) several other studies have been undertaken. Studies have shown that honey has many benefits *in vitro*. Molan, (2001) observed honey helped to clear infections by boosting the human immune system. It has also been shown to stimulate B and T lymphocytes, activate neutrophils, and stimulate monocytes to release cytokines TNF-a, 1 and IL-6 (messengers that activate many of the immunue responses to infection).

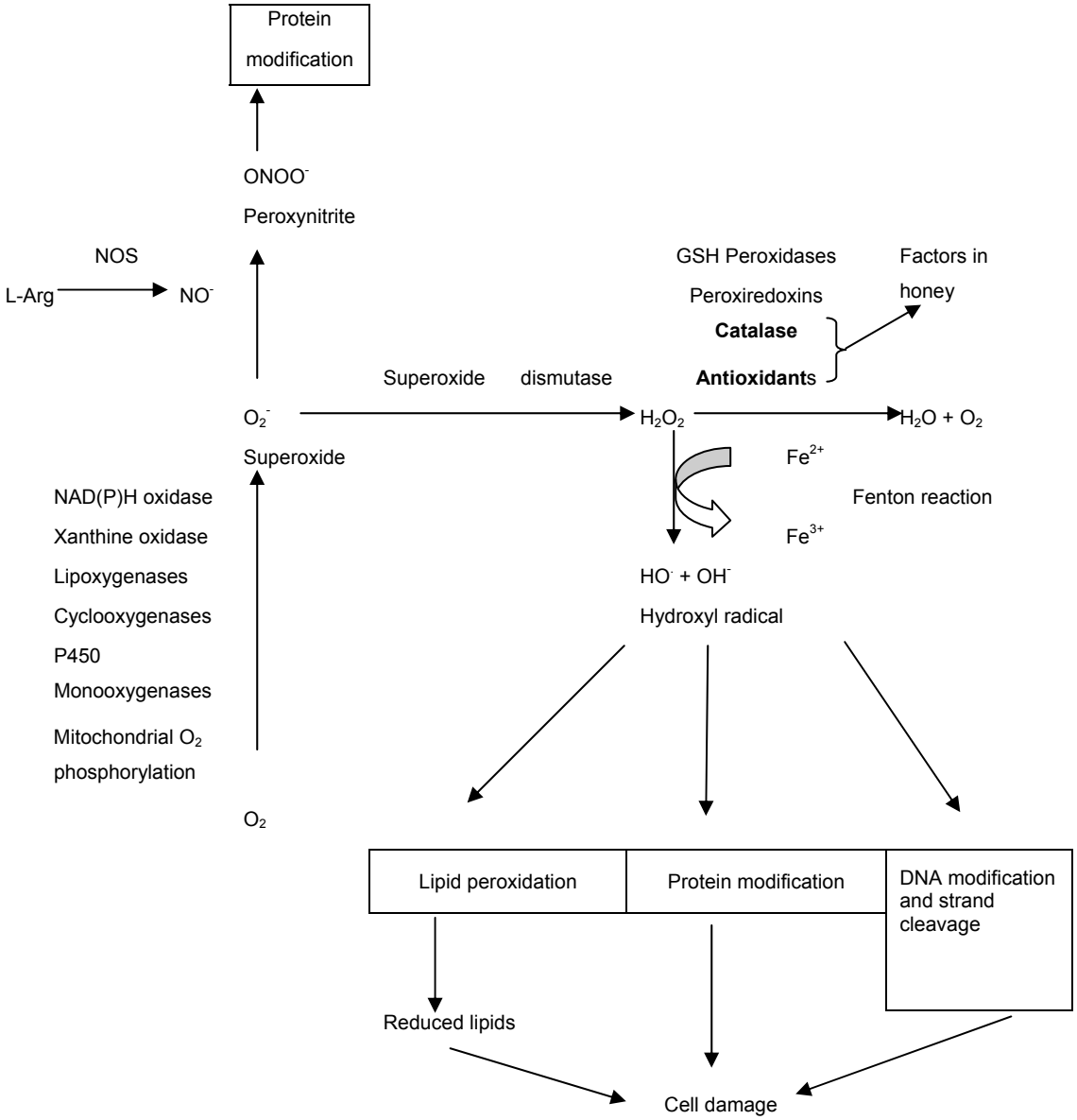


Figure 2.6: Different ROS produced during skin injury, the effect of inherent and honey derived enzymatic activity as well as the effect of polyphenols derived from honey for wound healing. Figure adapted from Schäfer and Werner, (2008).

Kassim *et al.,* (2010) have demonstrated that methanol and ethyl acetate extracts of ellagic acid and flavonoids in Malaysian honey were found to significantly reduce tumour necrosis factor (TNF) toxicity in the murine fibrosarcoma fibroblast L929 cell line. Of these two extracts the ethyl acetate extract effectively reduced NO production in lipopolysaccharide (LPS) stimulated RAW264.7 murine macrophages. The authors assume that the inhibition of the inflammatory process is due to the presence of quercetin, caffeic acid, chrysin and ellagic acid, of which all are polyphenols that have been shown in other studies to have anti-inflammatory effect (Kassim *et al.,* 2010).

2.6.1.4 Honey and its effect on cellular proliferation

Low concentrations of honey promote tissue growth, while higher concentrations result in a cytotoxic effect on growth. Kannan *et al.,* (2009) observed in a study done using Tualang honey that at concentrations greater than 0.8% there was significant inhibition of human osteoblast cell line, CRL 1543 using medium supplemented with 10%, 5% and 2% fetal bovine serum. This has lead to the identification of specific constituents in honey that can be used in anti-cancer therapy. Li *et al.,* (2011) reported that chrysin a flavonoid present in honey increased the sensitivity of human carcinoma cell lines: colorectal cells HCT-116 (colorectal), Hela (cervical) and Hep2 (liver) to the cytotoxic effects of TNF-related apoptosis-inducing ligand (TRAIL). TRAIL induces apoptosis in a wide range of tumour cells without having an apoptotic effect on normal cells. This effect is caspase dependent and is due to the activation of caspase 8. Chrysin inhibits drug metabolizing enzymes namely CYP1A1 and P-form phenolsulfotransferase which are both involved in carcinogen bioactivation. Although chrysin is absorbed across the GIT mucosa, the rapid gluconidation and sulfation found in the human adenocarinoma colonic Caco-2 may limit its ability to prevent carcinogen bioactivation or the killing of cancer cells. Fauzi *et al.,* (2011) studied the effect of 0 - 10% honey on the MDA-MB-231, MCF-7 (both human breast adenocarcinoma), Hela (cervical carcinoma) and MCF-10A (normal human mammary) cell lines and found that Tualang honey had a EC_{50} of 2.4 - 2.8% for the cancer cell lines but no killing was observed in the MCF-10A cell line. This effect on the cancer cell lines occurred via apoptosis. Tsiapara *et al.,* (2009) observed variable effects using ethyl acetate extracts of Greek honey. The same study found that Thyme honey reduced the viability of Ishikawa and PC-3 cells but stimulated the viability of MCF-7 cells.

In an *in vitro* (human T24, 253J and murine RT4 bladder cancer cell lines) and an *in vivo* murine animal model, honey at a concentration of 6% effectively reduced the survival rates of all cell lines and the average tumour volume in the animal studies. Jaganathan *et al.,* (2010) after showing that honey induced apoptosis in colon cancer cell lines investigated the effect of a honey sample with a high phenolic content and eugenol (a polyphenolic constituent of honey) on the growth of Ehrlich and solid ascite tumours that are mammary murine adenocarcinoma in origin. A 25% (v/v) honey solution and 80 - 125mg/kg eugenol intraperitoneal injection for 9 days resulted in a significant inhibition of growth for both tumour types.

2.6.1.5 Honey and the vascular system

Honey has been shown to suppress human low density lipoprotein (LDL) peroxidation by the superoxide anion radical, which offsets inflammation, cancers and atherosclerosis. LDL oxidation is an early event in atherosclerosis, and flavonoids are known to inhibit LDL oxidation through metal chelation and free radical scavenging mechanisms (Hegazi *et al.,* 2007). Beretta *et al.,* (2007) observed that a fortified fraction of native honey effectively protected endothelial cell cultures (EA.hy926) against H_2O_2- and AAPH- induced oxidative damage. By lowering the free radical concentration in serum, preventing the oxidation of LDL and maintaining the integrity of the endothelium antioxidants such as those found in honey may significantly lower and reduce the risk for the development of cardiovascular disease, although no animal or clinical studies involving the use of honey have been found.

2.6.1.6 Honey and its antioxidant effect

The antioxidant content and activity of honey from many regions of the world has been described. Many of the phenolic acids and flavonoids found in these honeys have been identified, as shown in Figure 2.5 (Estevinho *et al.,* 2008; Kaškonienė *et al.,* 2009; Pyrzynska and Biesaga, 2009; Truchado *et al.,* 2009), but only few studies involving the identification of polyphenols in honeys from Africa have been undertaken. These include honeys from Sudan (Makawi *et al.,* 2009), major flavonoids found include: quercetin, kaempferol, apigenin, hesperetin and isorhamnetin and honeys from Tunisia (Martos *et al.,* 1997), major flavonoids found include: myricetin and quercetin. The studies of African honeys are mainly on antioxidant content and activity, these include honeys from: Algeria (Ouchemoukh *et al.,* 2007), Burkina Faso (Meda *et al.,* 2005), Cameroon (Joseph *et al.,* 2007) and Morocco (Malika *et al.,* 2005). Most information available is related to the antioxidant effects on wound healing.

Some authors have evaluated the ability of honey or honey derived polyphenols on membrane oxidation and DNA oxidation (Beretta *et al.,* 2007; Viuda-Martos *et al.,* 2008), however, almost no studies could be found that studied the effect of these antioxidants in cell culture, therefore, the purpose of this study is to address the following gaps found in literature; determine the antioxidant activity of honey from the Southern Africa region and determine these antioxidant effects *in vitro*/cell culture. No studies have been undertaken to determine the anti-oxidant activity of honey from Southern Africa.

2.7 Aims

Southern Africa is a region of unique floral biodiversity and little is known regarding the antioxidant content and activity of the honeys from this region. The physiochemical properties, the antioxidant content and activity of honeys collected from this region will be determined. Furthermore the ability of these honeys to protect cellular macromolecules such as DNA and the erythrocyte membrane will be investigated. This will be followed by further evaluation of antioxidant activity of these honeys in cell culture. To achieve this, all methodologies required for the determination of honey quality, total phenol and flavonoid content as well as the chemical, biological and cellular assays that will be required for this study will be established.

The aims of this study were therefore to:

1. Establish all methodologies to determine: physicochemical properties, antioxidant activity and cellular oxidative effects of the honeys.
2. Collect 13 honeys from different regions of Southern Africa during 2008 and 2009.
3. To determine if these honeys fulfill the regulatory standards compiled in a Codex Alimentarius Standard in terms of quality. (CODEX STAN12-1981 revision 2001)
4. Determine protein and the enzymatic antioxidant content specifically catalase activity in all honeys.
5. Determine non-enzymatic antioxidant content by measuring the total polyphenolic content (TPC) and total flavonoid content (TFC) of all honeys.
6. Determine the correlation between the colour of the honeys and their TPC or TFC.
7. Determine antioxidant activity using the 2,2-diphenyl-2-picrylhydrazyl (DPPH), Trolox equivalent antioxidant capacity (TEAC) and oxygen radical antioxidant capacity (ORAC) assays.
8. Calculate the Relative Antioxidant Capacity Index (RACI) using data generated from the DPPH, TEAC and ORAC assays.
9. Determine the ability of all honeys to protect of plasmid DNA and erythrocytes against 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH)-induced oxidative damage.
10. Determine the effect of all honeys at 0.008% and 0.8% on the cellular growth of SC-1 (mouse fibroblast) cell line, using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and combined Neutral red (NR)/Crystal violet (CV) assays.

11. Determine the protective effects of all honeys at 0.025% and 2.5% against the oxidative damage on the SC-1 (mouse fibroblast) and Caco-2 (human colonic adenorcarcinoma) cell line, using the 2',7'-dichlorofluorescin diacetate (DCFH-DA) assay.
12. Determine if there is signification correlation between the assays used to measure antioxidant activity and the biological and cellular assays.

Finding's of aim 1 – 6 are presented in Chapter 3, aims 7 - 9 in Chapter 4 and lastly aims 9 - 12 in Chapter 5.

2.8 Hypotheses

Hypothesis 1: Honey collected fulfill the regulatory standards compiled in a Codex Alimentarius Standard (CODEX STAN12-1981 revision 2001). Furthermore honeys that are dark in colour (high absorbance at 450 nm) have a high total polyphenol content (TPC) and total flavonoid content (TFC).

Hypothesis 2: Darker honeys with high TPC and TFC are associated with high antioxidant activity measured using the chemical based assays, in addition darker honeys with high TPC and TFC are also associated with high antioxidant activity measured using the biological assays.

Hypothesis 3: Honeys with high antioxidant activity and the ability to protect plasmid DNA and the erythrocyte membrane against oxidative damage will also be able to protect the SC-1 and Caco-2 cell lines against AAPH-induced oxidative damage.

Hypothesis 4: There is a significant positive correlation between all parameters measured.

CHAPTER 3: THE PHYSICOCHEMICAL PROPERTIES AND ANTIOXIDANT CONTENT OF A SELECTION OF SOUTHERN AFRICAN HONEYS

3.1 Introduction

The physicochemical properties of honey are dependent on its physical and chemical compositions, and these properties are used to determine its quality (Silva *et al.,* 2009). The quality of honey is mainly determined by sensory, chemical, physical and microbiological characteristics. Globally, honey quality criteria are specified in regulatory standards compiled in a Codex Alimentarius Standard (CODEX STAN12-1981 revision 2001). The Codex Alimentarius Standard for honey quality include the following chemical and physical parameters: acidity (pH), moisture content, mineral content, acidity, hydromethylfurfural (HMF) content, diastase activity, sugar content, and water insoluble solids content (Finola *et al.,* 2007).

The pH of honey is attributed to the presence of organic acids (such as gluconic acid and inorganic ions such as phosphate and chloride) (Ahmed *et al.,* 2007). pH influences the texture, stability and shelf life of honey and is also a useful index of microbial growth. The percentage moisture depends on climatic factors and harvesting season and is carefully regulated in order to prevent fermentation. The ash content indicates the botanical origin of the honey whether it is floral, honeydew or a mixture thereof (Silva *et al.,* 2009). Factors that contribute to ash content are climate and the soil composition of the area where the nectar producing plant is located (Finola *et al.,* 2007).

One of honey's components includes 70% saccharides, the main sugars being fructose and glucose. This ratio is dependent on the nectar source and is indicative of honeys tendency to granulate. A low ratio is usually found in honeys that easily granulate (Ouchemoukh *et al.,* 2007; Finola *et al.,* 2007).

Honey also contains amino acids and proteins including enzymes. Proline is the predominant amino acid and is derived from salivate secretions of the honey bee during the conversion of nectar into honey (Ouchemoukh *et al.,* 2007). It indicates the total amino acid content, honey quality and maturity. Together with the percentage moisture content, proline content can be used to detect sugar adulteration. Honey contains 0.5% protein and this includes major jelly protein 1 and enzymes such as α-glucosidase (invertase), α- and β-amylase (diastase), glucose oxidase, catalase and acid phosphotase. The enzymes present in honey are mostly responsible for the conversion of nectar and honeydew to honey and

these are: α-glucosidase (invertase), α- and β-amylase (diastase), glucose oxidase, catalase and acid phosphotase. Enzymes are sensitive to heat; excessive heat causes denaturation and enzyme deactivation. The measurement of α- and β-amylase also known as diastase activity is used to determine whether the honey has been heated extensively (Meda *et al.,* 2005; Silva *et al.,* 2009).

Enzymatic and non-enzymatic antioxidants are the main contributors to antioxidant activity of honey, especially due to the presence of catalase and polyphenols. The function of catalase within biological systems is to protect against oxidative damage induced by H_2O_2. Catalase catalyzes the conversion of H_2O_2 to H_2O (Ou and Wolff, 1996). The colour of honey is an indicator of the presence of phenolic compounds. Generally, the darker the honey the more the quantity of phenolic compounds available (Silva *et al.,* 2009). The colour of honey also partly reflects the content of pigments such as carotenoids, polyphenols and ash content (Beretta *et al.,* 2005). The most common polyphenols found in honey are flavonoids (chrysin, pinocembrin, pinobanksin, quercetin, kaempferol, luteolin, galangin, apigenin, hesperetin, myricetin) and phenolic acids (caffeic, coumaric, ferrulic, ellagic, and chlorogenic acid) (Bertoncelj *et al.,* 2007). The antioxidant activity found in honey is also dependent on the geographical location, season and nectar source. Southern Africa has a unique floral biodiversity and this should be reflected in the honeys of this region. No studies have been undertaken regarding the antioxidant content and activities of honeys from this region. The main objective of the research presented in this chapter is to determine the physiochemical properties of a selection of honeys collected with this region. Secondly, to determine whether darker honeys have a high total polyphenolic and flavonoid content.

The specific objectives of this chapter are:

1. To collect honeys that are representative of regions of Southern Africa with unique floral biodiversity, as well as regions with an established floral agricultural background.
2. To determine if the honeys collected fulfill the Codex Alimentarius Standard for honey quality.
3. To measure the physicochemical properties as well as the catalase activity, the total polyphenol (TPC) and flavonoid (TFC) content of honeys collected within the Southern Africa region.
4. Finally, to determine if there is a significant correlation between all parameters measured.

3.2 Materials

Honey samples

Thirteen samples representative of unique floral regions of Southern Africa were collected from 2008-2009. These were from the Western Cape (WC), WCa, WCb and WCc, Eastern Cape (EC) ECa, ECb, and ECc, South Eastern Mozambique (SEM) SEMa, SEMb, and SEMc and the Agricultural honeys (A) A-E1and A-E2 (eucalyptus) and Agric-L (litchi) and A-O (orange).

Reagents, equipment and disposable plasticware

Potassium ferricyanide ($K_3Fe[CN]_6$), sodium hydroxide (NaOH), sodium phosphate dibasic dihydrate ($Na_2HPO_4 \cdot 2H_2O$), sodium phosphate (NaH_2PO_4), sodium chloride (NaCl), ninhydrin, proline, glucose (Glc), and fructose (Fru) were of analytical quality and were obtained from Merck Chemicals, Modderfontein South Africa (SA). Coomasie Blue, bovine serum albumin, L(-)proline, ninhydrin, hydrogen peroxide, potato starch, Folin-Ciocalteu's reagent, sodium carbonate anhydrous (Na_2CO_3), gallic acid and catechin were obtained from the Sigma-Aldrich Company, Atlasville, SA.

Equipment used included: Lambda LS5OB spectrophotometer from Perkin Elmer, Boston, MA, USA supplied by Separations Scientific, Honeydew, SA, a BioTek plate reader purchased from Analytical and Diagnostic Products (ADP) Johannesburg, SA was used. Freeze dry system LABCONCO from ALCATEL CIT, France and a Weiss-Gallenkamp Loughborough, United Kingdom muffle furnace supplied by Labotec, Midrand, S.A, was also used. A Hermle Z300 centrifuge, a Crison GLP 21 pH Meter and Eppendorf pipettes from Eppendorf AG Hamburg, Germany were all supplied by the Scientific Laboratory Equipment Company (LASEC), Cape Town, SA.

Disposable plasticware included: disposable plasticware included: 96 well plates, 50 ml, 15 ml tubes and pipette tips (10, 25, 100, 200, and 1000 µl) which were obtained from Greiner Bio-one also supplied by LASEC.

Laboratory facilities

All research was conducted in the research facilities of the Departments of Anatomy and Pharmacology of the Faculty of Health Sciences, University of Pretoria.

3.3 Methods

Sample preparation

Honey samples were obtained during 2008 to 2009 either from established honey farmers or from rural farm stalls. The honeys were transported to the laboratory and were stored at 4^0C in the dark. For all experiments a 50% stock honey solution was prepared. Each sample was filtered through a Whatman 1 filter paper to remove any solid material and then volumes of 5 ml were stored at -20^0C in the dark.

3.3.1 Determination of physicochemical properties

pH

The pH of a 10% (w/v) solution of honey in ddH_20 was determined with a pH meter.

Moisture content

To determine the percentage moisture, a volume of honey was weighed (mass before freeze drying, (MBF)). The sample was freeze dried for 4 days and then the mass was determined again (mass after freeze drying (MAF)). The percentage moisture was calculated as follows:

% Moisture = (MBF-MAF/MBF) X 100

Ash content

The percentage ash was determined by ashing 5g of honey (mass before ashing (MBA)) at 550ºC for 5 h in a muffle furnace (Finola *et al.,* 2007). The mass of the remaining material after ashing was measured (mass after ashing (MAA)) and the percentage ash was calculated as follows:

%Ash = (MBA-MAA/MBA) X 100

Saccharides

The Perkins test is a reducing sugar test that uses potassium ferricyanide as its oxidant. Glucose (Glc) and fructose (Fru) differ in the rate at which these monosaccharides cause the discolouration of a basic potassium ferricyanide solution. Potassium ferricyanide in the

presence of sodium carbonate oxidizes reducing material (principally sugars) in the extract and, in turn, is reduced to potassium ferrocyanide ($K_4Fe[CN]_6$) (Pandita *et al.,* 2007; Brushwood, 2000). From this a spectrophotometric method was developed and this was adapted to a 96 well format and was used to determine the ratio of Glc and Fru in honey. A stock solution of potassium ferricyanide was prepared by dissolving 1g potassium ferricyanide in 20% aqueous NaOH. A working solution was prepared by diluting the stock solution 1:10. Stock solutions each of 1g/100ml, 55 mM of Glc and Fru in ddH_2O was prepared and working solutions each of 5.5 mM were prepared before use.

In separate experiments the rate at which 100 µl Glc and 100 µl Fru causes the discolouration of 1.5 ml of the potassium ferricyanide working solution at 405 nm was determined. Measurements were against 1.5 ml of the potassium ferricyanide working solution and 100 µl ddH_2O. Complete discolouration was observed after 5 and 30 min for Fru and Glc, respectively. No further discolouration was observed for Glc after 5 min and no further discolouration of Fru occurred after 30 min. Therefore, from the absorbance readings 0 min to 5 min the concentration of Fru and after 30 min the concentration of Frc and Glc as well as the ratio of Fru:Glc could be determined.

This method was then adapted to a 96 multiwell format where to 25 µl volumes of 0.01% solutions of honey, 150 µl of the ferricyanide working solution was added. Absorbance was measured at 405 nm at 0 min to 5 min after adding the ferricyanide working solution the plate was then covered and placed in the dark for 25 min before absorbance measurements were taken. Controls contained 150 µl of the ferricyanide working solution and 25 µl ddH_2O. The method was validated using Glc and Fru solution with known ratios. The ratio of Fru:Glc was calculated as follows.

$$\textbf{Ratio Glc:Fru} = A_{0min}-A_{5min} : (A_{0min}-A_{30min})-(A_{0min}-A_{5min})$$

Proline content

The proline content was determined using a modified method of Meda *et al.,* (2005). A stock solution of 1mg/ml proline in ddH_2O was prepared. Using this stock solution a 0 - 0.2 mg/ml proline standard curve was prepared. For the determination of proline content of the honeys, 50 µl of a 10% (w/v) honey solution was added to the wells of a 96 well plate. To the standards and honey samples 100 µl of a 0.33g/100ml ninhydrin prepared in 2% acetic acid was added. The samples were mixed well and the absorbance was measured at 450 nm.

Protein content

Optical density

Protein was determined by measuring the optical density (OD) and the Bradford method (BF). The OD of a 10% w/v honey solution versus a suitable control (solvent blank) was measured at 280 nm and 205 nm using a Perkin Elmer spectrophotometer. Protein concentration was calculated using the following formula:

$$\textbf{Protein concentration (in mg/ml)} = A_{205}/(27 + A_{280}/A_{205}))$$

Bradford method

Bovine serum albumin (BSA) was used to prepare a stock solution of 50 µg/ml and this was used to prepare a standard curve of 0 - 4.33 µg/ml. A 10 µl volume of a 10% (w/v) honey solution was added to the wells of a 96 - well plate. Then 100 µl of Coomassie Blue (100 mg Coomassie Brilliant Blue mixed with 50 ml, 95% ethanol and 100 ml concentrated phosphoric acid made to a final volume of 200 ml with ddH_2O) solution was added. The samples were mixed well and the absorbance was measured at 630 nm.

Diastase activity

Diastase activity was measured using a 1% potato starch in a phosphate buffered solution (PBS) (0.2M Na_2HPO_4, 0.2M $NaH_2PO_4 \cdot H_2O$, 0.15M NaCl, pH=7.4). In a cuvette, 900 µl of 2% (w/v) honey solution, 900 µl of PBS and 900 µl of 1% starch were added together and then mixed well. The blank cuvette contained 900 µl of PBS, 900 µl of 1% starch and 900 µl ddH_2O. Every 6 min the absorbance was measured at 235 nm. The rate at which absorbance decreased over time was evaluated and from this data, diastase activity was calculated and expressed in Schade units.

Colour/absorbance

The colour of honey is due to the presence of Maillard reaction products (MRPs) and polyphenols. At 560 - 720 nm strong absorbance is due to the presence of MRPs while at 420 – 450 nm absorbance is due to the presence of polyphenols (Brudzynski and Miotto, 2011). Absorbance is therefore defined as the difference between the absorbance readings

at 450 nm and 720 nm and is expressed as milli-Absorbance units (mAU) (Beretta *et al.,* 2005).

3.3.2 Determination of enzymatic antioxidant content

Catalase activity

Catalase activity is proportional to the rate of H_2O_2 degradation which was monitored at 240 nm. A concentration of 20 mg/ml catalase (from beef liver, Boehringer Mannheim, Germany), was used to prepare a standard curve of 0 - 0.238 mg/ml. The gradient of the degradation curves was calculated and these values were used to prepare a standard curve. To 2 ml PBS, 100 µl of 10% honey was added. The contents of the cuvette were mixed well before 5 µl of a 30% H_2O_2 (0.071%) solution was added. Buffer was used as control. The change in absorbance was measured at 240 nm.

3.3.3 Determination of non-enzymatic antioxidant content

Total polyphenol content (TPC)

The Folin-Ciocalteu (F-C) assay is based on a reduction-oxidation reaction during which the phenolate ion is oxidized under alkaline conditions while reducing the phosphotungstic-phospho-molybdic complex in the reagent to a blue coloured solution (Waterman and Mole, 1994). Total polyphenol content (TPC) was determined using the F-C method of Amin *et al.,* (2006) which was modified for a 96 well format. Gallic acid (0 - 0.03 mg/ml) was used to prepare the standard curve. To 10 µl volume of a 10% (w/v) honey solution a 50 µl volume of F-C was added, followed by a 50 µl volume of a 7.5% sodium carbonate solution. The mixture was mixed well and the absorbance was read at 630 nm. For each sample a blank consisting of 10 µl of a 10% (w/v) honey solution with 100 µl PBS was run to correct for any colour interference. TPC was expressed as mg/100g gallic acid equivalents (GAE).

Total flavonoid content (TFC)

The aluminium chloride assay evaluates total flavonoids, and is based on the formation of a red aluminium complex where flavonoid acts as a bidental ligand, forming complexes with the C-4 keto group and either the C-3 or C-5 OH group of flavones and flavonols (Amaral *et al.,* 2009; Chang *et al.,* 2002). Total flavonoid content (TFC) was measured using a modified method of Amaral *et al.,* (2009). Catechin (0 - 0.21 mg/ml) was used to prepare a standard

curve. A 10 µl of a 10% (w/v) honey solution was added to each well of a 96 well microplate. To each well, 30 µl of a 2.5% sodium nitrite, followed by a 20 µl of a 2.5% aluminum chloride and then 100 µl of a 2% sodium hydroxide were then added. The mixture was mixed well and the absorbance was read at 450 nm using a BioTek plate reader. For each sample a blank consisting of 10 µl of a 10% (w/v) honey solution with 150 µl PBS was run to correct for any colour interference. TFC of each honey sample was expressed as mg/100g catechin equivalents (CE).

Data management and statistical analysis

All data is an average of three experiments and each measurement was done at least in triplicate, thereby generating 9 data points. The results are expressed as mean ± standard error of mean (SEM) of three experiments where each experiment point is the average of 3 assays. Several parameters such as protein, catalase and TPC 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) test was used for comparison of means using statistica software Version 9.0 (StatSoft, Tulsa, OK). Correlation analysis was also run with the same statistical package, and Microsoft Excel 2007.

3.4 Results and discussion

Southern Africa (Figure 3.1) is a region of unique biodiversity with many endemic plant species. The southern coastal Fynbos region (Western Cape)(WC) has 8920 flowering plant species, the south eastern region (Eastern Cape)(EC) is a transitional region between this and the subtropical floral regions and consists of 1814 endemic species. The southern eastern region of Mozambique (SEM) includes southern coastal woodlands, forests and mangroves with 162 endemic species. Within the Southern African region there are tracts of land that are extensively farmed and this includes sunflower, canola, grain, and fruit (litchi (L) and orange (O)) as well as plantations of pine and eucalyptus (E) trees. Within these biospheres honey is produced either commercially or it is collected by rural communities and sold on the roadside (Cowling, 1983; Goldblatt and Manning, 2002).

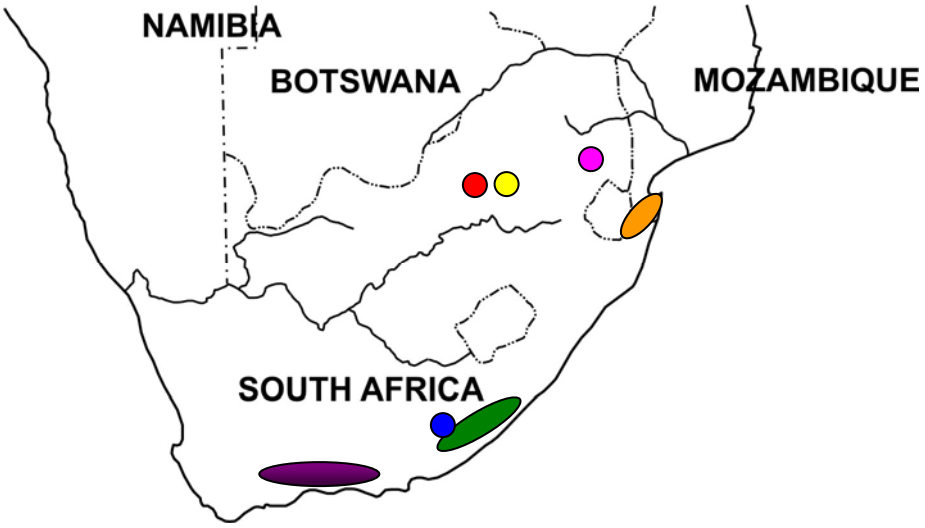


Figure 3.1: Map of Southern Africa showing the regions where honey samples were collected. Purple =Western Cape (samples WCa, WCb and WCc), Green = Eastern Cape (ECa, ECb and ECc), Orange= South East Mozambique (SEMa, SEMb and SEMc). Blue =Agricultural eucalyptus (A-E1), Yellow=eucalyptus (A-E2), Orange=Orange (A-O), Pink= Litchi (A-L).

This study was undertaken using 13 samples and serves as an exploratory study as no previous investigations regarding the antioxidant content and activity of Southern African honeys has been undertaken. Although the number of samples used is small, this study will provide essential information for further more detailed investigations. Nevertheless this sample size is larger than those used by Ouchemoukh *et al.,* (2007) having a sample size of 11, Malika *et al.,* (2005) having a sample size of 8 and Joseph *et al.,* (2007) having a sample size of 9, and meaningful data can therefore be obtained from this number of samples.

3.4.1 Physiochemical properties

Due to the wide range of sources of honey used in this study it was necessary to determine whether these honeys conform to the requirements of Codex Alimentarius Standard. Methodologies for the determination of pH, %moisture, ash content, Glc:Fru ratio, proline content, protein content and diastase activity were established. For the 13 honeys these parameters were measured and the data is presented in Table 3.1.

The pH of honey is due to the presence of organic acids such as gluconic acid as well as inorganic ions such as phosphate and chloride. All honey samples analyzed were acidic in nature, with pH values varying from 3.87 - 5.12 with the highest being ECc and lowest being ECa. These pH values are comparable to 3.50 - 4.43 for Algerian (number of samples analysed (n) =11) (Ouchemoukh *et al.,* 2007), 3.80 - 4.50 for Moroccan (n=8) (Malika *et al.,* 2005), 4.83 - 5.16 for Cameroonian (n=9) (Joseph *et al.,* 2007), 3.8 - 5.0 for Indian (n=7),

(Ahmed *et al.,* 2007; Saxena *et al.,* 2010), 3.7 - 4.4 for the Spanish, Andalusian (n=29) (Serrano *et al.,* 2004) and 3.45 - 4.70 (n=38) for Portuguese honeys (Silva *et al.,* 2009).

The percentage of moisture found in honey is a function of climatic factors and the season in which it was harvested. By also measuring the proline content it can be determined whether adulteration has occurred (Silva *et al.,* 2009). Dilution would result in an increase in moisture and a decrease in proline content. The percentage moisture content of the honey samples ranged from 10.09 - 20.73%, the highest being SEMc and lowest ECb. In literature, the range was 14.64 - 19.04% for Algerian (Ouchemoukh *et al*., 2007), 17.8 - 21.8% for Moroccan (Malika *et al.,* 2005), 16.49 - 18.00% for Cameroonian (Joseph *et al.,* 2007), 17.2 - 21.6% for Indian (Saxena *et al.,* 2010), 14.90 - 18.60% for Andalusian (Serrano *et al.,* 2004) and 13.52 - 19.70% for the Portuguese honeys (Silva *et al.,* 2009). The percentage moisture for all the honeys except SEMc (20.73%) were below 20%, as required by the Codex Alimentarius Standard for honey. However, according to the European Commission on honey (Bogandov, 1999), the maximum percentage moisture is 21%, therefore this sample was included in all further studies.

Ash content is an indication of mineral content and is a parameter for botanical origin and can be used to identify if the honey is floral, mix or honeydew in origin (Silva *et al.,* 2009; Ouchemoukh *et al.,* 2007). Blossom honeys have mineral content of ≤ 0.6% and honey dew and blossom blends ≤ 1.2% (Ouchemoukh *et al.,* 2007). The ash content of the honey samples evaluated ranged from 0.01-0.65g%, the highest being SEMc, and using the above mentioned criterion, all honey samples are of floral blossom origin except SEMc which is probably a honey dew blend. When compared to honeys of other origins such as Algerian ranged from 0.06 - 0.54% (Ouchemoukh *et al.,* 2007), Moroccan with 0.18 - 0.57% (Malika *et al.,* 2005), Cameroon with 0.36 - 0.66% (Joseph *et al.,* 2007), Indian with 0.08 - 0.39%, 0.03 - 0.43% (Ahmed *et al.,* 2007; Saxena *et al.,* 2010) and Portuguese with 0.09 - 0.53% (Silva *et al.,* 2009), the honeys used in this study had a similar range of ash contents.

The high monosaccharide content and the ratio of Glc:Fru is one of the unique characteristics of honey and this makes a food source as a natural sweetener or preservative. In this study a modification of the Perkins test was used to determine the Glc:Fru ratio based on the differences in the rate with which potassium ferricyanide reacts with glucose and fructose. The saccharide ratio, Glc:Fru ranged from 0.85:1 to 1.31:1, the highest ratio being ECb and lowest SEMb which indicates that ECb is more likely to granulate.

Table 3.1: Physicochemical parameters analyzed in honey samples

Parameters required by the Codex Alimentarius Standard for honey quality									Enzymatic antioxidant activity, TPC and TFC content			
Type	**pH**	**Moisture**	**Ash**	**Glc:Fru**	**Proline**	**P-OD**	**P-BF**	**Diastase**	**Catalase**	**A450**	**TPC**	**TFC**
		%	**%g**		**mg/100 g**	**mg/100 g**	**mg/100 g**	**ml/g/min**	**µmol H_2O_2/g**	**mAu**	**mg GAE/100 g**	**mg CE/100 g**
ECa	3.87	11.00	0.01	1.29	18.42	256.00	750.57	13.77	4.35	468.33	77.28	14.44
A-E1	4.18	14.52	0.01	1.08	19.11	360.00	753.44	12.72	8.70	638.33	77.81	25.73
A-E2	4.53	11.45	0.25	1.07	19.54	551.00	925.85	13.02	38.48	1095.00	97.14	30.16
WCa	4.38	14.45	0.19	1.04	28.28	755.00	961.22	13.77	9.95	1778.33	112.50	38.75
WCb	3.95	13.44	0.01	1.27	19.67	435.00	836.81	13.72	6.95	676.67	75.94	19.44
WCc	4.24	12.11	0.01	1.08	25.59	723.00	1054.25	13.20	13.28	1316.6	101.40	42.78
ECb	3.96	10.09	0.03	1.31	24.62	248.00	654.50	13.36	10.82	317.50	68.85	21.10
SEMa	4.53	18.47	0.13	1.07	39.23	1581.00	1286.40	12.76	11.68	2101.67	108.81	42.41
A-L	4.10	13.18	0.01	1.08	17.87	520.00	686.56	12.89	12.68	1036.55	82.24	22.44
SEMb	5.01	17.05	0.17	0.85	45.24	1724.00	981.95	14.40	14.60	2164.00	167.96	51.60
SEMc	4.93	20.73	0.65	1.27	29.06	1131.00	1062.29	15.01	19.89	1791.52	122.39	33.00
A-O	4.12	13.19	0.01	1.23	21.46	362.00	616.84	13.27	23.87	468.33	77.74	22.25
ECc	5.12	16.60	0.09	1.06	15.57	844.00	951.16	14.00	15.15	1466.76	126.71	35.97
Mean	**4.38**	**14.33**	**0.12**	**1.13**	**24.90**	**730.0**	**3308.73**	**13.53**	**14.65**	**1178.43**	**99.75**	**30.77**
Max	5.12	20.73	0.65	1.31	45.24	1724.0	4834.86	15.01	38.48	2164.00	167.96	51.60
Min	3.87	10.09	0.01	0.85	15.57	248.00	2302.48	12.72	4.35	317.50	68.85	14.44

Amino acids occur in honey at a concentration range of 20 – 300 mg/100 g (Cometto *et al.,* 2003). These amino acids may arise from different sources such as nectar, the honey bee and pollen. Because nectar derived amino acids are the only source of essential amino acids to the bee, the profile of amino acids present in the honey is considered to be a good indicator of floral or production area. Proline is the most abundant amino acid found in honey and Cometto *et al.,* (2003) reported that an average of 70% of all amino acids for 5 honey samples was proline. The high levels of proline found in honey makes it an ideal marker for sugar adulteration and ripeness with 18 mg/100 g the minimum value accepted for genuine honey in honey control laboratories (Bogandov *et al.,* 1999). Amino acid concentrations in a wide range of biological matrixes can be determined using the ninhydrin reaction. The majority of amino acids after reacting with ninhydrin produce a purple product while the reaction with α-amino acids such as proline results in the formation of a yellow dye complex. In this study, proline levels ranged from 15.57 - 45.24 mg/100 g, the highest being SEMb and the lowest being ECc. These values for the honey samples analyzed were within range when compared to the Algerian honeys (20.2 - 68.0 mg/100 g) (Ouchemoukh *et al.,* 2007), low when compared with Burkina Fasan honey (43.78 - 216.94 mg/100 g) (Meda *et al.,* 2005) and within the range for Andalusian honeys (3.70 - 98.66 mg/100 g) (Serrano *et al.,* 2004). ECc (15.57mg/100 g) would be considered an adulterated honey as its value falls below 18 mg/100g. A-L is not considered adulterated as it is borderline with a value of 17.87 mg/100 g.

The protein content of honey samples was determined using two different methods, the optical density and the Bradford methods. The absorbance at 205 nm is due to the peptide bond and at 280 nm due to aromatic ring structure and more specifically the presence of conjugated double bonds. Conjugated double bonds are also present in polyphenols (Fig 2.3) which results in an underestimation of protein content, as seen in the equation; an increase in value of the denominator, means a decrease in the value of protein content.

$$\textbf{Protein concentration (in mg/ml)} = A_{205} / (27 + A_{280}/A_{205}))$$

The range for the protein content of the honeys analysed was 248 - 1724 mg/100 g, the highest being SEMb and lowest ECb. From Table 3.1, it can be seen that the concentration of protein in most samples determined by the optical density method is less than that determined by the Bradford method. Robinson *et al.,* (1979) reported that of the Buiret, Bardford and Lowry methods that were used for the determination of protein, the Bradford method was the best for the determination of protein in the presence of high levels of polyphenols. The Bradford method makes use of Coomassie Blue as dye. Coomassie Blue

binds to protein and van der Waals and hydrophobic interactions occur between the basic amino acids (Arg, to a lesser degree Lys, His) and the aromatic amino acids (Trp, Phe, Tyr) of protein. In this study the protein content of the honeys was 616 - 1286 mg/100 g with the highest level measured for SEMa and the lowest for A-O. These levels are similar to 370 - 940mg/100 g reported for the Algerian honey (Silva *et al.,* 2009) but higher than 48.00-229.30mg/100g reported for Indian honey (Saxena *et al*., 2010).

Proteins including enzymes undergo denaturation after extensive heating. In order to determine whether honey was subjected to excessive heating, the activity of the enzyme diastase (α- and β-amylase) is measured (Silva *et al.,* 2009). The function of α- and β-amylase in the honey bee and the production of honey is to hydrolyze soluble starch into maltose and a little glucose (Bilderback, 1973). This reaction by α- and β-amylase is the basis of the method used to quantify diastase activity. Diastase activity ranged from 12.72 – 15.01 ml/g/min, the highest being SEMc and lowest AE-1. These results are similar when compared to 3 - 38 reported for Portugal honeys (Silva *et al.,* 2009), 1.47 - 49.42 reported for the Spanish honeys (Serrano *et al.,* 2004) and lower when compared to 22 - 45 reported for Canadian honeys (Sporns *et al.,* 1992).

Table 3.2 shows a strong correlation between proline and protein content, but a poor correlation between with diastase activity. Proline is an essential amino acid required for protein synthesis which includes enzymes such as diastase. Excessive heating causes the denaturation of diastase and subsequently lower activity. It can therefore be expected that following heating there will be a poor correlation between protein content and enzyme activity. SEMc had the highest activity and AE-1 had the lowest activity, nevertheless all samples fulfilled Codex Alimentarius Standards regarding diastase activity. A strong correlation was found between proline and protein-optical density (Protein-OD) ($r = 0.86$, $p < 0.001$) (Figure 3.2a) while for proline and protein-Bradford method (Protein-BF) the correlation was average. The correlation between Protein-OD and P-BF ($r = 0.80$, $p < 0.001$) was strong (Figure 3.2b). For Protein-OD and diastase activity the correlation was poor ($r = 0.36$, $p < 0.001$) (Figure 3.2c) as well as for Protein-BF and diastase activity ($r = 0.19$, $p < 0.001$) (Figure 3.2c). The graphs showing linear regression equations and Pearson's product moment coefficient (r^2) are presented in Figures 3.1 a-d.

Table 3.2: Correlation matrix of physicochemical properties of honey

	Protein-OD	Protein-BF	Diastase
Proline	**0.86**	0.61	0.28
Protein-OD		**0.80**	0.36
Protein-BF			0.19

Bold=highly significant

Of the 13 samples that have been evaluated 12 samples strictly fulfill the requirements of Codex Alimentarius Standard of honey. SEMc did not qualify using this criterion was as it had a percentage moisture content of 20.73%, however, it qualified according the European commission standards of honey that require a minimum moisture content of 21%. ECc, did not qualify as it had a proline content of 15.87mg/100g, the required being 18mg/100g, therefore it is considered an adulterated honey.

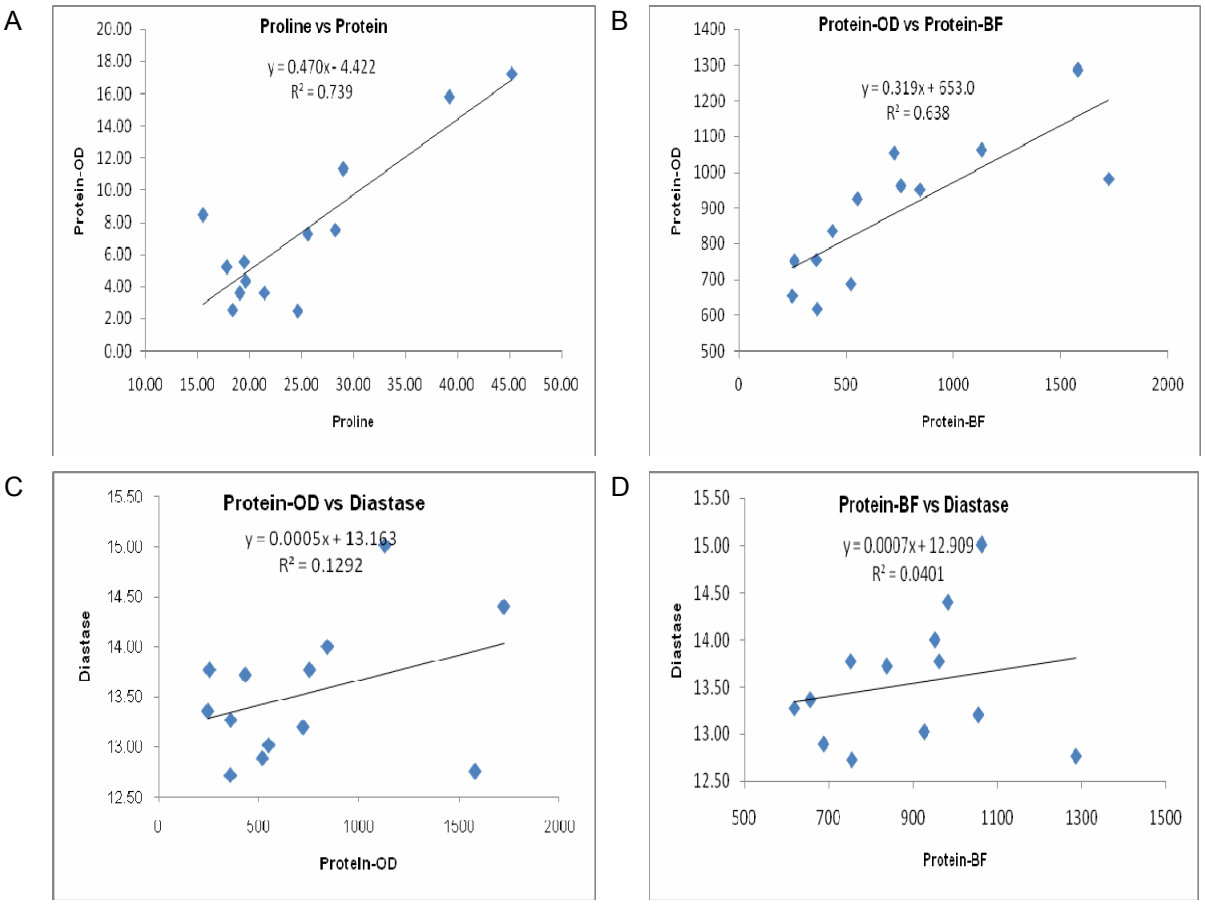


Figure 3.2: Correlations between A) proline and protein-OD, B) protein-OD and protein-BF, C) protein-OD and diastase activity and D) Protein-BF and diastase activity.

A further purpose of this study was to determine the antioxidant activity of the samples of honeys that fulfill the Codex Alimentarius Standard requirements. Antioxidant activity is either enzymatic or non-enzymatic with the enzymatic component being due to catalase activity and non-enzymatic due to the presence of polyphenols.

3.4.2 Enzymatic and non-enzymatic antioxidant content

Catalase activity, total phenolic and flavonoid content

Catalase activity in each of the honeys was determined by measuring the ability of honey to

degrade H_2O_2. Catalase activity ranged from 4.35 - 38.48 µmol H_2O_2/g, the highest being A-E2 and lowest ECa.

Non-enzymatic activity is due to the presence of polyphenols and includes flavonoids and phenolic acids. Few studies regarding the quantification of phenolic acids and flavonoid content of African, South American and Asian honeys have been done. Sudan honey was found to have quercetin, hesperetin, kaempferol, apigenin and isorhamnetin as their most common flavonoids (Makawi *et al.,* 2009), whereas Tunisian honeys had quercetin and myricetin as their common flavonoids (Martos *et al.,* 1997). These honeys show some similarity when compared to European honeys which also have an abundance of quercetin and kaempferol (Estevinho *et al.,* 2008; Kaškonienė *et al.,* 2009; Pyrzynska and Biesaga, 2009; Truchado *et al.,* 2009).

Generally, polyhenolic content is associated with the colour of the honeys. The trend is that darker honeys (honeys with high absorbance readings) have higher polyphenolic content and activity. The colour absorbance of the honey samples ranged from 317.50 - 2164 mAu, the highest being SEMb and lowest ECb. These values are within range when compared to Indian honeys (524 – 1678 mAu) (Saxena *et al.,* 2010), and high when compared to Burkina fasan honeys (28.74 - 59.52 mAu), Strawberry tree honey (78.96 mAu), and honeydew honey (25.56 mAu) (Beretta *et al.,* 2005).

The total polyphenolic content (TPC) can be measured following the reaction of polyphenols to the Folin-Ciocalteu (F-C) reagent. Polyphenols, specifically phenolic acids (such as caffeic acid, ferrulic acid, benzoic acid and gallic acid (Figure 2.3) reduce the F-C reagent. The phenolic content can be however overestimated as the F-C reagent also reacts with amino acids (Stratil *et al.,* 2006). The TPC of honey samples ranged from 68.85 - 167.96 mg GAE/100 g, the highest being SEMb and lowest ECb. These values are similar to 64 -1304 mg GAE/100 g, as reported for the Algerian honeys (Silva *et al.,* 2009) 32.59 - 114.75 GAE mg/100 g as reported for the Burkina Fasan honeys (Meda *et al.,* 2005) and 28.74 - 59.52 GAE mg/100 g as reported for a different study of Burkina Fasan honeys (Beretta *et al.,* 2005). When compared to honeys of European and Asian origin, these values are

considerably higher than 5.20 - 78.96 mgGAE/100 g reported for Italian honeys (Beretta *et al.,* 2005) and 47.00 - 98.00 mg GAE/100 g as reported for the Indian honeys (Saxena *et al.,* 2010) and 21.39 - 59.58 mg GAE/100 g as reported for Cuban honeys (Alvarez-Suarez *et al.,* 2010a). This was also found for the acacia, lime, sunflower and honeydew honeys with values of 2.00 - 39. 00 mg, 16.00 - 38.00 mg, 20.00 - 45.00 mg and 23.00 - 125.00 mg GAE/100g respectively, as reported for these honeys from Romania (Liviu-Al *et al.,* 2009).

Flavonoids are commonly found in foods with high antioxidant content, therefore have been associated with antioxidant activity. Total flavonoid content (TFC), was measured using aluminium chloride, due to the aluminium complex formed between flavonoids C-3 or C-5 hydroxyl and the C-4 keto group (Amaral *et al.,* 2009). This method may underestimate flavonoid content as it is only specific for flavones (apigenin, luteolin, kaempferol, quercetin, myricetin, rutin) and flavonols (catechin). The TFC of honey samples ranged from 14.44 - 51.60 mg CE/100 g, the highest being SEMb and lowest, ECa. These values are much higher when compared to 1.09 - 2.52 mg CE/100 g as reported for Cuban honeys (Alvarez-Suarez *et al.,* 2010a) and 0.17 - 8.35 mg QE/100 g as reported for Burkina Fasan honeys (Meda *et al.,* 2005). However, because the units of measurement for the Burkina Fasan honeys were quercetin equivalents (QE) and not catechin equivalents (CE), a real comparison could not be made for these honeys.

Table 3.3: Ranking of honeys based on A450, TPC and TFC

Honey	A450	TPC (mg GAE/100g)	TFC (mg CE/100g)
SEMb	**2164.00**	**167.96**	**51.60**
SEMa	**2101.67**	**108.81**	**42.41**
SEMc	**1791.52**	**122.39**	33.00
WCa	1778.33	112.50	38.75
ECc	1466.67	**126.71***	35.97
WCc	1316.67	101.40	**42.78**
A-E2	1095.00	97.14	30.16
A-L	1036.55	82.24	22.44
WCb	676.67	75.95	19.44
A-E1	638.33	77.81	25.73
ECa	468.33	77.29	14.44
A-O	468.33	77.74	22.25
ECb	317.50	68.85	21.10
Average	1178.43	99.75	30.77
Max	2164.00	167.96	51.60
Min	317.50	68.85	14.44

Bold =samples with highest levels
*ECc considered an adulterated honey

The highest A450 was associated with honeys with a high TPC and high TFC, except for SEMc which had a relatively low TFC. Samples with the highest A450 were SEMb, SEMa and SEMc, TPC were SEMb, SEMc and SEMa, and for TFC was SEMb, WCc and SEMa. From this data SEMb followed by SEMa are clearly the honeys with the highest TPC and TFC and potentially the highest antioxidant activity.

The colour, A450 was correlated with TPC or TFC (Table 3.4). Strong correlations for A450 vs TPC (r=0.87), A450 vs TFC (r=0.89) and TPC and TFC (r=0.87) were found. A poor correlation was found between catalase activity vs TPC and TFC; showing that there is no relationship between enzymatic and non-enzymatic antioxidant content

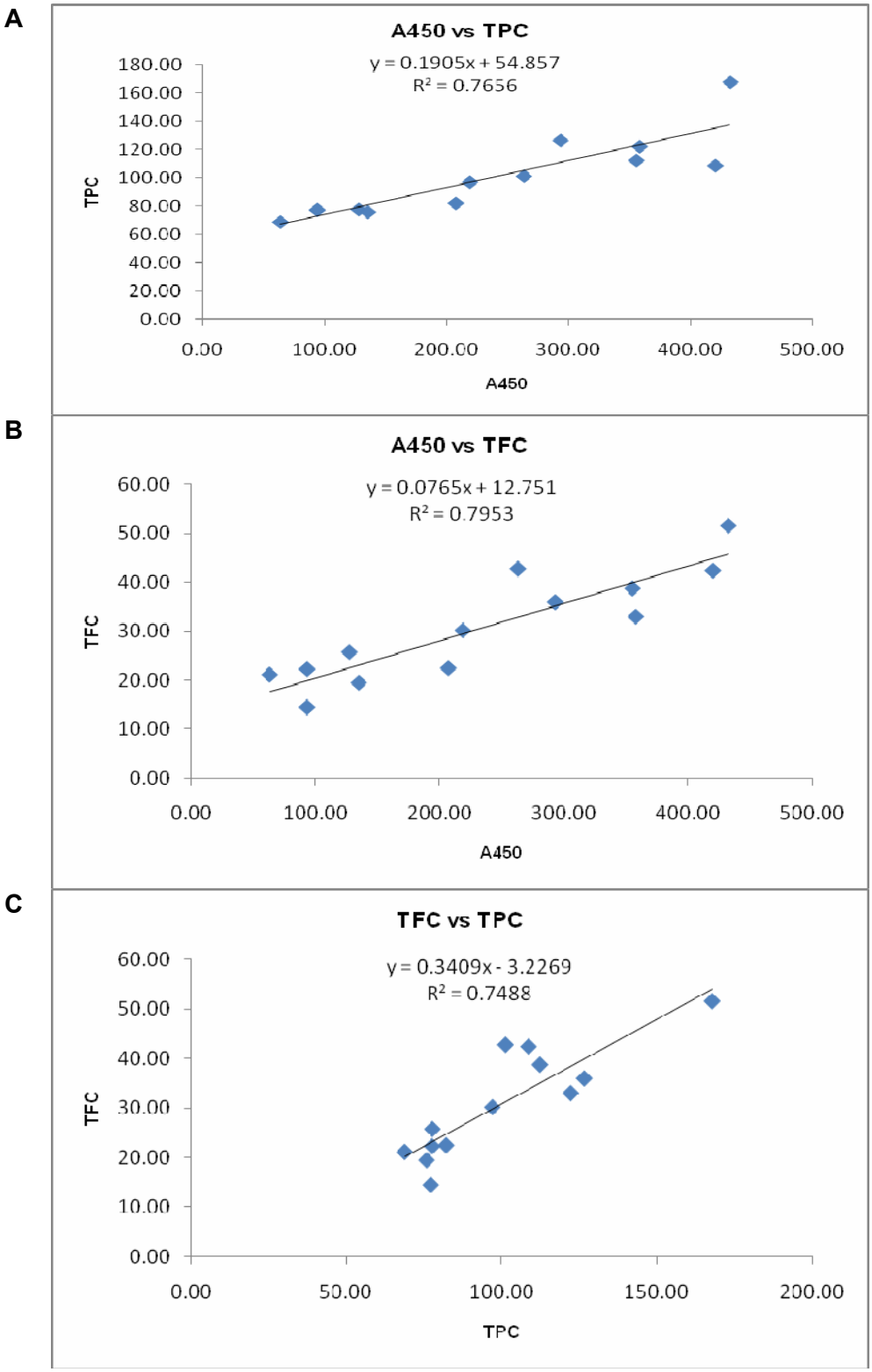


Figure 3.3: Correlations between A) A450 and TPC, B) A450 and TFC and C) TPC and TFC.

Table 3.4: r, r^2 and p values of honey samples for A450, TPC, TFC and catalase	
A450 vs TPC	r=0.87, r^2=0.77, p=0.00004
	Significant*
A450 vs TFC	r=0.89, r^2=0.79, p=0.00003
	Significant
TPC vs TFC	r=0.87, r^2=0.75, p=0.00000002
	Significant
Catalase vs TPC	r=0.16, r^2=0.02, p=0.0000001
	Not significant
Catalase vs TFC	r=015, r^2=0.02, p=0.0008
	Not significant

*Significance determined on a 95% confidence level, using 11 degrees of freedom.

3.5 Conclusion

The honey samples except for ECc strictly fulfilled the requirements of Codex Alimentarius Standard for honey.

For all honeys evaluated, SEMb and SEMa had the highest TPC and TFC while A-E2 had the highest catalase activity. Colour of honey samples is usually correlated with high antioxidant content and activity. For the honeys evaluated SEMb had the highest A450 and the highest TPC and TFC values. SEMa which had the second highest A450 also had the second highest TPC and TFC value, which leads to the conclusion that these honeys will also have high antioxidant activity. For the lighter honeys, ECb had the lowest A450 and the lowest TPC. ECa which had the second lowest A450 had the lowest TFC, which leads to the conclusion that these honeys will also have low antioxidant activity.

CHAPTER 4: THE ANTIOXIDANT ACTIVITY AND BIOLOGICAL EFFECTS OF A SELECTION OF SOUTHERN AFRICAN HONEYS

4.1 Introduction

Due to the presence of phenolic acids (caffeic acid, ferrulic acid, benzoic acid, and gallic acid) and flavonoids (quercetin, luteolin, chrysin, myricetin, pinocembrin, pinobanksin and kaempferol), honey has high antioxidant activity. The major flavonoids in honey are chrysin, pinocembrin and pinobanksin which are potent antioxidants (Gheldof *et al.,* 2002). TPC and TFC assays only provide information regarding antioxidant content and therefore antioxidant activity should be determined.

Antioxidant activity is measured using different assays; all the assays have the same basic principle which is electron transfer. These methods fall into two major categories: assays based on single electron transfer (ET), monitored through a change in colour as the oxidant is reduced, and assays based on a hydrogen atom transfer reaction (HAT), where the antioxidant and substrate (probe), compete for free radicals (Huang *et al.,* 2005).

ET reactions such as the trolox equivalent antioxidant capacity assay (TEAC), ferric reducing ability of plasma assay (FRAP), copper reduction assay (CUPRAC) and 2, 2-diphenyl-2-picrylhydrazyl assay (DPPH) have the same basic chemistry, as shown in the following reaction (Huang *et al.,* 2005):

$$\mathbf{M\,(n) + \bar{e}\ (from\ AH) \longrightarrow AH^{\bullet+} + M\,(n\text{-}1)}$$

This reaction involves two components, the antioxidant (AH) and the oxidant probe (M(n)). The probe is reduced, oxidizing the antioxidant and as a result the probe changes colour which is directly proportional to the concentration of the antioxidant. The slope of the curve reflects antioxidant reducing capacity expressed as trolox, gallic acid or catechin equivalents depending on the standard used. Due to the absence of oxygen radicals (i.e. a probe is reduced) it is often questioned how the reaction relates to antioxidant capacity, therefore the assumption is made that antioxidant capacity equals reducing capacity (Huang *et al.,* 2005). HAT reactions including, crocin bleaching assay, total peroxyl radical-trapping antioxidant parameter (TRAP) and the oxygen radical absorbent capacity assay (ORAC) (Tabart *et al.,* 2009), follow the reaction (Huang *et al.,* 2005):

$$ROO^{\bullet} + AH \longrightarrow ROOH + A^{\bullet}$$
$$ROO^{\bullet} + LH \longrightarrow ROOH + L^{\bullet}$$

The HAT reaction is the key step in the radical chain reaction and therefore more relevant to radical chain breaking antioxidant capacity (Huang *et al.,* 2005). In the ORAC assay, 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) generates peroxyl radicals, and the ability of the antioxidants to protects an indicator molecule, (i.e. fluorescein) is measured. The chemistry of this reaction is the same as that used in the plasmid, erythrocyte as well as the cellular DCFH-DA assay (Chapter 5) used to determine the ability of honeys to protect biological and cellular systems against oxidative damage.

In many studies more than one assay is used to measure antioxidant activity,as several factors may contribute to the over or underestimation of antioxidant activity. For example ET reactions are pH dependent where in acidic conditions the reducing capacity may be suppressed due to pronation of the antioxidant compounds, while at a high pH proton dissociation of phenolic compounds would enhance a samples reducing capacity (Huang *et al.,* 2005). The inherent colour of honey due to the flavonoids and MRP's may cause interference at the wavelength of interest (e.g. DPPH assay) and result in an overestimation of antioxidant activity. Often antioxidant activity is determined using the TEAC or DPPH assays together with the ORAC assay as the latter is considered to be more relevant. The advantage of the ORAC assay is that free radicals are generated at a constant rate and being a fluorimetric assay it is more sensitive than the colorimetric assays, DPPH and TEAC.

Once data has been generated using these assays it is impossible to determine which assay is the best measure of antioxidant activity due to the above mentioned factors, therefore Sun and Tanumihardjo, (2007) developed the Relative Antioxidant Capacity Index (RACI) which is a relative index and does not present a specific antioxidant property but rather a measure of "total antioxidant activity". This index is used to identify which sample has the highest antioxidant activity by taking all antioxidant activity data into account.

All antioxidants assays are chemistry based and provide little information on the effect thereof on biological systems. To measure the antioxidant effect on biological systems, the first level of screening involves the use of simple biological systems such as DNA and cell membranes as these macromolecules play an essential role in cellular function (Wei *et al.,* 2006; Kalpana *et al.,* 2008). The plasmid assay gives an indication of how antioxidants protect DNA, whereas the erythrocyte assay give a measure of the effect of antioxidants on the cell membrane using a simple cell type without organelles or a nucleus, the erythrocyte.

These assays are more rapid than cell culture methods (4 h vs 3 days) however blood used in the erythrocyte assay is derived from different donors and this may give variable results.

The specific objectives of this chapter are:

1. To determine antioxidant activity of honey samples evaluated in chapter 3, using the DPPH, TEAC and ORAC assays.
2. To determine the degree of correlation between colour, TPC, TFC with each of the antioxidant assays, the plasmid and erythrocyte assays.
3. To determine the Relative Antioxidant Capacity Index of each honey sample and to be able to identify those samples with the highest antioxidant capacity.
4. To determine whether high antioxidant activity is associated with high biological protection (i.e. protection of plasmid DNA and the erythrocyte membrane), against AAPH-induced oxidative damage.

4.2 Materials

The same samples and reagents used in Chapter 3 will be used in this chapter. Other reagents include: 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH), 2, 2-diphenyl-2-picrylhydrazyl (DPPH), ABTS (2,2'-Azo-bis (3-ethylbenzothiazoline-6-sulfuric acid) diamonium salt), flourescein sodium salt were obtained from Sigma-Aldrich Company, Atlasville, SA. The organic solvent methanol was of analytic grade and was purchased from Merck, Johannesburg, SA. The pBR322 plasmid DNA was from Promega, WI, USA and was supplied by Whitehead Scientific, Cape Town, SA.

Equipment used included: a FLUOstar OPTIMA plate reader from BMG labtechnologies, Offenburg, Germany. For electrophoresis an Amesham Power Supply_EPS 301 system coupled to a gel running system, model A1 class II (Owl Scientific, Inc, Woburn, MA USA) was used. Gels were visualized using a UV-transilluminator Vilber Loumat ELP 66 coupled to a Canon PowerShot Camera, Japan. All other equipment, disposable laboratory ware and laboratory facilities was the same as was used in Chapter 3.

4.3 Methods

4.3.1 Determination of antioxidant activity

DPPH radical-scavenging assay

DPPH radical scavenging assay was carried out according to a modified method of Awika *et al.,* (2003). Stock solutions of DPPH were prepared by dissolving 24 mg of DPPH in 100 ml methanol. The solution was then shaken in a sonicator for 20 min. The working solution was then prepared by diluting 20 ml of stock solution with 80 ml methanol. Trolox (25 mg/ml) was used to make the standard curve, which was linear between 0 and 800 µM. To 15 µl of a 10% (w/v) honey solution, 285 µl of DPPH was added. The microplate was then left to stand for 15 min in a dark place. To eliminate the possible effects of interference each sample served as its own control i.e. all components, no DPPH added. The plate was then read at 570 nm and the data was expressed as % inhibition (%I), IC_{50} (mg/ml) or µmol trolox equivalents per gram of sample (TE)/g.

TEAC assay

The TEAC assay was done according to Awika *et al.,* (2003) The $ABTS^{\bullet+}$ was freshly generated by adding 3 mM of potassium peroxodisulfate ($K_2S_2O_8$) solution to 8 mM ABTS and the mixture was left to react in the dark for at least 12 h at room temperature. The working solution was prepared by diluting ABTS stock solution with 0.2 M phosphate buffer, pH 7.4. Trolox was used as a standard, concentration range 0 - 1000 µM. A 2.9 ml volume of the working solution was added to 0.1 ml of 1% w/v honey solution. The reaction mixtures were left to stand at room temperature and the absorbance readings were taken at 734 nm after 30 min for the samples and 15 min for the standards, using the Lambda EZ150 spectrophotometer. To eliminate the possible effects of interference each sample served as its own control i.e. all components, no ABTS added. The results were expressed as µmol TE/g of sample.

ORAC assay

Procedures were based on a modified method of Ou *et al.,* (2002). AAPH was used as a peroxyl radical generator, trolox as standard (0 - 1000µM) and fluorescein as a fluorescent probe. PBS was used as a blank. To 160 µl volume of 0.139 nM flourescein working solution, 40 µl of PBS, or trolox (serial dilution) or 1% w/v honey was added. This was

followed by the addition of 40 µl of a 0.11 µM AAPH. Samples were mixed well and the microplate was placed into the plate reader and incubated at 37°C. The fluorescence was measured every 5 min for 4h. The assay protocol included: measurement start time of 0.0s, 10 flashes per cycle, 300 s cycle time, 485 nm for the excitation filter and 520 nm for the emission filter. The final ORAC values of the samples were calculated by using the net area under the decay curves (AUC). The results were expressed as µmol TE per g sample.

Relative Antioxidant Capacity Index (RACI)

RACI was determined according to the method of Sun and Tanumihardjo (2007). This was calculated as:

$$\textbf{Standard score} = (x-\mu)/\sigma$$

Where x is the raw data, µ is the mean and σ is the standard deviation

The standard score is a value that normalises the distribution and has a mean of 0 and a standard deviation equal to 1. RACI of each honey was created by averaging the standard scores from different antioxidant assays (DPPH, ORAC and TEAC), so that comparisons can be made without interference from different units, scales and distributions.

4.3.2 DNA protection assay

Conversion of the supercoiled form of plasmid DNA to the open-circular and further linear forms has been used as an index of DNA damage (Sung *et al.,* 2001).These forms can be separated by agarose gel electrophoresis due to their different electrophoretic mobility (Aronovitch *et al.,* 2007). Procedures for this assay were based on a modified method of Wei *et al.,* (2006). Briefly, the reaction mixtures containing 2.5 µg of pBR322 plasmid DNA, 10% (w/v) honey samples, 5.5 x 10^{-4} µM AAPH were made to 22.5 µl with phosphate buffer solution (PBS, pH 7.4) and incubated at 37 °C for 90 min. For the control samples, PBS replaced the honey samples. After incubation, samples were mixed with an equal amount (22.5 µl) of gel loading buffer (0.13% bromophenol blue and 40% sucrose) and the reaction mixtures were immediately loaded into a 1% agarose gel in 40 mM Tris, 20 mM glacial acetic acid and 0.5 M EDTA pH 8.0 (TAE) and containing 50 µg/ml ethidium bromide (EtBr). Electrophoresis was undertaken using a horizontal slab gel apparatus in a TAE buffer for 2 h at 60 V. Gel imaging and data analysis followed the method of Benherlal and Arumughan, (2008). Stained gels were placed on a UV trans-illuminator at 254 nm and photographed to

tagged image file format (TIFF) and was captured in RGB format before being imported into the image processing Gel Pro Analyzer, version 3.0 software (Media Cybernetics, Silver Spring, MD, USA) to quantify the density of the supercoiled DNA. The image was transformed to 8-bit grayscale format and then was normalized based on the blank lane by adjusting the brightness and contrast tool. The fluorescent region of each lane was then selected using free-hand selection tool and measured the average intensity of all pixels in the selected region. The numeric value of a pixel lay between 0 and 255 based on their signal strength. The values of all pixels in the selected area were computed and an average value per pixel was obtained. Average fluorescence in the control lane was considered 100% (0% DNA damage) and the difference between the control and treated DNA was evaluated as percentage damage.

4.3.3 Erythrocyte membrane protection studies

The erythrocyte cell membrane is a typical bilayer that can be used to determine the ability of the antioxidants contained in honey to protect this membrane against oxidative damage. Blood was collected from healthy, consenting donors as stipulated by the Students Ethics Committee regulations (SA2011). Using a sterile needle connected to a 5 ml EDTA vacuum extraction blood tube, 5 ml blood was collected from a vein. In the laboratory, the tube and isolated erythrocytes were labeled using laboratory based labeling system e.g. Erythrocytes: Researchers name: Date of collection. The erythrocytes were collected by centrifugation at 200xg and then the plasma and buffy coat was removed. The erythrocytes were then washed with isotonic buffered saline (isoPBS: 0.137 M NaCl, 3 mM KCl, 1.9 mM NaH_2PO_4, 8.1 mM Na_2HPO_4, pH 7.4) divided into 1 ml volumes and were kept at 4°C. For each experiment the erythrocytes were removed from the fridge and left on the laboratory bench to warm to room temperature and then were resuspended in isoPBS and were washed three times in isoPBS. A 10 µl volume of erythrocytes diluted in 100 µl isoPBS were then exposed to 40 µl of honey (10% w/v) or to trolox (stock solution 10mM, final concentration of 0.44 mM) used as standard for 30 min at 37°C. A volume of 40 µl AAPH final concentration of 24 mM was then added and the solution was incubated for a further 2 h. The samples were then removed from the incubator, centrifuged at 400xg and a 100 µl volume of supernatant was collected and placed into a well of a 96 well plate and the absorbance was measured at 570 nm. Two controls were included: erythrocytes, no AAPH or honey added and erythrocytes with AAPH. The results were expressed as mM TE/g.

Data management and statistical analysis

All data management and statistical methods used were the same as in chapter 3. Several factors were correlated. For example, it is widely stated in the literature that a high A450 correlates with high antioxidant activity. Therefore A450 was correlated with antioxidant activity determined using the TEAC, DPPH and ORAC assays. To achieve these above mentioned comparisons data was statistically evaluated using analysis of variance (ANOVA), using the same statistical analyses as were used in chapter 3.

4.4 Results and Discussion

4.4.1 Antioxidant activity

Antioxidant activity can be determined using either ET or HAT assays. Two ET-based assays namely the DPPH and TEAC assays were used and the ORAC assay a HAT-based assay was used for the determination of antioxidant activity.

The DPPH assay provides information on the reactivity of test compounds by means of a stable free radical. Due to its odd electron, DPPH which gives a strong absorption at 517 nm in visible spectroscopy has a deep violet colour. As this electron becomes paired off in the presence of a free radical scavenger (Figure 4.1), the absorption vanishes, resulting in decolourization (Russo *et al.,* 2004).

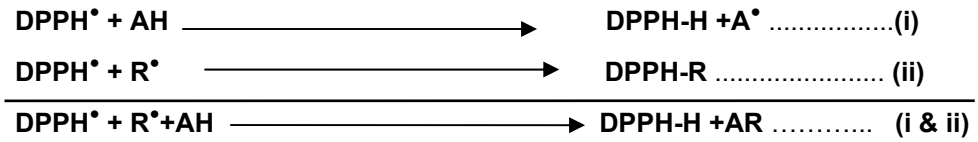


Figure 4.1: Schematic diagram of the DPPH reaction. Where AH is the antioxidant and R• is the radical.

A disadvantage of the DPPH assay is that the decolourization of DPPH is measured at 517 nm where coloured compounds such as carotenoids cause interference, resulting in difficulties regarding the interpretation of data (Alvarez-Suarez *et al.,* 2009). The antioxidant activity determined by DPPH is expressed in many studies as µmol TE/g, %I or IC_{50} (mg/ml). The %I measures the degree to which the sample inhibits or prevents the decolouration of DPPH. The IC_{50} is the concentration at which 50% of the decolouration of DPPH is inhibited. Whereas µmol TE/g is the standard method of data expression for many other antioxidant assays such as the TEAC and ORAC assays. For %I and µmol TE/g increased values are

associated with increased antioxidant activity, while a low IC_{50} is also associated with high antioxidant activity (Beretta *et al.,* 2005). For the honey samples used in this study, % inhibition (%I), IC_{50} and µmol TE/g was calculated (Table 4.2), which made it possible to compare this data with different studies. For the DPPH expressed as µmol TE/g and % I ECc had the highest levels, but since it is considered adulterated, WCa had the highest levels, and for DPPH expressed as $IC_{50,}$ A-E2 had the highest levels (Figure 4.2 a-c).

The DPPH data was correlated and a high degree of correlation was found between DPPH expressed as µmol TE/g and %I ($r = 0.991$, $p = 3.17 \times 10^{-11}$). A poor correlation was found between IC_{50} and µmol TE/g or %I (($r = 0.288$, $p = 0.339$ and $r = 0.227$, $p = 0.455$).

Using the IC_{50} assay the findings of this study, IC_{50}, 4.75 - 6.48 mg/ml was similar to 3.61 - 5.31 mg/ml as reported by Beretta *et al.,* (2005), for the Burkina Fasan honey, but was at the lower end of other studies with a range of 4.37 - 29.13 mg/ml as reported by Meda *et al.,* (2005), also for Burkina Fasan honeys. Antioxidant activity was within a lower range of 1.63 - 47.62 mg/ml as reported by Beretta *et al.,* (2005), for Italian honeys**,** while Bertoncelj *et al.,* (2007), reported 7.2 - 53.8 mg/ml for Slovenian honeys. With data expressed as %I, levels across a range of 2.84 - 38.51% were less than the values of 44 - 71% for Indian honeys as reported by Saxena *et al.,* (2010).

For correlation purposes with antioxidant content assays, only DPPH measured in µmol TE/g, was used. When the DPPH assay was correlated with A450, TPC, TFC and catalase, all assays except catalase showed strong correlation, with the highest degree of correlation observed with TPC ($r = 0.72$, $r^2 = 0.51$, $p = 0.00000002$) as shown in Table 4.2.

Table 4.1: DPPH values as µmol TE/g, % I and IC_{50}.

Sample	µmol TE/g	% I	IC_{50} (mg/ml)
A-E1	1.44	13.97	4.62
A-E2	2.52	27.40	**4.59**
A-L	1.15	9.17	4.81
A-O	0.99	8.36	5.20
ECa	0.51	3.55	6.48
ECb	0.42	2.84	6.37
ECc	**3.72***	**38.51***	4.78
SEMa	1.14	7.87	6.48
SEMb	**2.57**	**29.64**	4.84
SEMc	2.02	20.09	5.03
WCa	**3.07**	**32.07**	4.80
WCb	1.19	13.00	**4.63**
WCc	1.85	19.46	**4.75**

Bold = highest levels
*ECc considered an adulterated honey

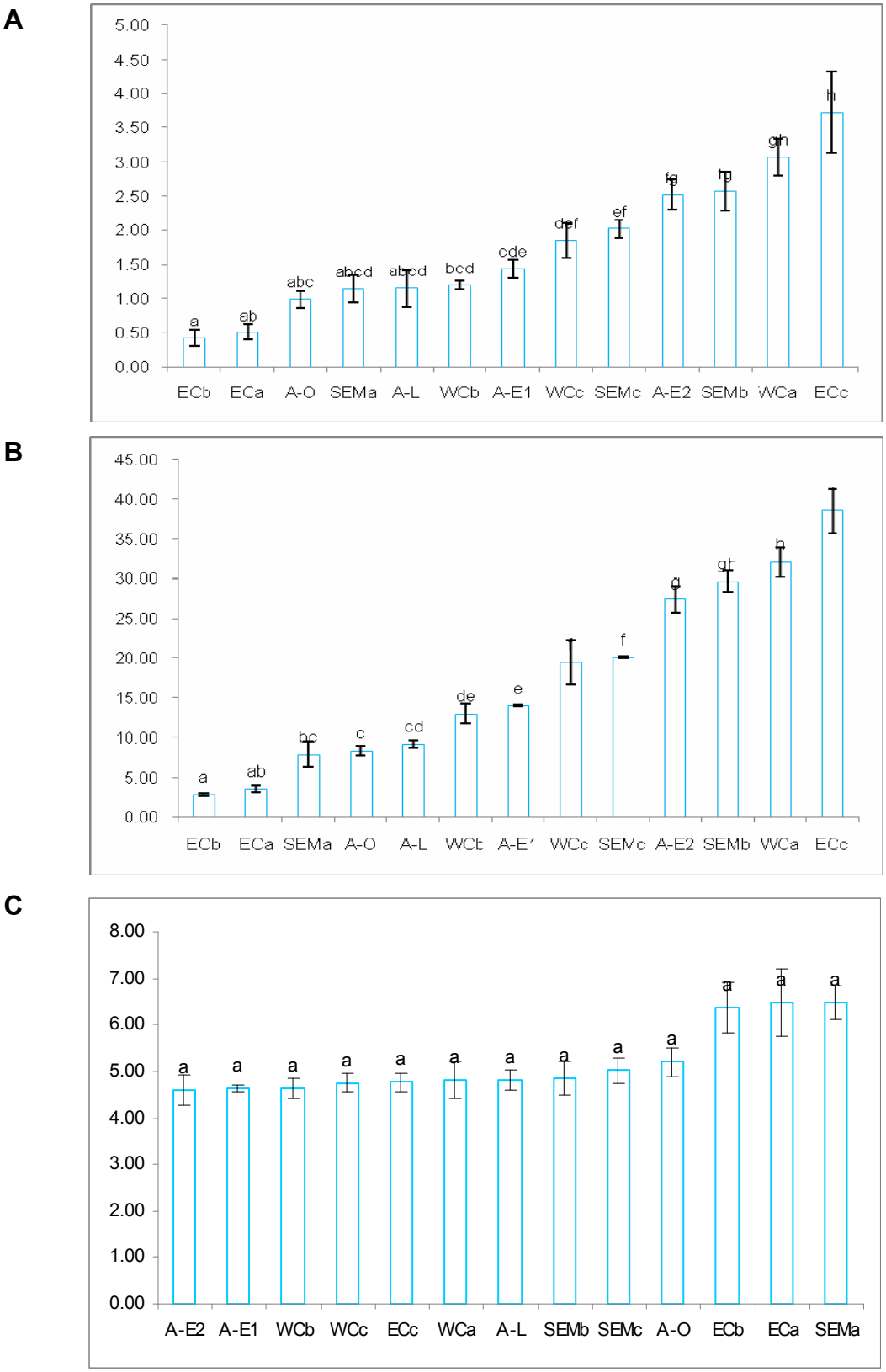


Figure 4.2: Ranking of honey samples from lowest to highest according to the DPPH assay. Data was expessed as A) µmol TE/g, B) % inhibition, and C) IC_{50} mg/ml. Data is an average of three experiments ± SEM. Means of honeys with different letters are significantly different, p≤0.001.

TEAC assay

The TEAC assay is often used for determining antioxidant activity and has the same basic principle as the DPPH assay. ABTS used in the TEAC assay is a stable nitrogen centred radical species. It is more versatile than DPPH, as both non-polar and polar samples can be assessed. In the TEAC assay, the absorption is maximum at 760 nm, a wavelength not often encountered with natural products (Dastmalchi *et al.,* 2007). Honey has an absorbance maximum at 740 nm and therefore to eliminate possible interference each sample served as its own control i.e. all components, no ABTS radical added. The results were expressed as µmol TE/g and is presented in Figure 4.3 and summarised in Table 4.3. The range of antioxidant activity in honey was 5.36 - 20.84 µmol TE/g. The lowest activity was found for ECc (not taken into account, due to possible adulteration) followed by ECa and the highest was for SEMb. When the TEAC values of these honeys were compared to TEAC values from other studies, honeys from the Southern African region had higher antioxidant activity than 1.03 - 2.94 µmol TE/g and 0.34 - 2.03 µmol TE/g reported for Cuban and Venezuelan honeys, respectively (Alvarez-Suarez *et al.,* 2010a).

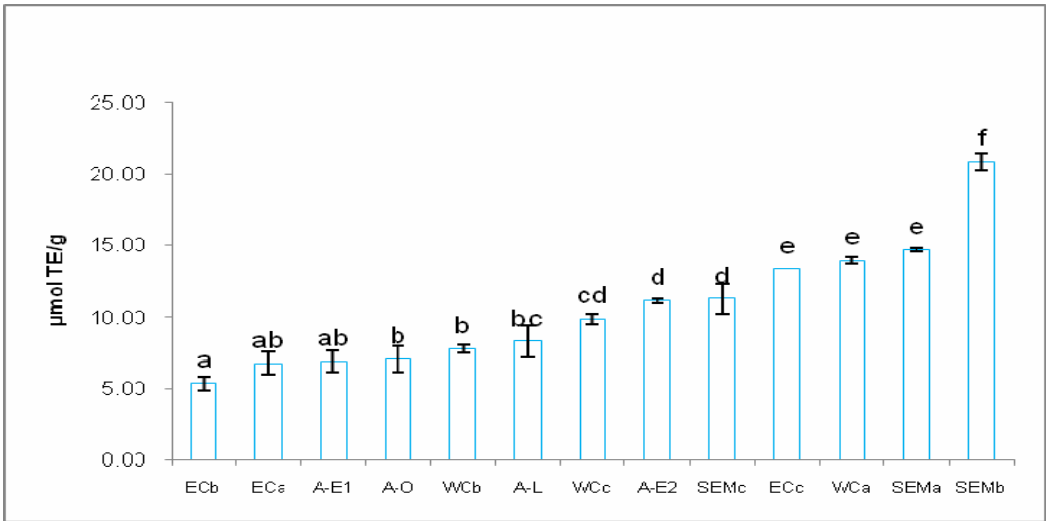


Figure 4.3: Ranking of honey samples from lowest to highest according to the TEAC assay. Data average of three experiments ± SEM. Means with different letters are significantly different, p≤0.001.

When the TEAC assay was correlated with A450, TPC, TFC and catalase, all assays except catalase showed strong correlations, with the highest being with TPC ($r = 0.95$, $r^2 = 0.90$, $p = 0.00000001$) as shown in Table 4.2.

ORAC assay

The ORAC assay is based on free radicals being produced by AAPH and the fluorescent probe is oxidized with resulting loss of fluorescence (Beretta *et al.,* 2005). In this assay, antioxidants compete with probes for the AAPH-induced peroxyl radicals, which results in the inhibition or retardation of oxidation of the probe (Tabart *et al.,* 2009; Huang *et al.,* 2005).

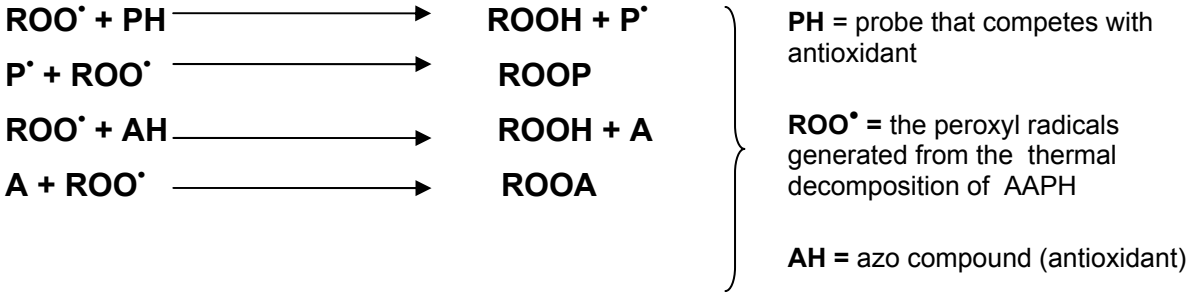


Figure 4.4: Schematic diagram of the principle of the ORAC assay, adapted from Huang *et al.,* (2005).

The above reaction is based on the assumption that one probe or antioxidant scavenges two radicals ($ROO^{\bullet}$), which fits in with the Halliwell's definition of an antioxidant: which is a molecule which when present in small concentrations compared to other biomolecules are able to protect and can prevent or reduce the extent of oxidative destruction of biomolecules (Huang *et al.,* 2005). In the ORAC assay, the probe that is used is flourescein, which is highly sensitive to oxidative induced changes in conformation and chemical integrity which results in a loss of fluorescence. The effect of the AAPH radical on fluorescein is shown in Figure 4.5. With increasing time there is an increase in radical formation with a resulting decrease in fluorescence. The effect can be counteracted with antioxidants such as trolox and those found in honey.

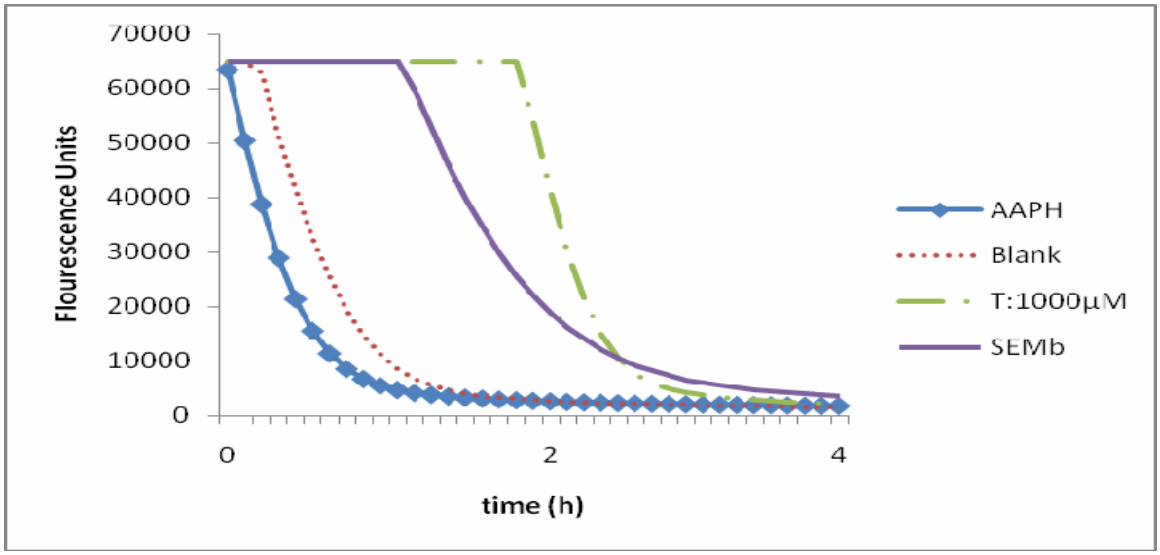


Figure 4.5: Representative quenching curves obtained in the study for fluorescein for the ORAC assay in the presence of AAPH, blank (water), Trolox: 1000µM, and the honey with the highest antioxidant activity (SEMb).

The advantage of this assay is that the use of AAPH as radical generator is also used in the the biological (the plasmid, erythrocyte) and cellular assays (DCFH-DA assay) as a source of peroxyl radicals and as a result direct comparisons can be made. Furthermore, the HAT reaction is a key step in the radical chain reaction within biological systems; therefore the HAT method is more relevant to the radical chain-breaking antioxidant capacity (Huang *et al.,* 2005).

The ORAC results are presented in Figure 4.6 and summarized in Table 4.3. The range of antioxidant activity was 3.71 - 49.26 µmol TE/g. When the ORAC values of these honeys were compared to other studies where the antioxidant activity of the honeys was determined with the ORAC assay it was found that these values were comparable or higher than 3.09 - 9.75 µmol TE/g reported by Rasmussen *et al.,* (2008), for American honeys, 1.75 - 6.89 µmol TE/g and 2.00 - 21.07 µmol TE/g reported by Beretta *et al.,* (2005), for Italian honeys, 11.07 - 18.23 µmol TE/g reported by Beretta *et al.,* (2005), for Burkina Fasan honeys and 4.59 - 12.89 µmol TE/g reported by Alvarez-Suarez *et al.,* (2010a), for Cuban honeys.

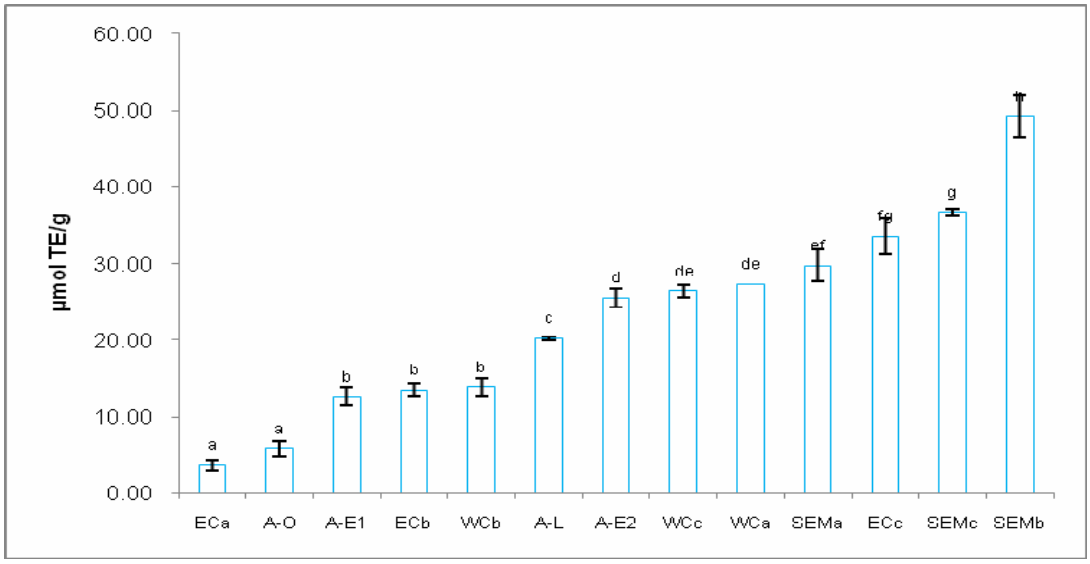


Figure 4.6: Ranking of honey samples from lowest to highest according to the ORAC assay. Data average of three experiments ± SEM. Means with different letters are significantly different, p≤0.001.

When the results ORAC assay were correlated with A450, TPC, TFC and catalase, all assays except catalase showed strong correlations, with the highest being with TPC (r = 0.94, r^2 = 0.88, p = 0.000000001) as shown in Table 4.2, possibly indicating that the phenolics could be contributing to the measured antioxidant activity of honeys.

Table 4.2: r, r^2, p values and significance levels of honey samples for A450, TPC, TFC and the antioxidant assays (DPPH, TEAC and ORAC)

	DPPH	TEAC	ORAC
A450	r=0.62, r^2=0.39	r=0.91, r^2=0.83	r=0.89, r^2=0.81
	P=0.00003	P=0.00003	P=0.00003
	Significant*	**Significant**	**Significant**
TPC	r=0.72, r^2=0.51	r=0.95, r^2=0.90	r=0.94, r^2=0.88
	P=0.00000002	P=0.00000001	P=0.000000001
	Significant	**Significant**	**Significant**
TFC	r=0.64, r^2=0.41	r=0.89, r^2=0.79	r=0.88, r^2=0.77
	P=0.0000004	P=0.0000005	P=0.0009
	Significant	**Significant**	**Significant**
Catalase	r=0.32, r^2=0.11	r=0.14, r^2=0.01	r=0.22, r^2=0.05
	P=0.0001	P=0.137	P=0.051
	Not significant	Not significant	Not significant

*Significance determined on a 95% confidence level, using 11 degrees of freedom.

Evaluation of the antioxidant activity revealed that with the DPPH assay, sample ECc, and with the TEAC and ORAC assays sample SEMb, had the highest antioxidant activity (Table 4.3).

Table 4.3: Summary of antioxidant activity of honeys

Honey	TEAC	DPPH	ORAC
	µmol TE/g	µmol TE/g	µmol TE/g
A-E1	6.89	1.44	12.63
A-E2	11.12	2.52	25.58
A-L	8.31	1.15	20.26
A-O	7.05	0.99	5.88
ECa	6.77	0.51	3.71
ECb	5.36	0.42	13.51
ECc	13.35	**3.72***	35.54
SEMa	14.66	1.14	29.73
SEMb	**20.84**	2.57	**49.26**
SEMc	11.28	2.02	36.73
WCa	13.99	**3.07**	27.26
WCb	7.77	1.19	13.92
WCc	9.86	1.85	26.42
Mean	10.56	1.74	22.58
Range	5.36 - 20.84	0.42 -3.72	3.71 - 49.26

Bold = highest activity
*ECc considered an adulterated honey

Honey is a complex mixture of sugars, enzymes and polyphenols such as: caffeic acid, ferrulic acid, benzoic acid, gallic acid, quercetin, luteolin, chrysin, myricetin, pinocembrin and kaempferol. The principles of the DPPH, TEAC and ORAC assays differ and each measure slightly different aspects of antioxidant activity. Factors that can contribute to differences in the data generated include rate of reaction, type of reaction that the antioxidant undergoes with the probe (e.g. the DPPH assay), detection limit of the assay e.g. colorimetric (DPPH and TEAC) vs fluorimetric assays (ORAC), as well as solubility of polyphenols (e.g. H_2O for the TEAC and ORAC assays and methanol for the DPPH assay) (Huang *et al.,* 2005).

Table 4.4: r, r^2 and p values for correlations between antioxidant assays

DPPH vs TEAC	r=0.67 , r^2=0.45, p=0.000002
	Significant*
TEAC vs ORAC	r=0.89 , r^2=0.80 , p=0.0005
	Significant
DPPH vs ORAC	r=0.72 , r^2=0.52 , p=0.00005
	Significant

*Significance determined on a 95% confidence level, using 11 degrees of freedom.

Correlations between these assays are shown in Table 4.4. All three assays when correlated to one another showed strong correlations. Assays that correlate best with each other are TEAC and ORAC, with an r = 0.89, r^2 = 0.80 and p = 0.0005.

Relative Antioxidant Capacity Index (RACI)

When data is generated using different antioxidant assays, it is difficult to identify honeys with the highest antioxidant activity as honey contains a complex mixture of polyphenols and flavonoids that differ in structure, concentration and activity. Each reacts differently in each antioxidant activity assay and a sample with high activity may have low activity when a different assay is used, e.g. WCa has the highest activity with the DPPH assay, while SEMb was found to have the highest activity with the TEAC and ORAC assays. Therefore to overcome this dilemma Sun and Tanumihardjo, (2007) developed an integrated approach for the evaluation of antioxidant capacity. Using statistical principles, food antioxidant data obtained from different assays was integrated into a single value, the Relative Antioxidant Capacity Index (RACI). Theoretically if each assay has the same value or unit, a sum or a mean could be calculated. For each antioxidant assay usually different units are used and for this reason the data is transformed into standard score and then the samples are compared and no studies could be found in the literature where the RACI for honeys has been determined, the study found was on vegetables (Sun and Tanumihardjo, 2007).

Using the RACI data it can be determined which assay best determines the antioxidant activity of the samples evaluated. Sun and Tanumihardjo, (2007) correlated RACI with each of the assays used to determine antioxidant activity and the correlations were found between RACI vs ABTS (0.79), RACI vs FRAP (0.84), RACI vs ORAC (0.56), RACI vs PAOXI (0.44) and RACI vs TRAP (0.87). In this study the RACI for each sample was calculated using the DPPH, TEAC and ORAC data.

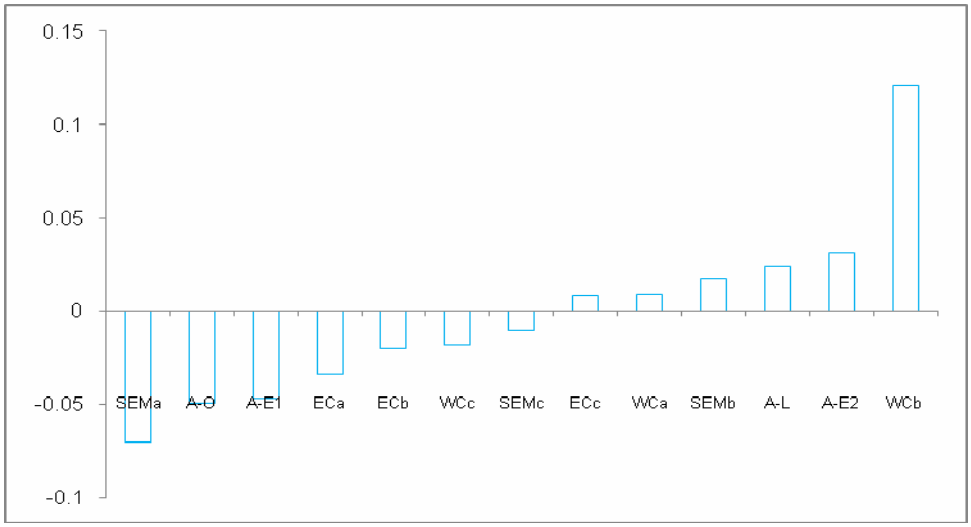


Figure 4.7: Graph representing RACI of three antioxidant assays used (TEAC, DPPH and ORAC).

Based on RACI data WCb was identified as having the highest relative antioxidant activity, while SEMb with the highest TEAC and ORAC values was identified as having the fourth highest RACI, as shown in Figure 4.7 By determining the correlation between RACI and a combination of assays, the two antioxidant assays's that contribute significantly to RACI can be identified. DPPH and TEAC were found to be the best antioxidant assays to use in combination (RACI = 0.99), as shown in Table 4.5. This may be due to the chemistry of the assays which are ET-based. For DPPH and ORAC assays a high level of correlation was found while a poor correlation between RACI and ORAC+TEAC was found.

Table 4.5: Correlations of RACI vs antioxidant assays

RACI vs combined assays	**RACI vs assays**
DPPH + ORAC $r^2 = 0.94$	DPPH $r^2 = 0.93$
DPPH + TEAC $r^2 = 0.99$	ORAC $r^2 = 0.21$
ORAC + TEAC $r^2 = 0.53$	TEAC $r^2 = 0.58$

4.4.2 Biological assays

Two main components of cells whose integrity is both essential to cell viability and growth is DNA (genomic and mitochondrial) and the cell membrane, the plasma membrane being the first site of exposure. Oxidants cause damage to several biomolecules such as membranes, protein, RNA and DNA which can eventually lead to cellular death via apoptosis and/or necrosis.

4.4.2.1 DNA protection studies

The plasmid assay is based on the principle whereby radicals such as AAPH cause supercoiled plasmid DNA to change to linear or circular forms. Single strand breaks are responsible for the formation of circular forms while double strand breaks lead to the formation of linear forms. Further damage leads to the further fragmentation of the linear form with the formation of smaller fragments. These forms have different rates of mobility in an agarose gel i.e. the supercoiled form (Figure 4.8, A) will migrate more rapidly than the linear forms (Figure 4.8, B). Devasagayam *et al.,* (1995), observed the ability of flavonoids to protect the pBR322 plasmid DNA against ROS and concluded that ROS causes two types of damage, base alterations and strand breaks with the former being more common but the latter being quantifiable. In this study the ability of each type of honey to protect the pBR322 plasmid against AAPH-induced oxidative damage was investigated. Due to the smaller volume of the supercoiled pBR322 plasmid DNA, this form migrated further than the linear form. The plasmid sample contains small amounts of linear DNA. Following exposure to AAPH all the plasmid DNA becomes linear and migrates less rapidly through the gel than the supercoiled form (Lane 3). Further fragmentation results in a band that has less fluorescence than the other bands. The presence of trolox protects plasmid DNA against oxidative damage (Lane 4-6) however high concetrations of trolox can cause strand breaks (Lane 7). To varying degress all honey samples were able to protect plasmid DNA against the strand breaking effects of AAPH (Lanes 7-20).

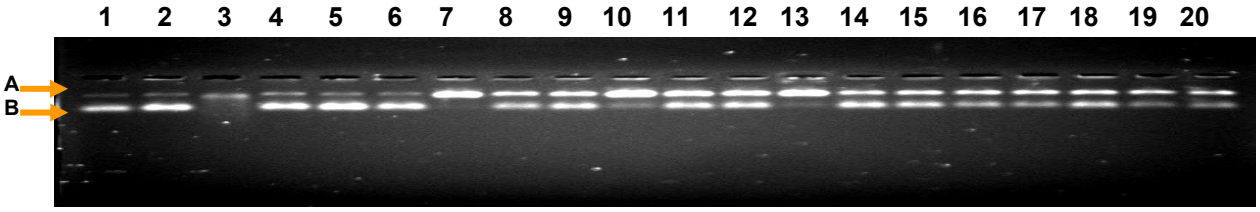


Figure 4.8: Agarose gel electrophoretic pattern of pBR322 DNA alone (1 and 2) and exposed to 5.5 x 10^{-4} µM AAPH and 3) alone and to 4) 140 µM trolox; 5) 280 µM trolox ; 6) 430 µM trolox; 7) 570 µM trolox; 8) SEMa; 9) WCa; 10) SEMb; 11) WCc; 12) SEMc; 13) ECc; 14) A-E2; 15) WCb; 16) SEMa; 17) A-E1; 18) A-O; 19) ECa; and 20) Ecb, all honey at a final concentration of 1%.

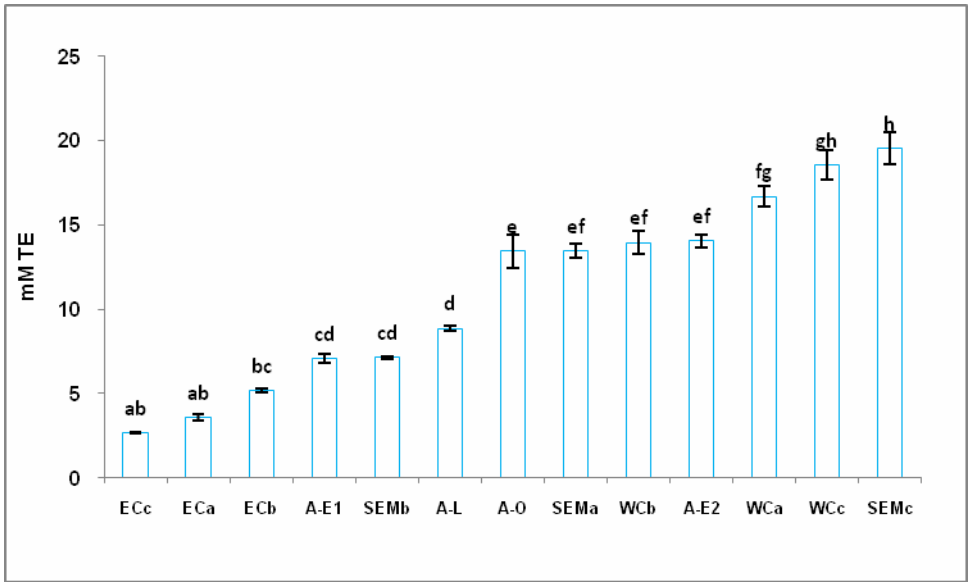


Figure 4.9: Ranking of honey samples ± SEM values, according to increasing protection of pBR322 plasmid DNA against strand breaks by AAPH. Means of honeys with different letters are significantly different, p≤0.001.

Quantification of this effect revealed that honeys; SEMc and WCc, provided the highest level of protection. In contrast ECc (not taken into account, due to possible adulteration) and ECa showed the lowest degree of protection (Figure 4.9). Devasagayam *et al.,* (1995), observed that natural antioxidants such as carotenoids and flavonoids were effective in preventing DNA damage caused by singlet oxygen. The flavonoids that showed protection were: myricetin (43.4%), luteolin (16.5%), apigenin (7.9%), and (+) catechin (30.3%) and protection to some degree was related to the structure of these molecules. Another flavonoid that also effectively protects plasmid DNA against H_2O_2-induced is hesperedin (Kalpana *et al.,* 2008). Honey contains several different polyphenols, at different concentrations, resulting in either an additive or synergistic protective effect against oxidative damage as has been described for EGCG and trolox (Wei *et al.,* 2006). The ability of honey to protect plasmid DNA indicates that the anitoxidants present in honey have the ability to protect both mitochondrial and genomic DNA against oxidative damage. This effect was not related to the colour, TPC, TFC as well as the antioxidant activity (DPPH, TEAC and ORAC) of these samples (Table 4.6).

4.4.2.2 Erythrocyte membrane protection studies

Erythrocytes contain no DNA and are an example of a typical cellular bilayer and haemolysis occurs as a direct result of damage to the cell membrane. This is the basis of an assay in which the ability of antioxidants to protect against ROS-induced disruption of the cell membrane is studied (Tabart *et al.,* 2009, Ximenes *et al.,* 2010). AAPH-induced haemolysis is the result of lipid peroxidation, oxidation of the membrane proteins that disrupts normal

membrane structure. AAPH also causes the depletion of the intracellular glutathione. In this study the ability of each honey sample to protect erythrocytes against AAPH-induced oxidative damage was investigated. SEMa, SEMc and ECa had the greatest ability to protect the erythrocytes against oxidative damage, while WCa, WCc and SEMb had the lowest ability (Figure 4.10). Differences between donors such as age, antioxidant status and lipid compsition all contribute the differences found between donors and this accounts for the large varaition in the standard error of mean in Figure 4.10. Of these samples SEMa is the second darkest honey and shows the highest protection and in contrast, SEMb with the highest A450 had the lowest protection against oxidative damage.

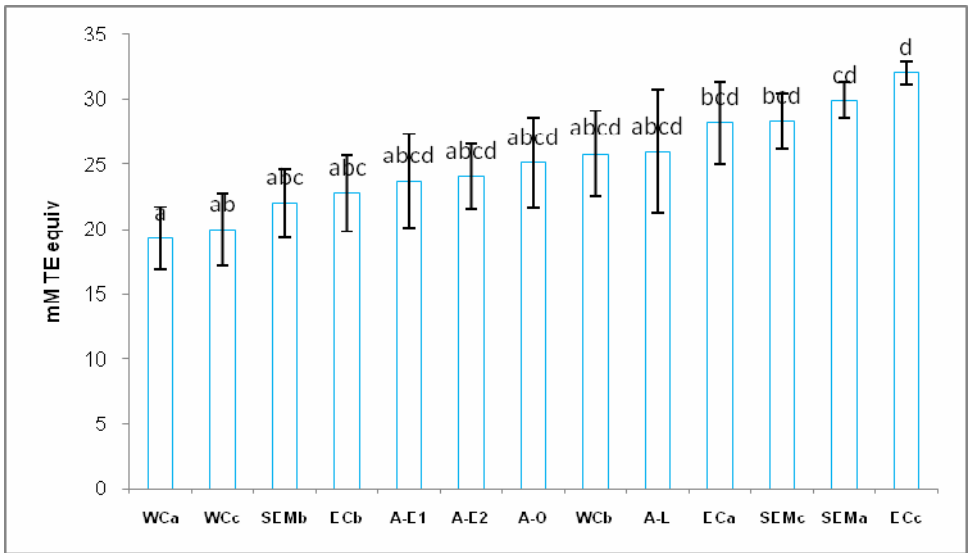


Figure 4.10: Ranking of honeys according to ability to protect human erythrocytes against AAPH induced haemolysis. Means of honeys with different letters are significantly different, p≤0.001.

No correlation was obtained between the haemolysis and plasmid assays (Table 4.6), as SEMc showed high potection with both assays, while ECa showed the lowest protection in the plasmid assay and WCa in the haemolysis assay. Furthermore no correlation was obtained between haemolysis assay and all other parameters (Table 4.6). These results concur with the study done by Tabart *et al.,* (2009), where the abliity flavonoid and phenolic acid componds to protect erythrocytes against oxidative damage was investigated. The correlation between haemolysis and DPPH, and TEAC in this study was very low, $r^2 < 0.08$, the highest correlation being with ORAC, $r^2 = 0.396$. Ximenes *et al.,* (2010), gave a probable explantion for this as the binding of flavonoids to the erythrocyte membrane as well as their ability to penetrate the membrane. These authors studied the protective effect of gallic acid and the esters thereof on AAPH-induced haemolysis and the depletion of erythrocyte glutathionine. It was found that inhibition of haemolysis was dosage related and the ester derivatives of gallic acid were more effective at preventing haemolysis than gallic acid. Two factors identified that were important for the prevention of haemolysis was radical

scavanging activity as well as the lipophilicity of antioxidants. In addition Ginsburg *et al.,* (2011), reported that cells bind polyphenols and thereby acquire enhanced oxidant-scavenging activity. Therefore the haemolysis assay measures the sum of these effects whereas antioxidant assays measure only the free radical scavenging effects. All honey samples were evaluated at a single concentration. In cellular systems several molecules that are antioxidants at low concetrations become pro-oxidants at high concentrations these include EGCG (Suh *et al.,* 2010) and gallic acid (Ximenes *et al.,* 2010). This may account for the differences seen for SEMa and SEMb in the plasmid assy, as well as for trolox which shows pro-oxidant activity at its highest concentration (Figure 4.8, lane 7).

Table 4.6: Correlation between assays used for the determination of antioxidant activity (DPPH, TEAC, ORAC) with the haemolysis assay and plasmid DNA assay.

	Haemolysis assay	Plasmid DNA assay
DPPH	r = 0.02, r^2 = 0.0005, p=0.00000000006	r = 0.11, r^2 = 0.01, p = 0.000006
	***Not significant**	**Not significant**
TEAC	r =0.029, r^2 = 0.00089, p=0.000001	r = 0.1, r^2 = 0.01, p = 0.78
	Not significant	**Not significant**
ORAC	r = 0.0098, r^2 = 0.0000096, p=0.6	r = 0.18, r^2 = 0.03, p = 0.007
	Not significant	**Not significant**

*Significance determined on a 95% confidence level, using 11 degrees of freedom.

4.5 Conclusion

Honey samples from Southern African region have high antioxidant activity that correlate with colour, polyphenol and flavonoid content. Of these honey samples SEMb had highest antioxidant activity according to the TEAC and ORAC assays, and A-E2 and WCb have the highest relative antioxidant capacity index. Samples SEMc and WCc best protected the pBR322 plasmid DNA while samples SEMc and SEMa best protected human erythrocytes against AAPH-induced oxidative damage. No correlation was obtained between these biological assays and colour, polyphenol and flavonoid content or antioxidant assays.

CHAPTER 5: CELLULAR EFFECTS OF THE ANTIOXIDANT ACTIVITY OF A SELECTION OF SOUTHERN AFRICAN HONEY SAMPLES

5.1 Introduction

To confirm the biological significance of the antioxidant effect of honey it was necessary to evaluate these honeys in cell culture, as *in vitro* systems mimic some of the original tissue characteristics such as cell-cell interactions and metabolism (Zucco *et al.,* 2004). Screening using cell systems will provide further information regarding cellular absorption, distribution, metabolism and excretion (ADME).

Chemical assays, such as those used in Chapter 4 provide information on the antioxidant potential of the samples but provides little information regarding antioxidant activity within a cellular environment. Differences between chemical and cell-based assay systems are summarized in Table 5.1.

Table 5.1: Summary of differences between chemical and cell based assays

	Chemical assays (e.g. ORAC)	Cell-based assays (e.g DCFH-DA)
Assay principles	Based on known chemical reactions between limited number of reagents	Based on interaction between added compounds and complex cellular systems
Data analysis	Quantitative	Qualitative
Linear dose-responses	Expected	Not expected

*Table adapted from Honzel *et al.,* (2008).

The cytotoxicity of honey related to inhibiting the growth of cancer cells as well as the ability to promote wound healing has been widely investigated *in vitro*. Few *in vitro* studies specifically related to the antioxidant activity of honey have been undertaken. This may be due to problems related to polyphenols binding protein such as albumin in serum which is used to grow cells *in vitro* (Zucco *et al.,* 2004), the chemical nature of reactive oxygen species (ROS), which are produced in large quantities at a biological site and therefore a whole arsenal of antioxidants are needed to cover the whole spectrum of ROS's (Blasa *et al.,* 2011). The reducing capacity of low molecular weight antioxidants (LMWA) which scavenge ROS by reduction (donating an electron), in the presence of metals (found in cell culture medium), LMWA become pro-oxidants (Blasa *et al.,* 2011). Antioxidant effects in various cell lines have been studied using the dichlorofluorescein diacetate (DCFH-DA) assay, for example, in neuronal cells (Kanski *et al.,* 2002), and in erythrocytes and is known as the CAP-e (cell-based antioxidant protection in an erythrocyte model) assay (Honzel *et*

al., 2008). In the CAP-e assay erythrocytes containing dichlorofluorescein diacetate (DCFH-DA) are exposed to antioxidants and then an oxidant such as AAPH is added. Oxidative damage causes the nonfluorescent DCFH-DA to form fluorescent diacetate (FDA) (Honzel *et al.,* 2008). The ability of antioxidants to prevent this increase in fluorescence is measured. This assay simply answers questions whether antioxidants enter and protect cells or do these antioxidants protect the surrounding environment of the cell? Blasa *et al.,* (2011), used a modified version of the CAP-e assay, the cellular antioxidant activity in red blood cells (CAA-RBC) assay and compared it with the chemical assay ORAC and concluded that the CAA-RBC assay is more predictive than ORAC, in quantifying absorption of antioxidants. In this study, instead of erythrocytes, the SC-1 and Caco-2 cell lines were used. The SC-1 cell line is a rapidly growing mouse fibroblast cell line, which is used in our laboratory for the evaluation of toxic effects. The human colon adenocarcinoma cell line, Caco-2 is a physiologically relevant cell type.

According to the definition of an antioxidant, antioxidants mediate their effect at low concentrations compared with those of an oxidizable substrate and significantly delays or prevents oxidation of that substrate including various types of molecules *in vivo* (Perez *et al.,* 2006). Therefore prior to testing honeys for antioxidant activity it was necessary to determine whether concentrations of AAPH (the oxidant used) and honey (the antioxidant) was cytotoxic and this was done using the SC-1 cell line.

The main aim of this study was to determine the protective effects of honeys from the Southern African region against oxidative damage in the SC-1 and Caco-2 cell lines.

The specific objectives of this study are:

1. To determine if the concentration of AAPH used in the short-term DCFH-DA assay translates into significant long-term (72h) cellular damage.
2. To determine the effect of 0.008% and 0.8% honeys on the SC-1 cell line, using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and the combined Neutral Red (NR) and Crystal violet (CV) assays.
3. Using the DCFH-DA assay, determine whether honeys, (0.025 and 2.5%) protects the SC-1 and Caco-2 cell line against AAPH-induced oxidative damage.
4. Finally, to determine the correlations between antioxidant content (Chapter 3) and, activity (Chapter 4) and cellular antioxidant protective assays.

5.2 Materials

Mouse fibroblasts (SC-1) and human colon cancer cells (Caco-2) were obtained from Highveld Biological Company, Johannesburg, SA.

The media and supplements used were: Eagles Minimum Essential Medium (EMEM) powder, foetal calf serum (FCS) and antibiotic solution (containing, streptomycin, penicillin and fungicide) were obtained from Highveld Biological Company, Johannesburg, SA. Dulbecco's Modified Essential Medium (DMEM), Neutral Red (NR), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Crystal Violet (CV) and dichlorofluorescein diacetate (DCFH-DA) were obtained from Sigma-Aldrich, Atlasville, SA. Fixatives, acids, salts and organic solvents such as: glutaraldehyde, acetic acid, formic acid, isopropanol were analytic grade and together with ethylene diamine tetra acetate (EDTA) and dimethyl sulphoxide (DMSO) 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. Water was double distilled and de-ionised (ddH_2O) with a continental water system and all medium, enzyme solutions and buffers were sterilized by filtration through a Millex 0.2 µm filter. Glassware was sterilized at 121°C for 20 min in a Prestige Medical Autoclave (series 2100).

Disposable plasticware included: 96 and 24 well plates, 25 cm^2 and 75 cm^2 tissue culture flasks, 50 ml and 15 ml centrifuge tubes, 10 and 5 ml pipettes and pipette tips (10, 25, 100, 200, and 1000 µl) and were obtained from either Greiner Bio-one supplied by LASEC, Cape Town, S.A or NUNC™ supplied by AEC-Amersham, Johannesburg, SA.

All laboratory facilities were the same as used in Chapter 3 and 4.

5.3 Methods

Honey samples

The same samples collected and evaluated in Chapters 3 and 4 were used. The honey samples were diluted with ddH_2O containing 1% antibiotics, to 5% (w/v) and 0.05% (w/v), sterile filtered, aliquoted and stored at -20ºC until needed.

Cultivation, maintenance and preservation of the SC-1 and Caco-2 cell lines

The SC-1 and Caco-2 cell lines were maintained in EMEM and DMEM respectively, each supplemented with 10% Foetal Calf Serum (FCS) (EMEM/FCS and DMEM/FCS) and a 1% antibiotic solution. An antibiotic stock solution was prepared by mixing 10,000 µg/ml Penicilin G (sodium salt), 10,000 µg/ml Streptomycin sulphate and 25 µg/ml Amphotericim B in 0.85% saline. A volume of 10ml of the working solutions was added to 1 litre of the prepared medium.

The cells were plated at $4x10^4$ cells per ml in 25 cm^2 and 75 cm^2 cell culture flasks and were maintained until confluency at 37°C at 5% CO_2. Once confluent, the cells were passaged with a 5% trypsin solution prepared in 100 ml phosphate buffered saline (PBS).

For the SC-1 cells, the cells were passaged by removing the medium from the confluent monolayer then adding 1 ml of a 5% trypsin solution and the flask was placed at 37°C for 1 - 2 min. A 5 ml volume of medium was then added to the trypsin solution containing detached cells, and transferred to a 15 ml centrifuge tube and the cells were collected by centrifugation at 7000xg for 2 min. The medium was removed and the cells were resuspended in 5 ml EMEM/FCS. The numbers of cells were determined by counting a 10 µl aliquot of cells using a haemocytometer.

For the Caco-2 cells, the cells were passaged by removing the medium from the confluent monolayer. The monolayer was rapidly then rinsed with a 5 ml, 0.53 mM EDTA/PBS solution, which was prepared by mixing a 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4 and 0.53 mM EDTA. The Caco-2 cells were then processed further as described for the SC-1 cell line.

These cell lines were either used for experiments described below or stored for later use at -70°C. For storage, the cells were suspended in cell culture freezing medium at a concentration of $2x10^5$ cells per ml. The freezing medium was prepared by adding 10% DMSO and 80% FCS to EMEM or DMEM. A volume of 1.8 ml was transferred to the freezing vials and stored by slow freezing (first placed on ice, then in -20°C and finally in a -70°C freezer). The cells were stored for a maximum of 6 months with minimal loss of viability.

For the following experiments, the vials containing the cells were thawed rapidly in warm water at 37°C. The cells were suspended in 5 ml medium supplemented with FCS, and

collected by centrifugation. The supernatant was removed and the cells were suspended in fresh medium and plated in 25 cm^2 culture flask.

5.3.1 *In vitro* cellular effects

The effect of AAPH on the SC-1 cell line using the MTT and combined NR/CV assay

Cells were plated at a cell concentration of $1x10^4$ cells per 500 µl in 24 - well flat bottom plates and were incubated for 24 h at 37°C and 5% CO_2 to allow the cells to attach to tissue culture surface before conducting experiments. The SC-1 cell lines were then exposed to 0 – 4.03 mM AAPH for 72 h.

Cell viability, lysosomal membrane integrity and cell number were determined using an MTT assay and a combined NR and CV assay. The procedure was as follows: For the MTT assay, a 1 mg/ml MTT solution was prepared in PBS. A 25 µl volume of the MTT solution was added to each well and cell culture plates were maintained for a further 3 h at 37°C. The medium was then carefully removed and the plates were blotted dry. The MTT formazan crystals were then dissolved with 200 µl of a isopropanol:HCl solution (24:1(1MHCl)) by shaking the plates for 5 min. The extracted formazan was then transferred into a 96 – well flat bottom plate. The absorbance was determined at 570 nm, on a Biotek plate reader. For the NR and CV combined assay, to each well 10 µl 0.15% NR prepared in PBS was added and incubated for 90 min at 37°C. The medium was then carefully removed and the plates were blotted dry. The cells were then fixed for 10 min with 200 µl of a 1% acetic acid and 1% formaldehyde solution in ddH_2O. The fixative was removed and the NR was solubilized with 200 µl of a 1% acetic acid and a 50% ethanol solution prepared in ddH_20. The dissolved NR was transferred into a 96 – well flat bottom plate, and absorbance was determined at 570 nm. The plates were then dried well either by placing them open on the benchtop for 48 h or placing plates in the microwave at 60% for 5 min, or until the dried layer of cells could be observed. The cells attached to the bottom of the plate were then stained by adding 500 µl of a 0.1% (w/v) CV solution prepared in 200 mM of formic acid, pH 3.5, to each well for 30 min. The microplate was washed with ddH_2O and dried as described above; the bound dye was dissolved in 10% acetic acid prepared in ddH_2O. The solution was transferred into a 96 – well flat bottom plate, and absorbance was determined at 630 nm. For the MTT, NR and CV assays data was expressed as percentage compared to the control (no AAPH added).

The effect of honey samples on the SC-1 cell line using the MTT and combined NR/CV assay

Cells were plated at a cell concentration of $1x10^4$ cells per 500 µl in 24 – well flat bottom plate and were incubated for 24 h at 37°C and 5% CO_2 to allow the cells to attach to tissue culture surface before conducting experiments. The SC-1 cell lines were then exposed to 100 µl volume of 0.05% w/v and 5% (w/v) honey samples for 72 h, final concentration 0.008 and 0.8% (w/v), respectively. Cell viability, lysosomal membrane integrity and cell number were determined using an MTT assay and a combined NR and CV assay, as explained above.

The protective effect of honey samples against oxidative damage on the SC-1 and Caco-2 cell lines

Cells (SC-1 and Caco-2) were plated at a cell concentration of $2x10^4$ cells per 100 µl in 96 – well flat bottom plates and were kept for 24 h at 37°C and 5% CO_2 to allow cells to attach to well surfaces before experiments were conducted.

Oxidative damage

Cytotoxicity due to oxidative damage was determined as shown in Figure 5.1A. For this a 20 µM solution of DCFH-DA was prepared as follows. A 1 mg/10 ml, 200 µM stock solution was prepared, from which a 20 µM working solution was prepared. A 40 µl volume of the DCFH-DA solution was added to each well. Fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 520 nm. The change in fluorescence from 0 - 60 min was measured every 2 min using a FLUOstar OPTIMA plate reader.

Total protective effects

Total protective effects were evaluated as shown in Figure 5.1B. For this 20 µM solution of DCFH-DA was prepared as described above. Volumes of 40 µl DCFH-DA solution were added to each well and cell culture plates were maintained for a further 1 h at 37°C. The medium containing the DCFH-DA solution was then carefully removed. Cell culture plates were washed once with PBS, and plates were blotted dry. A volume of 40 µl honey sample (0.05 and 5% (w/v)) was then added to each well of the cell culture plates followed by a 15 mM, 40 µl volume of AAPH, the AAPH having a final concentration of 7.5 mM, and the honey samples having a final concentration of 0.025 and 2.5% (w/v). Change in

fluorescence was measured immediately over 0 - 60 min, every 2 min. The gradient of the change in fluorescence was calculated, and the data was expressed as % damage where AAPH alone causes 100% damage.

Intracellular protective effects

Intracellular protective effects were evaluated as shown in Figure 5.1C. For this DCFH-DA was prepared as described above. Volumes of 40 µl of DCFH-DA solution was added to each well followed immediately by 40 µl of honey sample (0.05 and 5% (w/v)), final concentration 0.025 and 2.5% (w/v). Cell culture plates were maintained for a further 1 h at 37°C. The medium containing the DCFH-DA and honey was then carefully removed. Cell culture plates were washed once with PBS, and plates were blotted dry. To each well a 7.5 mM, 40 µl volume of AAPH was added. Change in fluorescence was measured as described above and the % damage was calculated as described above.

A. Cytotoxicity			
Cells + DCFH-DA	Uptake, 1 hr →	Add honey or AAPH	Immediately measure change in fluorescence, 1h
B. Total antioxidant protection			
Cells + DCFH-DA	Uptake, 1hr →	Add honey +AAPH	Immediately measure change in fluorescence, 1h
C. Intracellular antioxidant protection			
Cells + DCFH-DA + honey	Uptake, 1 hr →	Wash cells, add AAPH	Immediately measure change in fluorescence, 1 h

Figure 5.1: Cytotoxicity, total and intracellular methods used in evaluating the SC-1 and Caco-2 cell lines.

Data management and statistical analysis

All data management and statistical methods used were the same as in chapter 3. Several factors were correlated using analysis of variance (ANOVA), using samples as independent variables and the values determined as dependent variables. Fisher's least significant difference (LSD) test was used for comparison of means using statistica software Version 9.0 (StatSoft, Tulsa, OK). Correlation analysis was also run with the same statistical package, and Microsoft Excel 2007 (students T-test). P-values of 0.05 or less were considered significant.

5.4 Results and discussion

5.4.1 *In vitro* cellular effects

The effect of AAPH on SC-1 cellular function

Biological assays such as the erythrocyte haemolysis assay and the plasmid assay do not take into account absorption, distribution, metabolism and excretion. Cell lines provide this information at a cellular level and include absorption from the extracellular enviroment, distribution to different cellular compartments such as the cytoplasm, lysosomes and mitrochondria. The effect on cellular functioning can involve either the upregulation or down regulation of specific genes. As these genes are important for normal cell functioning after several population doublings, this can translate either into cellular death, cellular senescence or an increase in the rate of cellular growth (Kannan *et al.,* 2009; Li *et al.,* 2006). This translates in a decrease in cell number; a cytotoxic effect, no change and an increase in cell number; a mitogenic effect. Using the MTT, NR and CV assays compared to the control, for cytotoxicity a decrease and for mitogenic effect an increase in the measured parameters will be found. Each of these assaya measures different aspects of cellular function, the MTT assay measures mitochondrial activity and is an indication of cellular viability. NR is taken up and accumulates in the lysosomes of viable cells while CV stains protein and is an indirect measure of cell number (Chiba *et al.,* 1998).

For the DCFH-DA assay it was nessary to determine if the concentrations used translated into cellular damage. The SC-1 cells are a normal rapidly growing mouse fibroblast cell line that often used in our laboratory for the optimization of toxicity studies. The SC-1 cells were exposed to AAPH for 72 h, after which lysomal membrane integrity and cellular viability and number was determined using the NR, MTT and CV assays respectively (Figure 5.2A-C). An exponential decrease in cell viability and lysosomal membrane integrity was observed from 100% to 80%, and 60% and 40% for the MTT and CV and NR assays, respectively. Changes for all assays were significant at a concentration of 0.78 mM. CV binds protein is and indirectly considered to be an indicator of cell number and this is based on the assumption that the compound evaluated does not have an effect on cellular protein composition. Effect on cell number or rather the binding of CV to the proteins of the SC-1 cells was more dramatic with a significant change at 0.28 mM AAPH. AAPH is a powerful oxidant and the first cellular target is the cell membrane subsequent damage causes protein leakage (as is also seen in the haemolysis assay) and a decrease in CV staining. Both the lysosome and mitochondria are present in the cytoplasm often close to the cell membrane

and therefore would be secondary targets of AAPH-induced damage. For the antioxidant assays, the SC-1 and Caco-2 cells were exposed to 5 mM AAPH for 60 min, a concentration that results in significant changes in cell viability, cell membrane integrity and cell number.

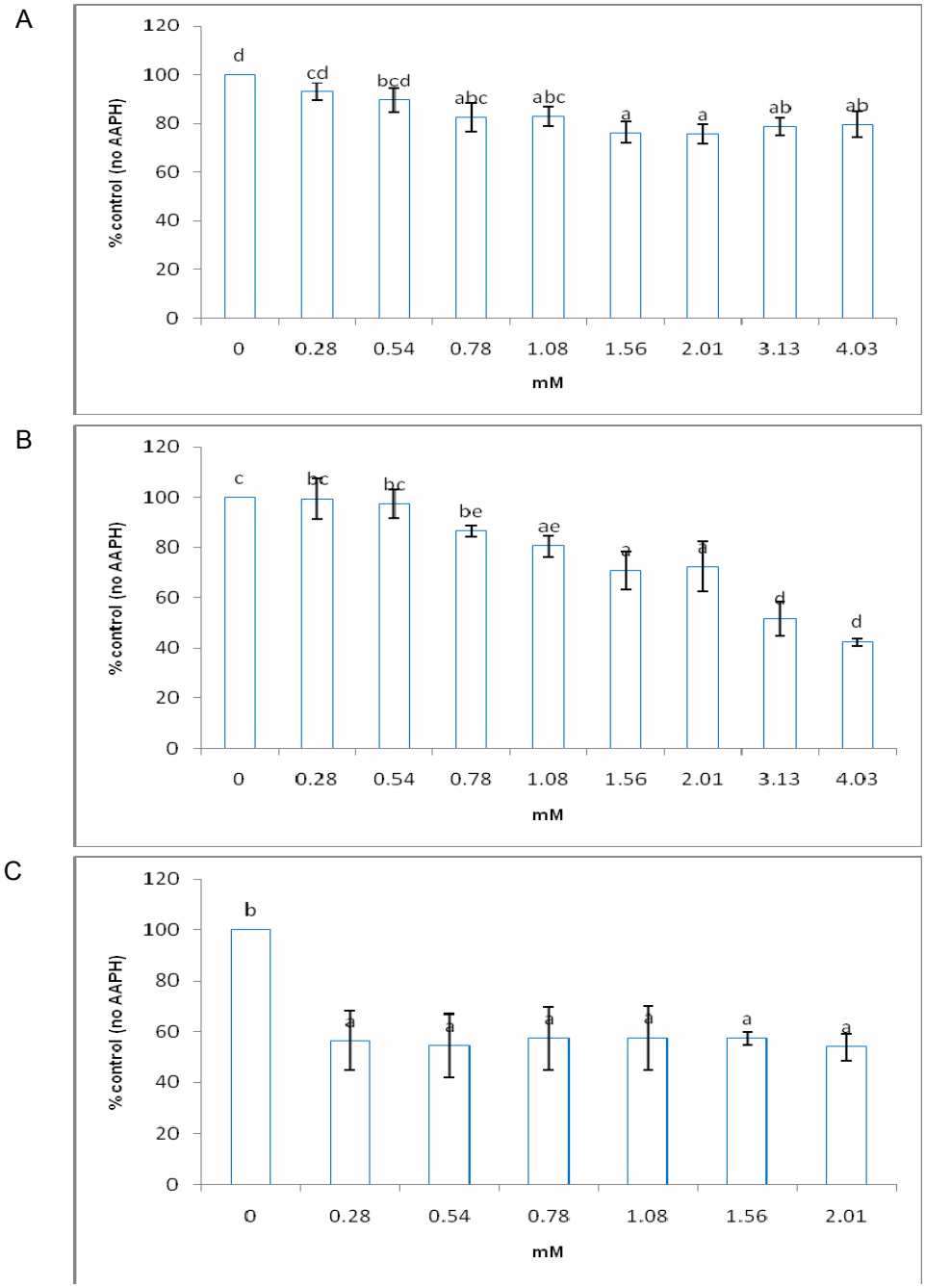


Figure 5.2: The effect of AAPH on the viability of SC-1 cells after exposure for 72 h. Lysosome membrane integrity as well as cell viability and number determined using the MTT, NR and CV assays respectively. Data is an average of three experiments ± SEM. Means of AAPH with different letters are significantly different, p≤0.001.

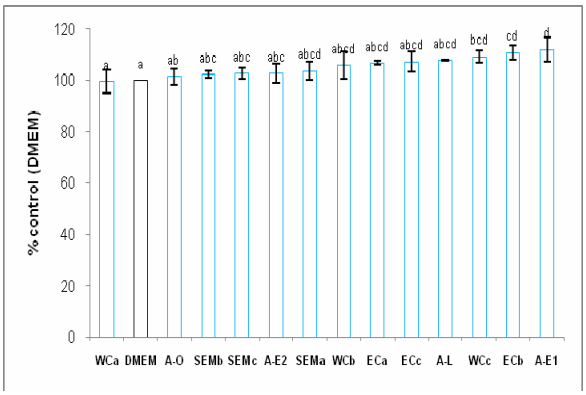
The effect of honey on SC-1 cellular function

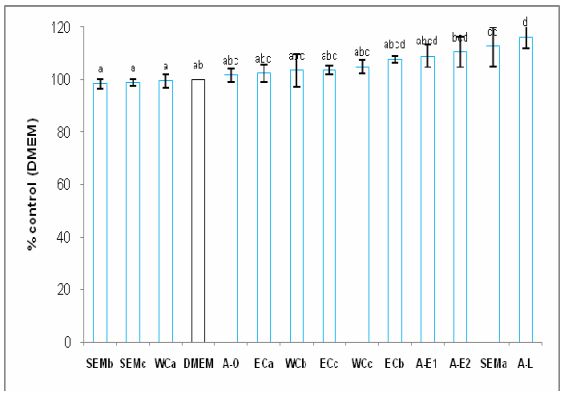
For antioxidant studies the samples evaluated should have no cytotoxic effects and for this purpose SC-1 cells were exposed to 0.008% and 0.8% honey solutions and the lysosomal membrane integrity and cell viability and number were determined. Honey is a complex mixture of different compounds including sugars, enzymes and phenolic compounds and due to the complexity of honey it can have several effects on cell cultures and this includes no effect, not toxic (NT), a toxic or mitogenic effect. Following 72 h exposure cell vaibility, lysosomal membrane integrity and cell number was determined with the MTT, NR and CV assays respectively (Figure 5.3 A-C). For each assay, data was expressed as percentage control (100%). Samples to the left of the control (DMEM containing 5% FCS) (see Figure 5.3), indicates a possible toxic effect (% < control) while to the right of DMEM a mitogenic effect (% > control). These results are summarized in Table 5.2.

An increase in cell viability (MTT assay) was observed for WCc, ECb, A-E1 at 0.8% and A-E2, SEMa, A-L at 0.008% (Table 5.2). An increase in lysosomal membrane integrity (NR assay) was observed for ECb at 0.008%. No increase in cell number (CV assay) was observed for any of the samples. For other samples no change in cell viability was observed, but a decrease was observed for the NR assay at 0.008% for A-L and at 0.8% for WCa and A-E2. No changes were observed for the CV assay at 0.008% but decreases were observed at 0.8% for ECa, SEMc, WCa, A-O, A-E1 and A-L.

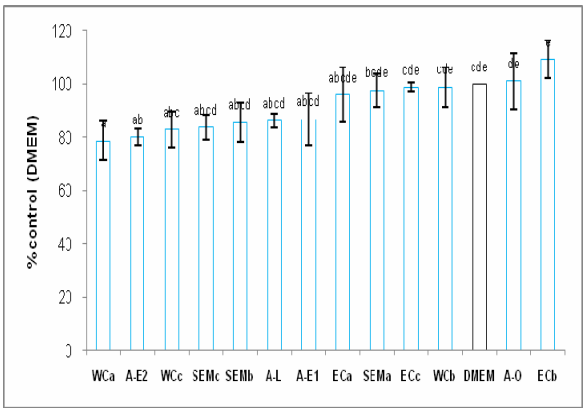
0.8%	0.008%

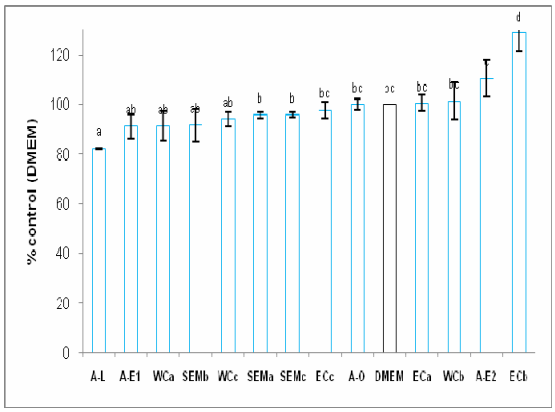
MTT



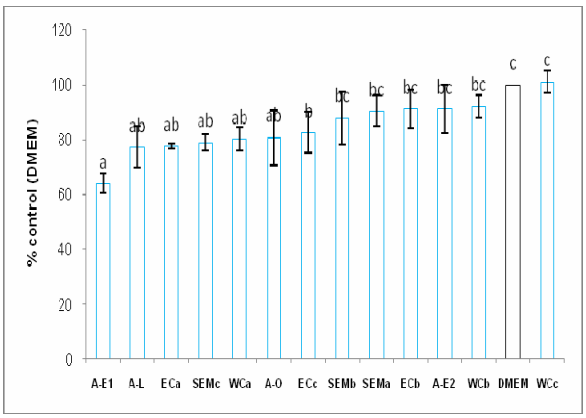


NR





CV



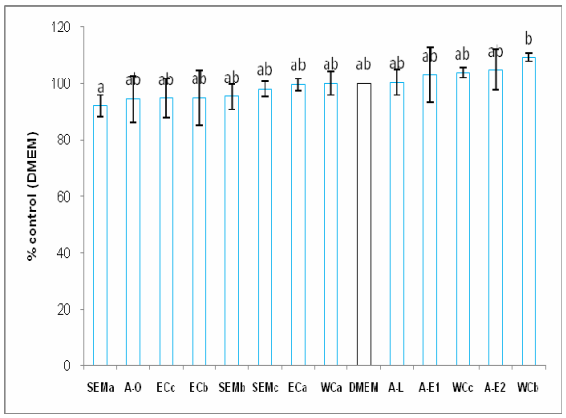


Figure 5.3: Graphs representing the effect of honey (0.8% and 0.008%) on SC-1 cells viability using different assays, MTT, NR and CV respectively after 72 h incubation. Average of 3 independent experiments, each assay point in quadruple. Data expressed as Mean ± SEM. Means of honeys with different letters are significantly different, p≤0.001.

Table 5.2: Summary of the effect of 0.008% and 0.8% on cellular vaibility, lysosomal membrane integrity and cell number

	Honey samples												
	AE1	**AE2**	**A-L**	**A-O**	**ECa**	**ECb**	**ECc**	**SEMa**	**SEMb**	**SEMc**	**WCa**	**WCb**	**WCc**
						0.008%							
MTT	-	↑ (10%)	↑ (16%)	-	-	-	-	↑ (12%)	-	-	-	-	↑ (5%)
NR	-	-	↓ (18%)	-	-	↑ (29%)	-	-	-	-	-	-	-
CV	-	-	-	-	-	-	-	-	-	-	-	-	-
	NT	NT		NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
						0.8%							
MTT	↑ (12%)	-	-	-	-	↑ (10%)	-	-	-	-	-	-	-
NR	-	↓ (20%)	-	-	-	-	-	-	-	-	↓ (21%)	-	-
CV	↓ (36%)	-	↓ (22%)	↓ (20%)	↓ (23%)	-	-	-	-	↓ (22%)	↓ (20%)	-	-
						NT	NT	NT	NT			NT	NT

NT=not toxic

Samarghandian *et al.,* (2011), observed that the viability of human renal cancer lines (ACHN) exposed for 24, 48 and 72 h to honey samples at a concentration of 0-1% varied from 80-100% (measured using the MTT assay) and at a higher concentration of 20% a dose dependent decrease in cell viability was observed. Kannan *et al.,* (2009), observed that in the human osteoblast cell line (CRL 1543), exposed to 0.02% honey for 72 h a significant increase in cellular proliferation from 100% (control) to 105.3%, using the MTT assay. With increasing concentrations of honey, these authors observed that cell viability decreased and at 1% honey it had decreased to almost 60%. Indications from these studies are that low concentrations of 0.02% causes an increase in mitochondrial functioning measured with the MTT assay. Li *et al.,* (2006), reported an upregulation of the superoxide dismutase, catalase, glutathione, glutathione reductase, glutathione peroxidase, glutathione S-transferase (GST), and NAD(P)H:quinone oxidoreductase-1 (NOQ1) following exposure of aortic smooth muscle cells to the antioxidant resveratol. Enzymes superoxide dismutase (Madesh and Balasubramanian, 1998), NAD(P)H:quinine (Prochaska and Santamaria, 1988) and glutathione S-transferase (York *et al.,* 1998) are mitochondrial enzymes and are able to metabolise MTT. In cell culture the upregulation of these enzymes would result in a measured increase in cell viability. This was reported by Kannan *et al.,* (2009), for the human osteoblast cell line (CRL 1543) exposed to 0.02% honey for 72 h. Likewise in this study exposure to 0.008% honey for 72h resulted in an increase of 105-129% compared to control, for A-E2, A-L, SEMa, WCc at 0.008% and ECb (0.8%). This is not a mitogenic effect as there is not an increase in cell number but rather an elevated cellular response (Li *et al.,*

2006). A similar response was also observed by Danz *et al.,* (2009), in Doxorubicin (DOX), a drug used as an antibiotic in the treatment of cancers. The authors found mitochondrial stabilization following exposure of neonatal rat ventricular myocytes and DOX-induced damaged cardiomyocyte to resveratol. This elevated cellular response observed in this study, may make cells resistant to the oxidative and electrophilic stress.

At 0.8% A-E1, A-E2, A-L, A-O, ECa, SEMc and WCa shows toxicity and this effect is 19-36%. Therefore the effect of honey in the SC-1 cell line is concentration dependent where low concentrations result in an improved cellular defense reponse and high concentrations cytotoxic effects. Variable effects between samples can be due to cell to flavonoid concentration ratio, cell and flavonoid type, degree of flavonoid metabolic conversion, and nature of preexisting or subsequent stress and cell culture conditions (Bestwick and Milne, 2001). This effect is further demonstrated using the effect of quercetin, a known natural antioxidant. This effects were studied using HL60 (lymphocyte) cells by Bestwick and Milne, (2011), and A549 (lung cancer) cells by Robaszkiewicz *et al.,* (2007). In the lung cancer cells, low concentrations of quercetin were found to have no effect on cellular proliferation whereas increased concentrations caused apoptosis and necrosis. A similar effect was also reported for quercetin in lymphocytes (Bestwick and Milne, 2011; Robaszkiewicz *et al.,* 2007).

To summarize, in the SC-1 cell line AAPH shows significant toxicity. For the honey samples at a concentration of 0.008% no cytotoxicity was found but at a concentration of 0.8% several samples did show cytotoxicity. These results were obtained following 72 h exposure; therefore the assumption can be made that at the same honey concentrations over a period of 1h no significant toxic effects may be observed. Attempts were made to develop an *in vitro* antioxidant assay using the MTT, NR and CV assays. This was unsuccessful and this could be due the binding of polyphenols to albumin the major protein found serum, the inherent free readical scavenging effects of albumin as well as the end point that is measured. For these reasons the DCFH-DA assay was used.

<u>The protective effect of honey samples against oxidative damage on the SC-1 and Caco-2 cell lines</u>

Oxidative stress is defined as a disturbance between pro-oxidant and antioxidant balance in favor of pro-oxidants leading to damage (Ashokkumar and Sudhadiran, 2008). In cellular systems, this antioxidant effect can be measured by using the dichlorofluorescein diacetate (DCFH-DA) assay. Cells are incubated with DCFH-DA which is nonfluorescent. This

compound crosses the cell membrane of intact cells, intracellulary it is hydrolyzed enzymatically to nonfluorescent dichlorofluoroscein (DCFH), oxidative damage results in the formation of fluorescein (DCF) which has strong fluoresence. The emitted fluorescence is directly proportional to concentration of the free radicals (Wang *et al.,* 1999). An advantage of this assay is that it is rapid (2 h) and allows usage in a multiwell format analysis in a serum free enviroment.

DCFH-DA + ROS $\xrightarrow{\text{Oxidised}}$ **highly flourescent dichloroflourescein, DCF**

In this study, the SC-1 and the Caco-2 cell lines were used. The latter although of tumour origin is still relevant as it is related to the organ of origin, namely the GIT. Oxidative damage in animal studies has been shown to cause colon cancer (Rosignoli *et al.,* 2008; Ashokkumar and Sudhadiran, 2008). Colon cancer is influenced by genetic, environmental and nutritional factors, e.g. increased fat intake which leads to increased bile acids. Studies have shown antioxidants have protective effects, against the acids that contribute to oxidative damage. Cell culture studies have shown that antioxidants also have a protective effect in the Caco-2 cell line. In a study using this cell line, Rosignoli *et al.,* (2008), studied the effect of antioxidants on Caco-2 exposed to deoxycholic acid (DCA), a bile acid that causes DNA damage. When Caco-2 cells were incubated with DCA in the presence of ß-carotene and α-tocopherol, DNA damage was reduced. Ashokkumar and Sudhadiran, (2008), evaluated luteolin (a flavone also found in honey) for its ability to protect mouse colon against azoxymethane (AOM)-induced oxidative damage. Damage was recorded as formation of aberrant crypt foci (ACF). From this study it was seen that AOM on its own caused formation of ACF's (42 per colon), whereas the number decreased when in combination with luteolin (23 per colon). Similar results were obtained by Prabhu *et al.,* (2009), using the radical dimethylhydrazine, (DMH, an electrophilic diazonium ion) and an antioxidant compound astaxanthin. Astaxanthin protected colon cells from oxidative damage caused by DMH.

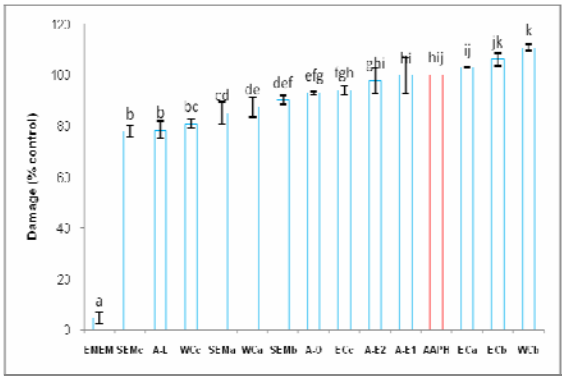
The possible oxidative effect of honey at a concentration of 0.025% and 2.5% was evaluated using the DCFH-DA assay (Figure 5.1B and C). No cytotoxicity due to oxidative damage was observed (data not shown). The lower concentration, 0.025% was between that of 0.008% and 0.8% used in the long term toxic studies (SC-1 cell line, MTT, NR and CV assays). A concentration of 2.5% was also used so that intracellular uptake could be assessed. The ability of honey to protect the SC-1 and the Caco-2 cell lines against AAPH-induced oxidative damage was then evaluated. Percentage damage less than 100% indicates protection i.e. all samples to the left of the red column (Figure 5.4 and Figure 5.5), the

EMEM column, which represents the effect when no AAPH or honey is added. Data to the right of the red column (AAPH, 100% damage), indicates honey that contains components that are cytotoxic.

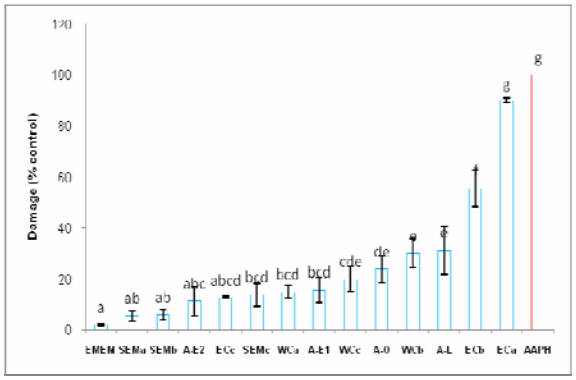
SC-1

2.5%

Intracellular

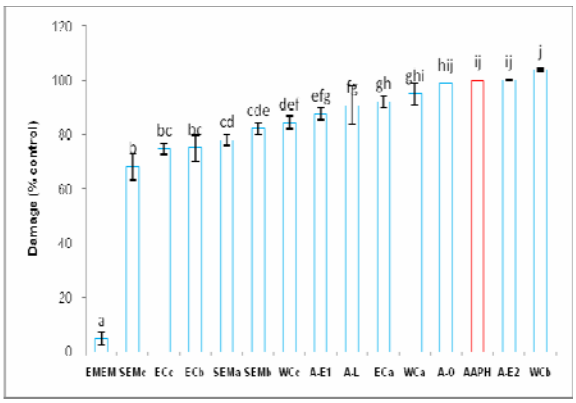


Total



0.025%

Intracellular



Total

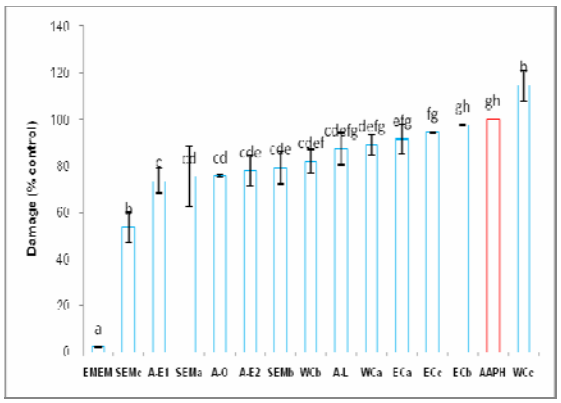


Figure 5.4: Graphical representation of the ability of 0.025% and 2.5% honey to protect SC-1 cells from AAPH-induced oxidative damage. Intracellular: SC-1 honey + DCFH-DA (60 min) washed, AAPH added. Total: SC-1 DCFH-DA (60 min), washed, honey + AAPH. Average of 3 independent experiments, each assay point in quadruple. Data expressed as Mean ± SEM. Means of honeys with different letters are significantly different, p≤0.001.

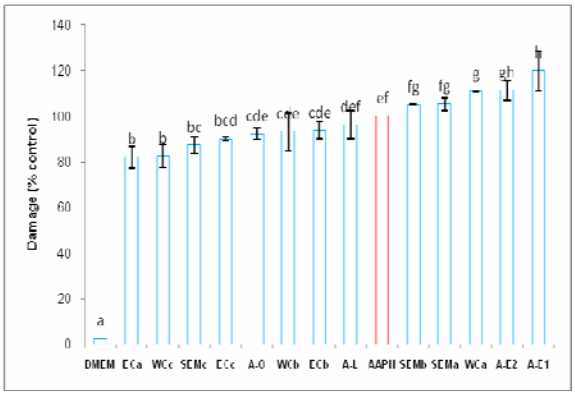
Total protection represents the ability of all antioxidants in the medium to protect the cells from oxidative damage. A fraction of these antioxidants do enter the cell and afford protection from the intracellular compartment. Intracellular protection is the protection that occurs due to antioxidants that are in the intracellular compartment of the cell or those antioxidants that have bound to the cell membrane. For the SC-1 cell line (Figure 5.4) oxidative damage effects were observed at a concentration of 2.5% and 0.025% for WCb (intracellular). At a concentration of 2.5% oxidative damage was observed for WCc (total). These findings were different from the long-term study using the MTT, NR and CV assays where WCb showed no cytotoxicty. Of the samples evaluated at 2.5%, 7/13 showed

intracellular protection and 12/13 total protection. The highest intracellular protection was observed for both SEMc and A-L (% protection (%P) = 20% at a concentration of 2.5%) and highest total protection was observed for both SEMa and SEMb (%P = 90% at a concentration of 2.5%). At a concentration of 0.025% honey, 9/13 samples showed intracellular protection and 11/13 total protection. The highest protection was observed in SEMc (%P = 75%, intracellular at a concentration of 0.025%) and SEMc (%P = 45%, total at a concentration of 0.025%).

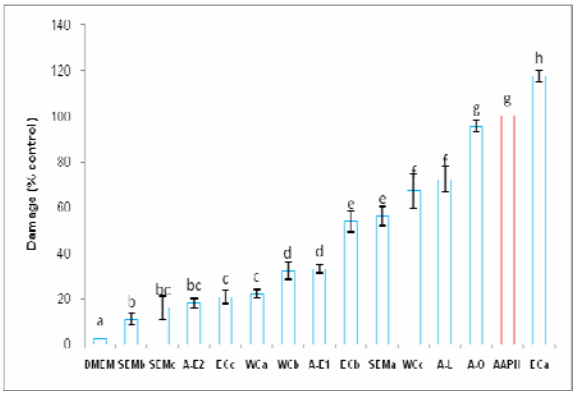
Caco-2

2.5%

Intracellular

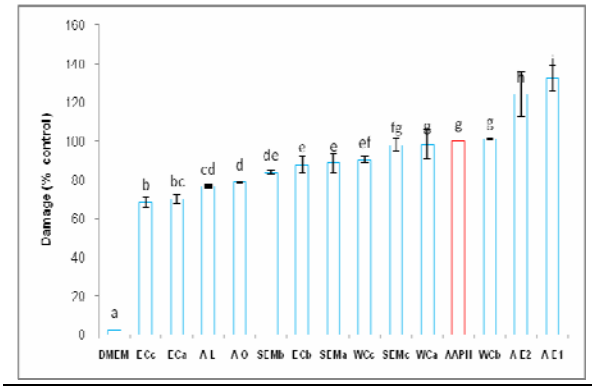


Total



0.025%

Intracellular



Total

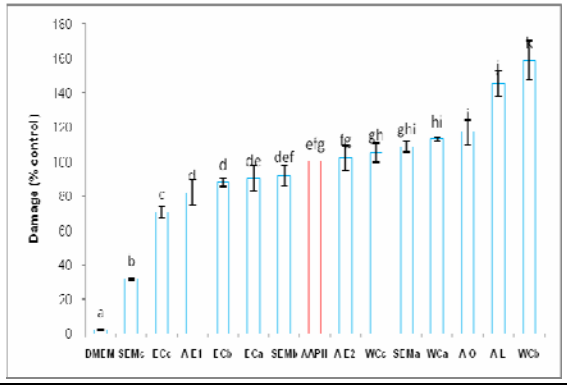


Figure 5.5: Graphical representation of the ability of 0.025% and 2.5% honey to protect Caco-2 cells from AAPH-induced oxidative damage. Intracellular: Caco-2 + honey + DCFH-DA (60 min) washed, AAPH added. Total: Caco-2 + DCFH-DA (60 min), washed, honey + AAPH. Average of 3 independent experiments, each assay point in quadruple. Data expressed as Mean ± SEM. Means of honeys with different letters are significantly different, p≤0.001.

In the Caco-2 cell line, the samples evaluated at 2.5%, 4/13 showed intracellular protection and 8/13 total protection. The highest intracellular protection was observed for ECa and WCc (%P = 15% at a concentration of 2.5%) and ECc and ECa (%P=32% at a concentration of 0.025%). The highest total protection was observed for SEMb (%P = 90% at a concentration of 2.5%) and SEMc (%P= 70% at a concentration of 0.025%). Increased

intracellular cytotoxicity was found for WCa, A-E1 and A-E2 at 0.025% and A-E1 and A-E2 at 2.5%. Increased total toxicity was found for A-L and ECa at 0.025% and ECb at 2.5%. This is different to the effect when honeys were added alone, whereby in the absence of AAPH no cytotoxicity was observed. However, in the presence of AAPH an additive or synegistic effect occured, where previous damage which was minimal was amplified.

Table 5.3: Summary of samples that show significant protection against oxidative damage.

Cell line	Protective effect			
	2.5%		0.025%	
	Intracellular	Total	Intracellular	Total
SC-1	**A-L**, A-O, SEMa, SEMb, **SEMc**, WCa, WCc (n=7)	All except ECa, Highest **SEMa** and **SEMb** (n=12)	A-E1, A-L, ECa, ECb, ECc, SEMa, SEMb, **SEMc**, WCc (n=8)	A-E1, A-E2, A-O, SEMa, SEMb, **SEMc**, WCb (n=7)
Caco-2	**ECa**, ECc, SEMc, WCc (n=4)	All except A-O, ECa, highest **SEMb** (n=11)	A-L, A-O, ECa, ECb, **ECc**, SEMa, SEMb, WCc (n=8)	A-E1, ECc, ECb, **SEMc** (n=4)

Bold=highest protective effect

The results show better protection in the total experiments than intracellular experiments in both the SC-1 and Caco-2 cell lines. This is because in the total experiments the honeys are in direct contact with the peroxyl radicals (AAPH) and have more time (2h) to protect against radical damage caused by these radicals, whereas in the intracellular experiments the honeys only have 1h incubation to enter the cells, after which the cells are washed (Figure 5.1) therefore, less honey derived antioxidants are present to protect the cells against radical-induced damage, showing that intracellular protection is a function of time, lipophilic ability of the antioxidants (ability to cross the membrane) and concentration of the antioxidant and cell density.

Overall, the Caco-2 cell line was more sensitive to the toxic effect of components present in honey than the SC-1 cell line. At 2.5%, intracellular, SC-1 showed protection with 7 and Caco-2 with 4 samples. Total, SC-1 showed protection with 12 and Caco-2 with 11 samples, and at 0.025%, total, SC-1 showed protection with 7 and Caco-2 showed protection with 4 samples, as summarized in Table 5.3. The sensitivity of a cell line is a function of the antioxidant capacity of the different compartments of the cell (cytoplasm, mitochondria and nucleus) (Koren *et al.,* 2008), as well as the rate and degree of absorption, metabolism, conjugation and secretion. The Caco-2 cell line is a well accepted model of human intestinal absorption and Walle *et al.,* (1999), have reported that although chrysin is absorbed by Caco-2, its metabolites undergo rapid glucuronidation and sulfation thereby reducing its antioxidant capacity due to its short half-life intracellularly.

Poor correlations were found between these results and those of Chapter 3 and 4. In a study by Girard-Lalancette (2009), in which the DCFH-DA and the ORAC assay was used to determine the antioxidant activity of fruit and vegetable juices in the L929 fibroblast cell line, reported differences in antioxidant ability between the two assays where quercetin showed high antioxidant activity with the ORAC assay but low oxidative intracellular protection with the DCFH-DA assay. Blasa *et al.,* (2011), using the cellular antioxidant activity in red blood cells (CAA-RBC) assay and compared it with the chemical assay ORAC also found no correlations between the two assays, further supporting the data that suggests that there is no correlation between the chemical assays and the biological assays.

5.5 Conclusion

Honey samples from the Southern African region at concentrations that show minimal cytotoxicity are able to protect the SC-1 and Caco-2 cell lines against oxidative damage.

CHAPTER 6: CONCLUDING DISCUSSION

Thirteen honey samples from the Southern African region were evaluated for physicochemical properties, antioxidant content and activity, as well as their ability to protect biological and cellular systems against oxidative damage.

The honey samples evaluated were of a high quality and physicochemical evaluation showed that all honey samples except ECc, had not undergone any adulteration. All honey samples except for ECc fulfilled the requirements of Codex Alimentarius Standard for honey and the European commission requirements. For all honeys evaluated, SEMb had the highest total polyphenolic content (TPC) and total flavonoid content (TFC) while A-E2 has the highest catalase activity. A strong correlation was found between colour, TPC and TFC.

Antioxidant activity was determined using the 2, 2-diphenyl-2-picrylhydrazyl (DPPH), trolox equivalent antioxidant capacity (TEAC) and the oxygen radical antioxidant capacity (ORAC) assays. Honeys identified with high antioxidant activity were ECc, SEMb, and SEMb with the DPPH, TEAC and ORAC assays respectively. Antioxidant activity also correlated well with colour, TPC and TFC. Data generated form the DPPH, TEAC and ORAC assays were used to calculate the relative antioxidant capacity index (RACI) and WCb was identified as the honey with the highest RACI. Poor correlations were found between colour, TPC or TFC and RACI.

Exposure of cells to the oxidant 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH), results in lipid membrane peroxidation, protein oxidation, glutathione depletion and eventual cell death via apoptosis or necrosis. Two major cellular targets are the cell membrane and DNA. The ability of the honey samples to protect these targets using the pBR322 plasmid and the erythrocyte membrane assays was evaluated. Of these, SEMc and SEMa best protected the erythrocyte membrane and the plasmid DNA, respectively against AAPH-induced oxidative damage. No correlation was found between these assays and other measurements of antioxidant content and activity. However SEMc was found to have the third highest TFC and second highest antioxidant activity measured with the ORAC assay while SEMa was found to have the third highest TPC and the second highest antioxidant activity measured with the TEAC assay.

Further evaluation then involved the effect of AAPH on honeys cellular function. Two types of experiments were undertaken. The first was to determine the cytotoxicity of AAPH and the honeys *in vitro* using the SC -1 cell line. Requirements of antioxidants studies are that the

oxidant must cause significant cellular damage while the antioxidant must be nontoxic and then in combination the antioxidant must be able to protect the cell against oxidative damage. In the SC-1 cell line the oxidant, AAPH after 72 h of exposure, caused a significant decrease in cell number, lysosomal membrane integrity and cell viability at 0.28 mM, 0.78 mM and 1.56 mM respectively.

Then the honeys were evaluated at a concentration of 0.008% and 0.8% for 72 h. Increased cellular viability (mitochondrial functioning, MTT assay) was found at a concentration of 0.008% for A-E2, A-L and SEMa and at 0.8% for A-E1, ECb and WCc. Only for ECb at 0.008% an increase in lysosomal membrane integrity/number was found. At both concentrations no increase in cell number was found. A decrease in lysosomal membrane integrity/number was found for A-L (0.008%) and WCa and A-E2 (0.8%). No decrease in cell number was found at a honey concentration of 0.008% while at a higher concentration of 0.8% ECa, SEMc, WCa, A-O, A-E1, A-L were found to be cytotoxic.

Several attempts were made to develop an antioxidant assay using the concentrations of AAPH that induced cell death and the concentrations of honey that did not induce cell death, but no effect was observed. Similarly using trolox, a known antioxidant, no effect was observed. Several reasons have been put forward to explain this effect and include the inherent antioxidant capacity of albumin, binding of polyphenols to serum constituents such as albumin, growth factors and the oxidation in cell culture medium of antioxidants such as EGCG, the solubility of antioxidants in cell culture medium and the number of double bonds and position of hydroxyl groups of antioxidants. Using the DCFH-DA assay the antioxidant and the polyphenol binding effects are eliminated. Furthermore a fluorometrically method is more sensitive to changes in cellular homeostais compared to colorimetric methods.

The short term, antioxidant effect of all honeys was then evaluated using the DCFH-DA assay as this assay is rapid and allows the use of multiwell format analysis of many samples and cell lines. The AAPH used showed significant toxicity *in vitro* was kept constant and the ability of each honey sample at 0.008 and 0.8% was evaluated. Although some samples did showed cytotoxicity (ECa, SEMc, WCa, A-O, A-E1, A-L at a concentraion of 1%, CV assay) after 72 h, the assumption was made thath this effect would be minimal after 2 h exposure as used in th DCFH-DA assay. At the concentrations of honey used for the DCFH-DA assay, the honeys did not induce any oxidative damage.

Two different antioxidant effects were measured, total and intracellular protection. Total protection measures the ability of all antioxidants to protect against oxidative damage,

whereas intracellular protection is more physiologically relevant as it measures the protective effect of the bioavailable antioxidants. Of the 13 honey samples evaluated in the SC-1 cell line at a concentration of 2.5% 12/13 and 7/13 showed total and intracellular protection, respectively. Of these the highest total protection was for SEMa (% Protection (%P) = 95) and SEMb (%P = 93). Intracellular protection was the highest for SEMc (%P = 21) and A-L (%P = 20). At 0.025% honeys, 7/13 and 8/13 honeys showed total and intracellular protection respectively. For both the highest protection was found for SEMc (%P = 43, total and %P = 30, intracellular). Total protective effect was measured over a period of 2 h and intracellular protective was measured over a period of 1 h. If antioxidant effects are time related and in these experiments a plateau had not been reached, therefore the intracellular effects would be even greater than reported.

In the Caco-2 cell line treated with 2.5% honey samples, 11/13 and 4/13 showed total and intracellular protection, respectively. Of these the highest total protection was for SEMb (% Protection (%P) = 90). Intracellular protection was the highest for ECa (%P = 28) and WCc (%P = 26). At 0.025%, 4/13 and 8/13 honeys showed total and intracellular protection respectively. The highest total protection was found for SEMc (%P = 62) and intracellular protection was ECc (%P = 28). The highest protection offered by honey was observed for the SC-1 cell line when compared to the Caco-2 cell line. Although no direct correlations were found between the antioxidant assays and the biological or cellular assays in this study it was found that consistently honeys with dark colour and high antioxidant content (TPC and TFC), acivity (TEAC, DPPH, ORAC and RACI) and best protected pBR322 plasmid, erythrocyte membrane and cell lines *in vitro* against oxidative damge.

Hypothesis 1: Honey collected fulfill the regulatory standards outlined in a Codex Alimentarius Standard (CODEX STAN12-1981 revision 2001), was supported as all honeys fulfilled these standards. Honeys that are dark in colour (high absorbance at 450nm) have a high total polyphenol content (TPC) and total flavonoid content (TFC), was supported by the observation that dark coloured honeys did indeed have the highest TPC and TFC content and a direct correlation was found between these parametres.

Hypothesis 2: High TPC and TFC are associated with high antioxidant activity measured using the chemical based was supported as honeys with high TPC and TFC had high chemical antioxidant activity. High TPC and TFC are associated with high antioxidant activity measured using the biological based assays, was not supported as honeys with high TPC and TFC had variable biological antioxidant effect.

Hypothesis 3: Honeys with high antioxidant activity and the ability to protect DNA and the erythrocyte membrane against oxidative damage will also be able to protect the SC-1 and Caco-2 cell lines against AAPH-induced oxidative damage, was mostly supported as majority of the honeys with high antioxidant activity, showed ability to protect DNA and erythrocyte membranes and also protected the SC-1 and Caco-2 cell line against AAPH-induced oxidative damage.

Hypothesis 4: There is a significant positive correlation between all parameters measured, was partially supported as there was correlation between the antioxidant assays, but none between the antioxidant assays and the biological and cellular assays The Null Hypothesis that there is no correlation between antioxidant assays and biological and cellular assays had to be accepted.

RECOMMENDATIONS

This is an exploratory study which found that honey collected within the southern Africa region had high antioxidant activity. To confirm this finding a larger number of samples either representative of a specific region (e.g. Western Cape) or a single source (e.g. only agricultural, eucalyptus, entire region) must be collected. The origin of these samples needs to be confirmed, by pollen analysis. The amino acid composition is function of region and amino acid finger printing using high performance liquid chromatography (HPLC) can also indicate floral origin. This will allow correlations to be made between agricultural and wild types and the different geographical regions where the honey was collected.

Although the statement is made that these honeys have high antioxidant activity compared to other studies, a direct comparison with a sample with known activity is ideal. Therefore inclusion with these samples a honey sample with known activity such as Manuka (*Leptospermum scoparium*) honey from New Zealand will be meaningful.

Secondly, High performance liquid chromatography (HPLC) and Mass spectrometry (MS) analysis of honeys with high antioxidant activity, will allow the identification and characterization of constituent polyphenols. This may lead to the identification of known polyphenols (i.e. known structure and antioxidant activity, such as chrysin) or the identification of new polyphenolic compounds, the structure and activity thereof would still need to be determined.

In addition for each polyphenol identified, activity relationship studies can be undertaken to identify specific cellular targets. Such studies have identified flavonols and flavones as

inhibitors of β-secretase (Bace-1), which is involved in the generation of the amyloid β peptide, the accumulation of which is associated with Alzheimer's disease.

Digestion and the associated high pH and proteolytic enzyme activity may alter the antioxidant activity of honey. Evaluation of the antioxidant activity of honey after digestion is necessary to determine whether honey is able to protect the gastrointestinal tract against oxidative damage. Furthermore the bioavailability of these antioxidants must also be determined as this is a function of matrix, storage conditions, heating effects, digestion as well as lipophilicity, absorption rate, serum half life and excretion. The effect of each of these parameters on the antioxidant activity will provide important information regarding health benefits of the antioxidants found in honey.

In animal studies the absorption, distribution, metabolism and excretion (ADME) of honey as such or isolated constituents should be determined and also be further evaluated in an oxidative stress disease model, e.g. a disease of the gastrointestinal tract, Crohn's disease such as the C3H/HeJBir murine model of colitis.

Wound healing, antitumour, antimicrobial and anti-inflammatory effects are just some of the known effects of honey. Indigenous communities also use honey for many other purposes and these should also be researched and be subjected to scientific evaluation.

Honey production occurs in rural areas, the information from this study will increase the commercial value thereof due to its possible health promoting properties thereby increasing the economic sustainability of these communities such as been done by HoneyCare Africa with their "Money for Honey" program.

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