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The effects of the trapping of methylglyoxal by flavonoids on antioxidant and antibacterial activity

by

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The effects of the trapping of methylglyoxal by flavonoids on antioxidant and antibacterial activity

By

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Abstract

Methylglyoxal (MGO) is a highly reactive dicarbonyl compound, formed as a metabolite from nonenzymatic and enzymatic reactions and is the leading precursor of advanced glycation end products (AGEs). AGEs contribute to ageing, type 2 diabetes mellitus (T2DM), and diabetes-related complications. However, MGO also has beneficial antibacterial activity and is the bioactive ingredient of medicinal honeys such as Manuka.

Flavonoids are a group of phytochemicals that are powerful antioxidants. Polyphenols including flavonoids have been reported to trap MGO, forming adducts thereby preventing AGE formation. However, there is little to no information on the effect of adduct formation on the antioxidant properties of flavonoids and the antibacterial activity of MGO.

In this study, catechin (CAT), chrysin (CHRY) and naringenin (NAR) at 0.1 mM and mixtures of each flavonoid with MGO (1:1) and (1:2) were evaluated for antioxidant and antibacterial activity. Antioxidant activity/capacity were evaluated with the total polyphenolic content (TPC), total flavonoid content (TFC), Trolox equivalent antioxidant capacity (TEAC) and the oxygen radical absorbent capacity (ORAC) assays. The bovine serum albumin (BSA)/MGO model was used to evaluate the effect on glycation. The 2', 7'-dichlorofluorescein diacetate (DCFH-DA) assay with the L929 cell line was used to evaluate cellular antioxidant activity. Cytotoxicity was determined in the L929 cell line using the crystal violet (CV) and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium (MTT) assays. *Escherichia coli* (*E. coli*) and *Bacillus subtilis* (*B. subtilis*) were used to determine antibacterial activity using the microbroth-dilution assay and subsequent changes to morphology were evaluated using scanning electron microscopy (SEM).

A reduction in antioxidant content was observed for: CHRY (TPC), CAT and NAR (TFC) and in antioxidant activity for: CHRY (TEAC) and CAT (ORAC), when combined with MGO. Overall most of the antioxidant activity of the flavonoids was not affected by the addition of MGO. In the presence of BSA and MGO, all flavonoid:MGO combinations reduced formation of AGEs except NAR in combination with MGO.

All flavonoids alone and in combinations did not cause cellular oxidative damage while MGO and AAPH induced increased cellular damage indicating that MGO via AGE formation makes cells more sensitive to the effects of oxidants that form radicals. Only CAT reduced the oxidative effects of MGO/AAPH. For all combinations there was no effect on cell number, although cell viability was significantly reduced for CHRY and its combinations and for NAR and NAR:MGO1.

Flavonoids at 0.1 mM CAT, CHRY and NAR had no antibacterial activity against *E. coli* while inhibition was observed only with NAR against *B. subtilis*. MGO at 0.1 and 0.2 mM inhibited bacterial growth while in combination the antibacterial activity was significantly reduced. MGO as well as NAR caused major changes to bacteria morphology. In combination, the antibacterial activity of MGO was reduced, and ultrastructure changes associated with toxicity was also observed in most groups.

In conclusion, flavonoids do trap MGO and this effect does not significantly alter flavonoid antioxidant activity. However, the antibacterial activity of MGO is reduced. Future studies should focus on the chemistry and the effects involved and should include dosage dependent studies.

Keywords: antioxidant, antibacterial, flavonoids, methylglyoxal, mono- and di- MGO adducts, antiglycation, AGEs.

Declaration

I, Refilwe Joy Ndalane, declare that this research dissertation is my own work and has not been presented for any degree at any other University.

Signed.....

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"Give thanks to the Lord, for He is good; His love endures forever." Psalm 107:1

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List of Abbreviations and Symbols

#	
%	percentage
µL	microliter
3-DG	3-deoxyglycosone
°C	degrees Celsius
µM	micromolar
µg/mL	micrograms per millilitre

A

Å	Angstrom
AAPH	2,2'-azobis(2-amidinopropane) dihydrochloride
ABTS	2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)
AGE	advanced glycation end
$AlCl_3$	aluminium chloride
ATCC	American Type Culture Collection
AUC	area under curve

B

B. subtilis	*Bacillus subtilis*
BSA	bovine serum albumin

C

CAT	catechin
CEL	N-ε-(carboxyethyl)lysine
CHRY	chrysin
CML	N(6)-carboxymethyllysine
CV	crystal violet
CVD	cardiovascular disease

D

DCF	2′, 7′-dichlorofluorescein
DCFH	2’, 7’-dichlorodihydrofluorescein
DCFH-DA	dichlorofluorescein diacetate
ddH_2O	double distilled water
dH_20	distilled water
DHA	dihydroxyacetone
DMEM	Dulbecco's modified essential medium
DOLD	deoxyglucasone-lysine dimer
DPPH	2, 2-diphenyl-2-picrylhydrazyl

E

E. coli	*Escherichia coli*
EDTA	ethylenediaminetetra acetic acid ($C_{10}H_{16}N_2O_8$)
ED_{50}	effective dose
Em	emission wavelength
ET	electron transfer
EtOH	ethanol

F

FA	formaldehyde
FBS	foetal bovine serum
FC	Folin-Ciocalteu's
FCS	fetal calf serum

G

g	gram(s)
G100	growth rate at 100%
g/L	gram per liter
g/mol	gram per mol
GA	gallic acid
GA	glutaraldehyde
GAE	gallic acid equivalent
GO	glyoxal
GPx	glutathione peroxidase
GSH	glutathione

H

h	hour(s)
H_2O_2	hydrogen peroxide
HAT	hydrogen atom transfer
HCl	hydrochloric acid
HMDS	hexamethyldisilazane

I

isoPBS	isotonic phosphate buffered saline

K

$K_2S_2O_8$	potassium peroxodisulfate

L

L	litre
L929	mouse fibroblast cell line
LASEC	Laboratory Scientific Equipment Company
LB	Lysogeny Broth
LD_{50}	lethal dose
LDL	low-density lipoprotein

M

M	molar
MGO	methylglyoxal
MIC	minimum inhibitory concentration
mg	milligrams
mg/kg	milligram per kilogram
min	minute(s)
mL	millilitre
mM	millimolar
MOA	mechanism of action
MOLD	methyl glyoxal-lysine dimer
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MW	molecular weight

N

Na_2CO_3	sodium carbonate anhydrous
Na_2HPO_4	sodium hydrogen phosphate
NAD(P)H	nicotinamide adenine dinucleotide phosphate hydrogen
NADH	nicotinamide adenine dinucleotide
$NaHPO_4$	sodium hydrogen phosphate
NaOH	sodium hydroxide
NAR	naringenin
NDD	neurodegenerative disease
NF-ƙB	nuclear factor kappa beta
nm	nanometre
NO^-	nitrogen monoxide

O

O^-	singlet oxygen
O^{2-}	superoxide
OD	optical density
$ONOO^-$	peroxynitrite
ORAC	oxygen radical absorbance capacity

P

P-value	probability value
PBS	phosphate buffer saline
PC	positive control
PH	logarithmic scale for the measurement of the acidity or alkalinity of an aqueous solution

R

r	correlation
r^2	correlation of determination
ROS	reactive oxygen species

S

SEM	scanning electron microscopy
SOD	superoxide dismutase

T

T2DM	type 2 diabetes mellitus
TD_{50}	toxic dose
TE	Trolox equivalent
TEAC	Trolox equivalent antioxidant capacity
TFC	total flavonoid content
TI	therapeutic index
TPC	total polyphenolic content

U

UMF	Unique Manuka Factor
UV	ultraviolet

V

VC	vehicle control

X

xg	relative centrifugal force
XO	xanthine oxidase

Chapter 1: Introduction

With aging, biological systems deteriorate and oxidant by-products, known as reactive oxygen species (ROS) produced during normal metabolism as well as endogenous sources of ROS are a major contributing factor to the development of cardiovascular disease (CVD) (Lazarević *et al.*, 2018), cancer (Prasad *et al.*, 2017) and neurodegenerative disease (NDD) (Islam, 2017).

Endogenous antioxidant defenses protect cells and tissue against ROS and these include antioxidant elements such as glutathione (GSH) and antioxidant enzymes including superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx) (Valko *et al.*, 2007). Exogenous sources of antioxidants such as antioxidant vitamins (ascorbic acid (vitamin C) and tocopherol (vitamin E), polyphenols and peptides provide additional protection against ROS. Dietary sources of these molecules include oils, nuts, fruit and vegetables as well as other sources such as tea and honey. A major group of polyphenols are the flavonoids, which can be subdivided into the anthoxanthins (flavones and flavonols), flavanones, isoflavones, flavan-3-ol and anthocyanidins that can scavenge ROS and reduce associated oxidative stress.

Methylglyoxal (MGO) is derived from the fragmentation of triose-phosphates and by the metabolism of amino acetone and acetone (Choi *et al.*, 2016). In medicinal honey, such as Manuka honey, MGO levels are high. Due to the antibacterial activity of MGO these honeys are used to treat wounds. The mechanism of action is the inactivation of bacterial proteins by causing protein cross-linkages (Jenkins *et al.*, 2015) and this leads to flagella inactivation and the loss of bacterial membrane integrity (Rabie *et al.*, 2016). Levels of MGO, are raised in patients with type 2 diabetes mellitus (T2DM) (Dhananjayan *et al.*, 2017) and this MGO contributes to oxidative stress and associated diseases. Concern has been expressed regarding the treatment of wounds in these patients with medicinal honey having a high MGO content (Kamaratos *et al.*, 2014), as endogenous MGO can further exacerbate oxidative stress in these patients.

Not only can flavonoids scavenge ROS, but these molecules also have the ability to trap MGO (Lo *et al.*, 2006) that results in the formation of mono- and di- MGO adducts. Adduct formation results in the addition of an acetyl group to the C-terminal ring of flavonoids such as catechin. An extensive evaluation of current literature reveals that very little is known about the effects of adducts formation on the antioxidant activity of flavonoids. Likewise, with the trapping of MGO by flavonoids it is unknown whether the antibacterial activity of MGO is reduced. In addition, it is unknown whether trapping under physiological conditions has a significant effect on the antioxidant and antibacterial activity of flavonoids and MGO respectively.

Chapter 2: Literature review

2.1 Aging and reactive oxygen species

ROS are formed as a by-product of normal metabolic processes. Examples of these molecules are oxygen ions (O^{-2}), free radicals such as (superoxide [O^{2-}] and hydroxyl radicals), and peroxides (hydrogen peroxide (H_2O_2) (Morrell, 2008).

ROS are small and highly reactive molecules that play an important role in cell signalling when maintained at proper cellular concentrations. In contrast, at high concentrations ROS are harmful causing oxidative stress that can damage cellular structures such as deoxyribonucleic acids (DNA), proteins and lipids causing defective organelle function leading to cellular and tissue damage (Fulle *et al.*, 2004). Oxidative stress is a major role player in the development of conditions and diseases most of which are often age-related, such as cancer, Parkinson's disease, cataracts, CVD and aging. Therefore the identification of effective strategies to detect and eliminate excess ROS is important for survival in an oxygenated environment (Lin *et al.*, 2000).

The free radical theory of aging assumes that " there is a single basic cause of aging, modified by genetic and environmental factors, and postulates that the free radical reactions are involved in aging and age-related disorders" (Harman, 1981). Aging has been defined as "the progressive accumulation of changes with time that are associated with or responsible for the ever-increasing susceptibility to disease and death which accompanies advancing age" (Beatty *et al.*, 2000).

There have been a number of suggested ROS mediated processes that contribute to the aging process and these include (i) accumulative changes, such as point mutations in DNA and ineffective DNA repair mechanisms, (ii) macromolecule cross-linkages (iii) progressive breakdown in the accuracy of protein synthesis (iv) the attack of the immune system by self-antigens and (v) and free radical reaction damage (Harman, 1981). Recent literature suggests that various lifestyles such as drug addiction and stress (Shalev & Belsky, 2016) may accelerate biological aging by triggering early onset age-related diseases (Bachi *et al.*, 2017). Research has focussed on reducing the effects of these processes and the focus of this literature review is the contribution of antioxidants to alleviate oxidative stress induced by ROS and MGO.

2.2 Antioxidants

An antioxidant is defined as any substance, that when present at low concentrations compared with that of an oxidizable substrate significantly delays or inhibits the oxidation of that substrate (Halliwell & Gutteridge, 2015). Antioxidants may be endogenous and/or non-endogenous (exogenous) and are involved in various mechanisms to inhibit or alleviate oxidation and oxidative stress.

Endogenous antioxidants are inherent cellular molecules and usually act as the first line of defence against ROS. These include antioxidant molecules such as GSH and enzymes such as GPx, catalase and SOD. The depletion of GSH and/or inhibition of any of these enzymes results in ROS accumulation as shown in Figure 2.1. Activation of mitochondrial transport chain, NAD(P)H oxidase (NOX) and xanthine oxidase (XO) generates the superoxide radical anion ($O_2^{\cdot-}$); which is either transformed into peroxynitrite ($ONOO^-$) by its interaction with nitric oxide ($NO^{\cdot}$) or H_2O_2 by SOD. Catalase and/ or GP_x regulates the H_2O_2 concentrations whilst peroxiredoxins (PRX) play an important role in H_2O_2 removal especially in the presence of redox metals.

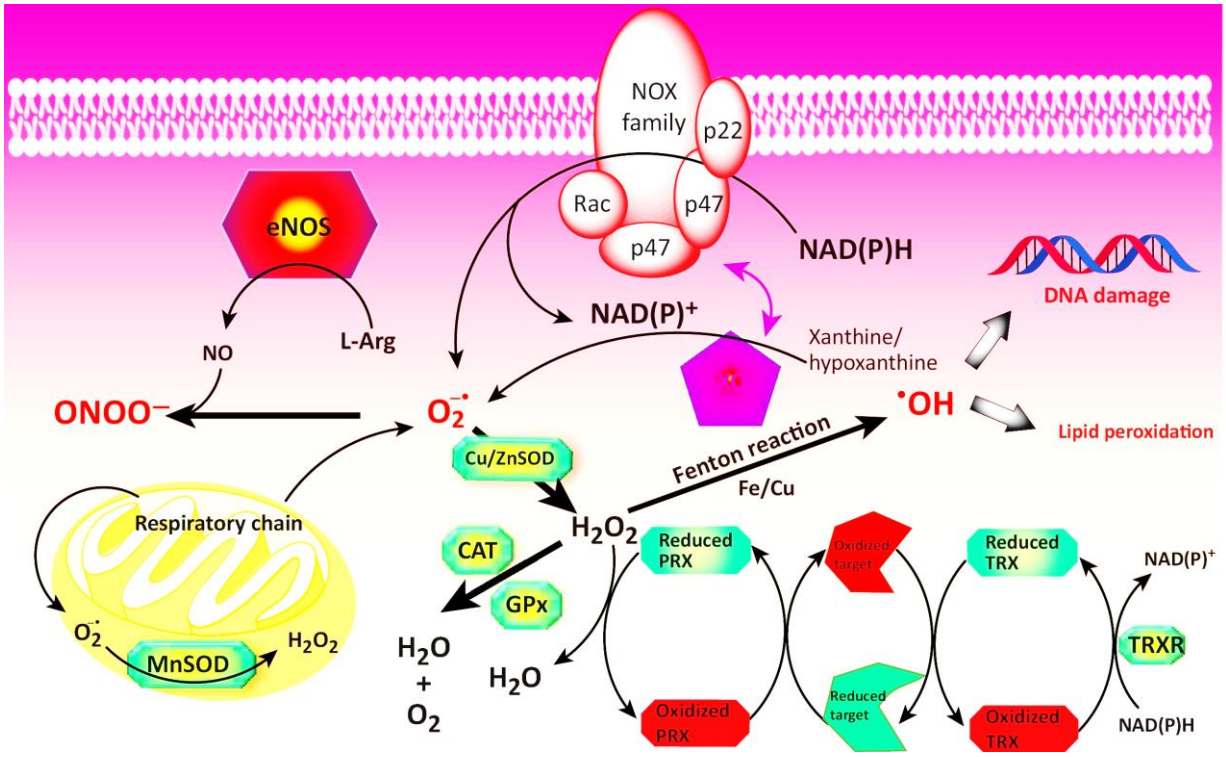


Figure 2.1: The regulation of ROS by endogenous antioxidants (Poprac *et al*., 2017). Reprinted with permission from the corresponding author M. Valko.

Exogenous antioxidants are non-enzymatic and are usually dietary in origin. These include polyphenols, carotenoids, ascorbic acid and vitamin E (Figure 2.2). An explanation on the mechanism of antioxidant scavenging as described by Poprac *et al*., (2017) using vitamin C

and E is provided. Vitamin E (α- tocopherol, T-OH) scavenges lipid peroxyl radicals ($ROO^{\cdot}$) to form vitamin E radical (α- tocopheroxyl radical, $T\text{-}O^{\cdot}$). Vitamin C (ascorbate, $AscH^{-}$) then donates its hydrogen to $T\text{-}O^{\cdot}$, generating T-OH and resulting in the formation of a vitamin C radical ($Asc^{\cdot-}$). The $Asc^{\cdot-}$ is transported to DHA and through the action of dehydroascorbate reductase (DHAR), $Asc^{\cdot-}$ is reduced to $AscH^{-}$ with the aid of GSH, forming oxidized glutathione (GSSG). GSSG is then reduced to GSH by glutathione reductase (GR) with the aid of NAD(P)H. Likewise polyphenols, via a similar mechanism, can also scavenge radicals.

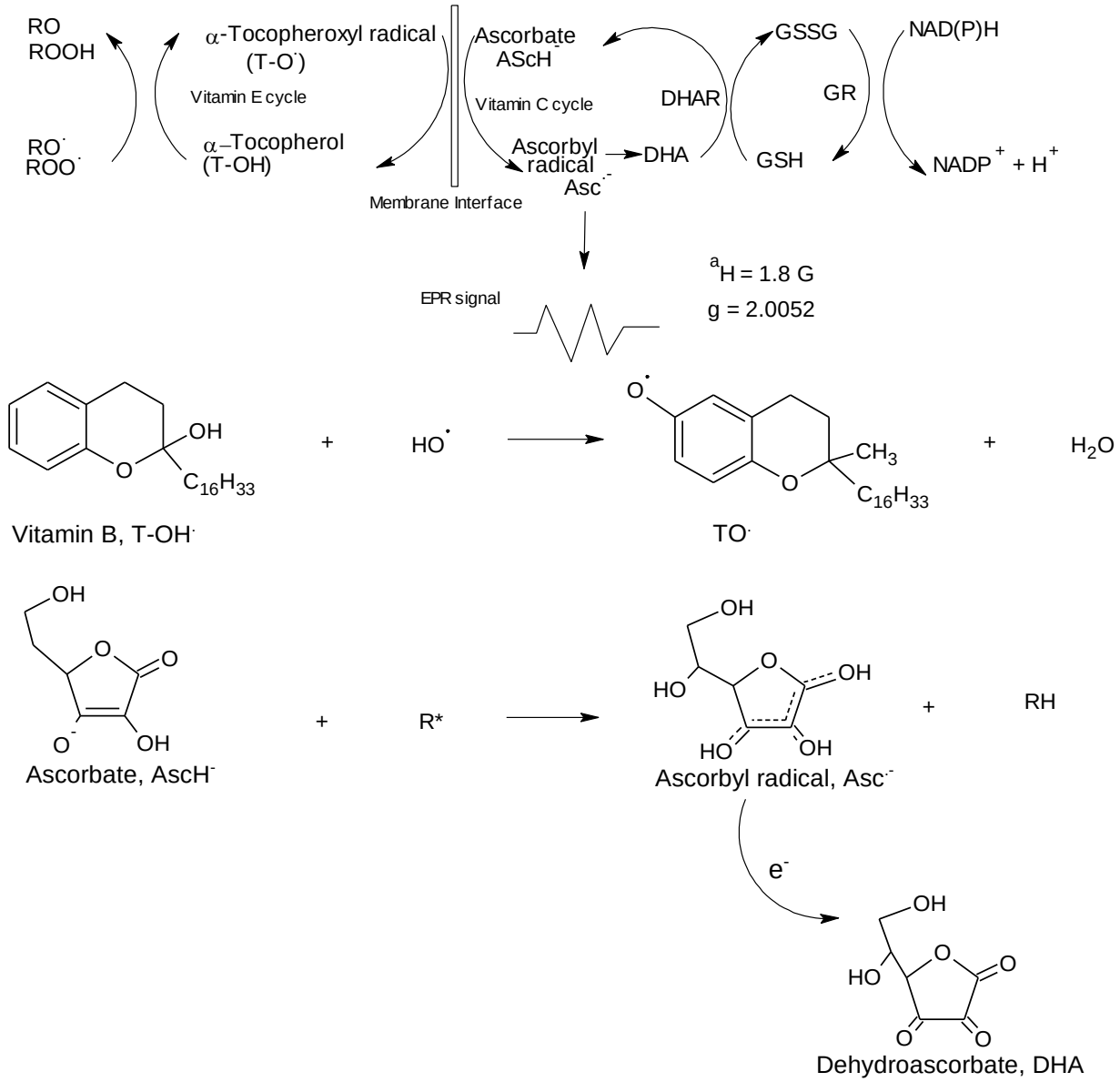


Figure 2.2: The regulation of ROS by exogenous antioxidant vitamins, adapted from Poprac *et al.,* (2017)

Polyphenols

Polyphenols are a structural class of mainly natural but also organic, synthetic or semi synthetic compounds that are characterized by either single or multiple phenol structural units (Quideau, 2006) as shown for theaflavin (Figure 2.3).

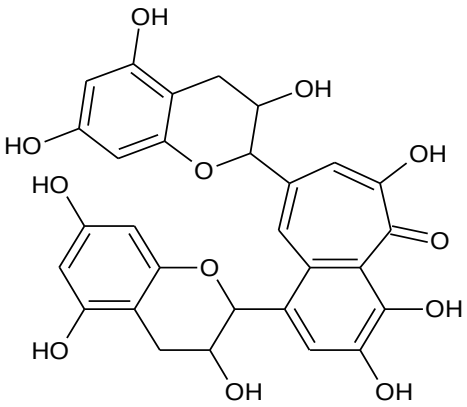


Figure 2.3: Structure of the polyphenol, theaflavin found in tea (Lin *et al*., 2000).

Polyphenols are secondary metabolites that are found throughout the plant kingdom. In plants, phenolic compounds are produced as a response to environmental factors and include protection against ultraviolet light (UV) light. These molecules also play an important role in plant growth and reproduction, and also strengthen plant material (Ghasemzadeh & Ghasemzadeh, 2001). In the diet polyphenols exhibit a wide range of biological activity (Ghasemzadeh & Ghasemzadeh, 2011) and these include anti- inflammatory (Araujo & Leon, 2001), anti-ulcer (Matsuda *et al.*, 2003), antitumour (Murakami *et al.*, 2004) antispasmodic (Ammon & Wahl, 1991) antioxidant (Ghasemzadeh & Ghasemzadeh, 2011) and antidepressant activities (Yu *et al.*, 2002). Polyphenols are subdivided into two major groups and these are phenolic acids and flavonoids. As the flavonoids are the focus of the present study, the structure and reactions of flavonoids will be described in greater detail.

2.2.1 Flavonoids

Flavonoids are synthesized from phenylalanine via the polypropanoid pathway and are classified into six subgroups that share several basic characteristics. Flavonoids are readily available in food sources such as chocolate, tea, fruits and vegetables as well as wine. All flavonoids have C_6-C_3-C_6 structural skeleton, consisting of a heterocyclic ring and two aromatic cyclic rings, as shown in Figure 2.4 (Stefek, 2011). Flavonoids have high antioxidant activity and function as reducing agents, free radical scavengers and quenchers of singlet oxygen formation (Ghasemzadeh & Ghasemzadeh, 2011).

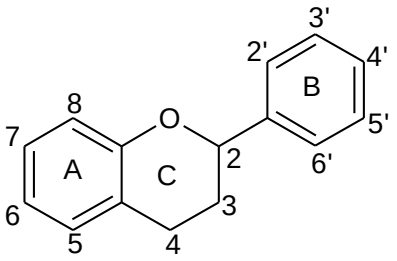


Figure 2.4: The general flavonoid structure with a common aromatic ring (A+B) bound by three carbon atoms forming oxygenated heterocycle (ring C) (Stefek, 2011).

The different classes of flavonoids are the flavan-3-ols, anthocyanins, flavonols, flavones, flavonones and isoflavones (Raffa *et al.*, 2017). The characteristic feature of these classes of flavonoids is based on the modification of the heterocyclic 4H-pyrance ring C, by different levels of oxidation and substituents (Raffa *et al.*, 2017) as indicated in Figure 2.5.

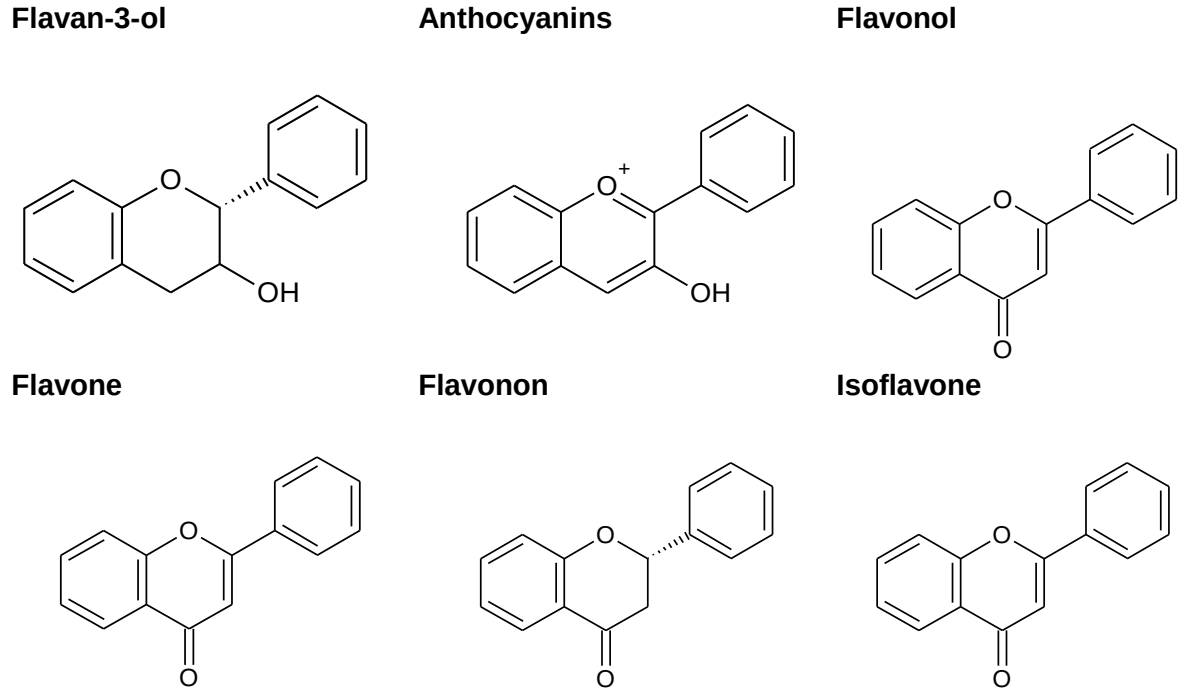


Figure *2.5*: The six subgroups of flavonoids (Akram, 2011).

This study will focus on catechin (CAT) a flavanol, chrysin (CHRY) a flavone and naringenin (NAR) a flavonone. These flavonoids were chosen due to the common and different structural composition which will allow for a broader collaborative study; they differ in the number of hydroxyl groups attached to their aromatic ring B and the absence or presence of a ketone group on the oxygenated heterocycle ring C (Figure 2.5 and 2.6). Catechin is a flavanol and has two hydroxyl groups on ring B and no ketone group on ring C while there are 2 hydroxyl groups on ring A, as shown in Figure 2.6. Catechin is found in high concentrations in a variety of plant-based foods and beverages including tea (Wang *et al.*, 2017). The beneficial effects

of the consumption of CAT is the increase in plasma antioxidant activity, dilation of the brachial artery (Ota *et al.*, 2016), and increased resistance of low density lipoprotein (LDL) to oxidation (Matumoto-Pintro *et al.*, 2017). Cellular effects include antimicrobial activity, anti-proliferative, anti-inflammatory and antioxidant properties (Monika *et al.*, 2017).

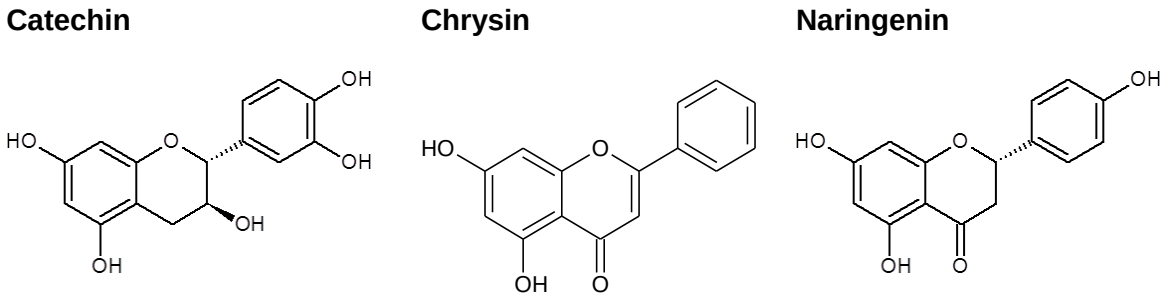


Figure 2.6: The chemical structure of the flavonoids catechin (CAT) a flavanol, chrysin (CHRY) a flavone and naringenin (NAR) a flavonone that was used in the present study.

In contrast chrysin (CHRY), a flavone, lacks hydroxyl groups on ring B, it has a ketone group on ring C and two hydroxyl groups on ring A (Figure 2.6). This flavonoid is found in plants i.e. honeycomb and mushrooms (Jung, 2016). It has a varied biological activity profile which includes antioxidant, anti-anxiolytic effect, anti-diabetogenic activity and antivirus effects (Zheng *et al.*, 2003). Cellular effects include its antineoplastic effects as this flavone has been reported to inhibit the growth of human lung adenocarcinoma cells by inducing apoptosis (Jung, 2016).

Naringenin (NAR), a flavonone has a single hydroxyl group on ring B and a ketone group on ring C and two hydroxyl groups on ring A (Figure 2.6) and is the predominant flavanone found in grapefruit and is responsible for the bitter taste of grapefruit juice (Felgines *et al.*, 2000). Other sources include oranges (Pereira *et al.*, 2017). The bioactivity of NAR includes antioxidant, anticancer, antimutagenic, anti-inflammatory, antifungal and antiviral activity (Ke *et al.*, 2017) as well as photo-protective activity (Joshi *et al.*, 2017).

Related to antioxidant efficacy, flavonoids portray an important structure-activity relationship (SAR) (Pietta, 2000). Flavonoids are based on the flavan nucleus, and the number, position and type of substitution/s influences radical scavenging and chelating activity. This accounts for the diverse and multiple mechanisms of flavonoid action. With SAR the most stable flavonoid has a carbonyl functional group and/or double bond in the heterocycle ring while the substantial prooxidant, antioxidant and chelating activity is due to the presence of multiple hydroxyl groups while the presence of methoxy groups increases membrane partition, lipophilicity and steric effects (Heim *et al.*, 2002).

2.2.1.1 Formation of reactive carbonyl compounds

Reactive carbonyl compounds arise from various exogenous and endogenous sources of MGO (Loeffler *et al.*, 2006). MGO, also known as pyruvaldehyde, oxopropanal and/ or acetylformaldehyde, is an organic compound (from a family of dicarbonyls) that is often used as a reagent in organic synthesis, as a flavoring agent and in tanning (Figure 2.7). The Maillard reaction, carbohydrate auto-oxidation and degradation, are a few of the exogenous sources of MGO and is formed during processing, cooking and prolonged storage (Nemet et al., 2006).

Reactive carbonyl compounds such as MGO and glyoxal are formed endogenously by enzymatic and non-enzymatic pathways (Nemet *et al.*, 2006). In *in vitro* cell, and *in vivo* animals studies MGO has been identified as a byproduct of various metabolic pathways and is formed from 3-aminoacetone in threonine catabolism, during lipid peroxidation and by enzymatic elimination of phosphate from glycerone phosphate, glyceraldehyde-3-phosphate or 3-amino acetate (Rondeau & Bourdon, 2011). Under physiological conditions, MGO is generated by non-enzymatic elimination of phosphate from glyceraldehyde-3-phosphate or dihydroxyacetone phosphate (Seidler, 2013).

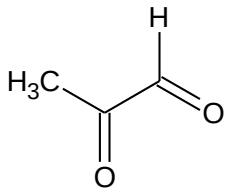


Figure 2.7: Structure of 2-oxopropanal or methylglyoxal (MGO) (Granvogl et al., 2012).

In T2DM patients, MGO is produced enzymatically by MGO synthase and cytochrome P450 IIE1 (Rondeau & Bourdon, 2011). It is also produced by enzymatic oxidation of ketone bodies (acetoacetate and acetone), generating MGO via the catabolism of acetol by cytochrome P450 IIE1 isozymes (Kalapos, 2013).

2.2.1.2 Methylglyoxal and the formation of AGE

MGO is involved in the formation of AGE which are a complex and heterogeneous group of compounds that have been implicated in diabetes related complications (Jensen *et al.*, 2016). In diabetes, MGO levels increase due to high amount of three- or four-carbon sugars derived from glucose in diabetic patients and older people. The increase in sugars leads to the accumulation of AGEs, thus causing age-related disorders, obesity, apoptosis and inflammation (Verma & Manna, 2016). For this reason, MGO is regarded as an important contributor to the development of diabetic complications (Szwergold, 2017).

AGEs form via the Maillard reaction where reducing sugars such as glucose react non-enzymatically with amino groups in proteins, lipids and nucleic acids through a series of reactions forming Schiff bases and Amadori products to produce AGE, as summarized in Figure 2.8 (Singh *et al.*, 2001). Glucose is coupled to a nucleic acid, a protein amino group or lipid through a series of reactions to yield an unstable Schiff base and Amadori products. Amadori products and reactive intermediates (GO, MGO and 3- deoxyglycosone (3-DG), are also produced by glycolysis, lipid peroxidation or glucose auto-oxidation and react non-enzymatically with arginine or lysine residues to form AGEs (Figure 2.8) (Lin *et al.*, 2016). Polyphenols can prevent AGE formation, by preventing lipid peroxidation and inhibiting oxidative pathways or binding MGO as described in Section 2.3.1.

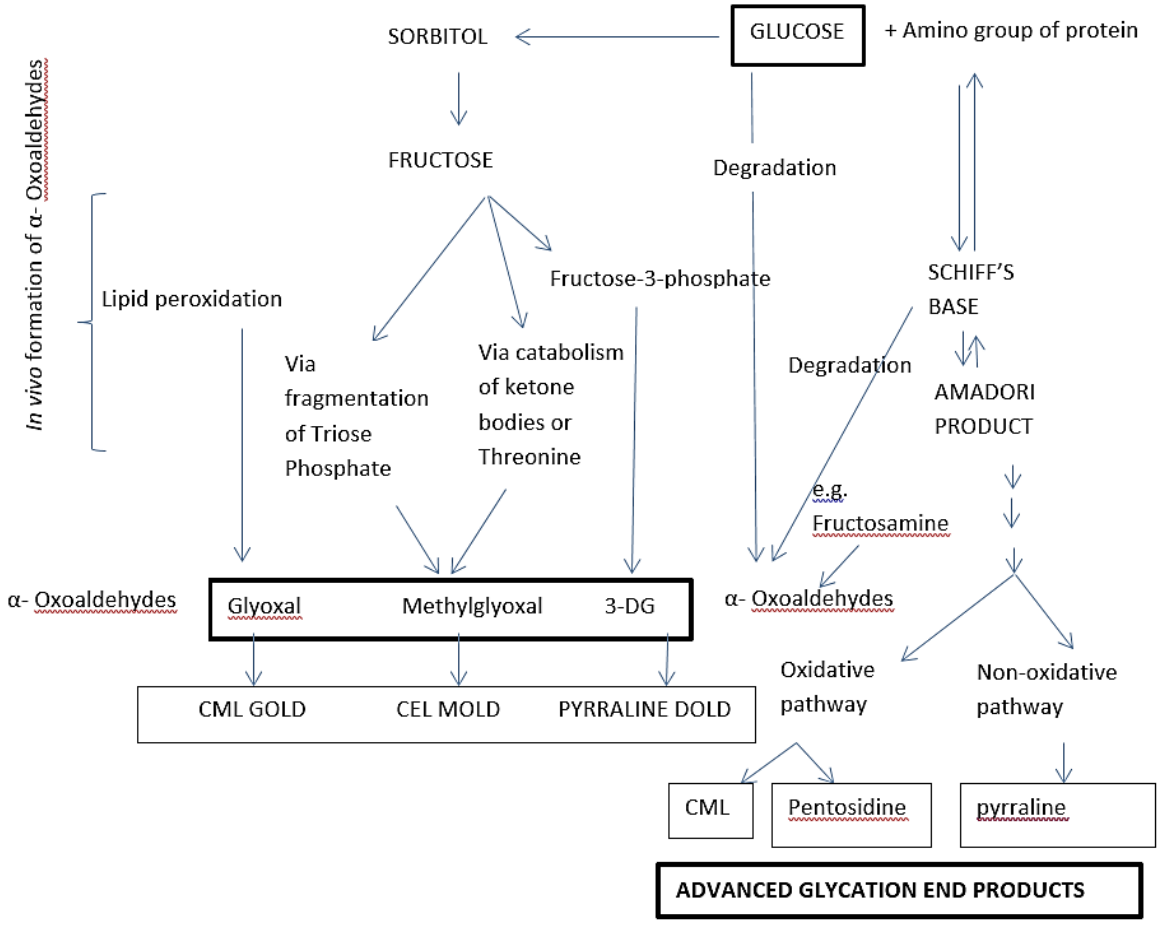


Figure 2.*8*: AGE formation pathway by alpha-oxoaldehydes (glyoxal, MGO and 3-deoxyglycosone). 3-deoxyglycosone (3-DG); methylglyoxal (MGO); N-ε-(carboxymethyl) lysine (CML); N-ε-(carboxyethyl) lysine (CEL); deoxyglucasone-lysine dimer (DOLD); methyl glyoxal-lysine dimer (MOLD); glyoxal lysine dimer (GOLD) (Singh *et al.,* 2001).

2.3 Antibacterial activity

Antimicrobials are agents or substances that hinder microbial activity by either microbicidal (kill microbes) or microbiostatic (inhibit microbial growth) activity (Al-Adham *et al.*, 2013). Antimicrobials can inhibit protein synthesis by interacting with bacterial ribosomes, interacting with DNA, and inhibiting nucleotide synthesis, blocking DNA synthesis by inhibiting DNA gyrase and topoisomerase IV, activity. In addition, antimicrobials can also inhibit enzymes required for folic acid synthesis, and transpeptidases required for the formation of peptidoglycan. Antimicrobials can induce cross-linking of structures in the cell wall and form complexes with peptidoglycan.

In Manuka honey, MGO and to a lesser degree the polyphenols in honey have antibacterial activity (Mandal & Mandal, 2011). MGO interferes with protein synthesis thus preventing DNA replication; it alters bacterial DNA structure, reacts with thiol groups in protein thereby altering protein structure, function and activity thereby inhibiting bacterial growth (Booth *et al.*, 2003; Rabie *et al.*, 2016).

Manuka honey is classified based on the presence of leptosperin, dihydroxyacetone (DHA) and MGO; the 'non-peroxide' peroxide antibacterial activity of Manuka honey is termed Unique Manuka Factor (UMF) (UMFHA, 2019). Methylglyoxal has been identified as the main antibacterial component of Manuka honey (Stewart *et al.*, 2014). A UMF value is obtained by grading Manuka honey based on MGO content with a range of 38.4 – 761 mg/kg (Mavric *et al.*, 2008). The minimum inhibitory capacity (MIC) range of MGO on *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Bacillus subtilis* (*B. subtilis*) was reported as 0.4 – 1.2 mM and 0.2 – 0.4 mM, respectively. The MGO content of therapeutic Manuka honey is UMF>10, which is ≥263 mg/kg and since a 1.1 mM MGO solution is equivalent to 72.27 mg/kg MGO this implies that honey of UMF>10 would cause lysis of both Gram-negative and positive bacteria (Rabie *et al.*, 2016a). Although Manuka honey of UMF>10 heals wounds, concern is expressed because in diabetic patients MGO levels are increased (Han *et al.*, 2007) and although in these patients blood sugar levels may be controlled, patients still possess high MGO levels that form per unit glucose (Jensen *et al.*, 2015).

Various flavonoids have been reported to have antibacterial activity and this is not surprising since some flavonoids are formed as antimicrobial barriers in plants as a response to microbial infection (Orhan *et al.*, 2010). A SAR also exists for the antibacterial activity of flavonoids. For example, Avila *et al*., (2018) identified that the antibacterial activity of chalcones is due to the hydroxylation at position 2' though it does affect structural stability (Ávila *et al.*, 2008). Various mechanisms of actions (MOA) have been identified for flavonoids and this includes bactericidal and bacteriostatic activity. These effects include damage to the cytoplasmic

membrane, which alters membrane fluidity and causes cell wall lysis (Ikigai *et al*., 1993; Bernard *et al.*, 1997; Tsuchiya & Iinuma, 2000). Inhibition of NADH cytochrome c reductase adversely affects energy metabolism (Haraguchi *et al.*, 1998) while inhibition of topoisomerase reduces nucleic acid synthesis (Mori e*t al*., 1987; Bernard *et al.*, 1997; Plaper *et al.*, 2003). Further studies found that the flavolan, flavonol and flavan-3-ol classes damaged the cytoplasmic membrane by generating H_2O_2 (Cushnie & Lamb, 2005; Sirk *et al.*, 2008). Isoflavones inhibit nucleic acid synthesis through the inhibition of topoisomerase (Gradišar *et al.*, 2007; Wang *et al.*, 2010). Additional MOAs are the inhibition of FabG and FabI (Zhang & Rock, 2004) causing the inhibition of cell membrane synthesis, due to the inhibition of D-alanine-D-alanine ligase(Wu *et al.*, 2008).

CAT is reported to have antibacterial activity against various microorganisms such as *Erwinia carotovora, Erwinia amylovora, Xanthomonas campestris* and *Agrobacterium radiobacter;* however, the antibacterial activity of CAT but has little to no effect on previously tested Gram-positive bacteria, *Staphylococcus aureus (S. aureus)*, *Bacillus subtilis, Citrobacter freundii, Staphylococcus coagulans*, and also minimal activity against *Escherichia coli* (*E. coli*) (Veluri *et al.,* 2004). CHR has significant antibacterial activity against a number of Gram-positive and Gram-negative bacteria (Liu *et al.*, 2010). Antibacterial activity against Gram-negative bacteria is attributed to membrane interaction effects where membrane fluidity is decreased (Wu *et al.*, 2013). NAR is reported to have antibacterial activity, at 0.1 mM NAR strongly inhibited Gram-negative bacterium *Vibrio harveyi,* did not significantly inhibit *E. coli* while the effect on *B. subtilis* was minimal (Ulanowska *et al.*, 2006).

2.3.1 Trapping interaction between flavonoids and methylglyoxal

Methylglyoxal is highly reactive, it attacks amine groups in peptides and proteins resulting in AGE formation thus leading to carbonyl stress (Wu & Yen, 2005). MGO induces H_2O_2 in the mitochondrial respiratory chain, activates nuclear factor beta (NF-ƙB) and induces the genes responsible for proliferation and inflammation (Wang & Ho, 2012), all leading to the AGE formation. Synthetic compounds that inhibit AGE formation such as aminoguanidine are commercially available however; these drugs have some- to severe side effects. Therefore, the need for naturally derived AGE inhibiting compounds is necessary and the flavonoid aglycones are promising agents (Yang *et al.*, 2017).

Some studies report that flavonoids could inhibit the formation of α- dicarbonyl compounds (MGO and GO) by scavenging free radicals, whilst other studies prove that polyphenols efficiently inhibit MGO by trapping MGO to form mono- or di- MGO adducts as shown in Figure 11 (Liu *et al.*, 2017). The mechanism in which MGO is trapped by flavonoids is due to an increase in nucleophilicity of the unsubstituted carbons at the A ring, at a slightly alkaline pH,

which facilitates the addition of MGO at positions C6 and C8 (Wang and Ho, 2012). Phenolic compounds exhibiting a CAT-like structure such as genistein (Figure 2.9) have the strongest ability in direct trapping of MGO (Wang & Ho, 2012).

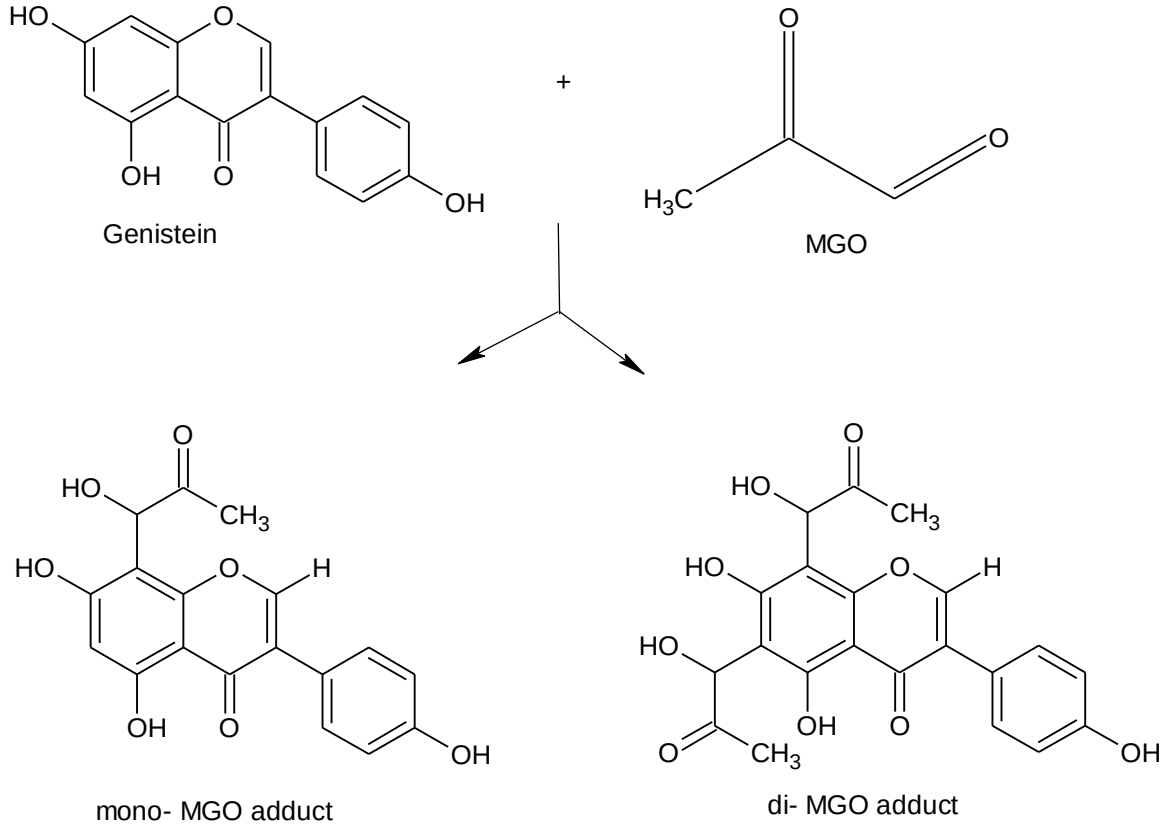
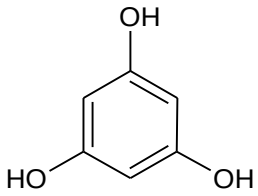


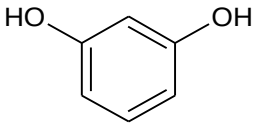
Figure 2.9: The trapping of MGO by genistein to form mono- and/or di- MGO adducts. Due to an increase in nucleophilicity of the unsubstituted carbons at the A ring (Figure 2.4) the addition of MGO at positions C6 and C8 occurs with the formation of a mono and/or di MGO adduct (Wang & Ho, 2012).

The MOA for MGO trapping by flavonoids has been studied in detail. Shao *et al*., (2014) selected some typical sub-components of flavonoids to investigate the A, B and C ring of MGO-trapping efficacy. When investigating the A ring, phloroglucinol and resorcinol were used, and findings were that phloroglucinol was a better MGO scavenger compared with resorcinol; suggesting that the C-5 hydroxyl group possibly enhances trapping efficacy. The hypothesis was tested using daidzein and genistein, which has an almost identical A ring structure to phloroglucinol and resorcinol, with similar B and C ring structures. It was found that after 4 h genistein could trap 94% of MGO whereas daidzein only trapped 54.5% of the MGO (Figure 2.10) (Shao *et al.*, 2014).

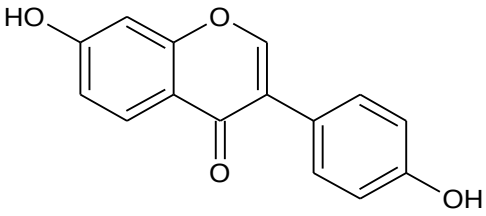
Phloroglucinol



Resorcinol



Daidzein



Genistein

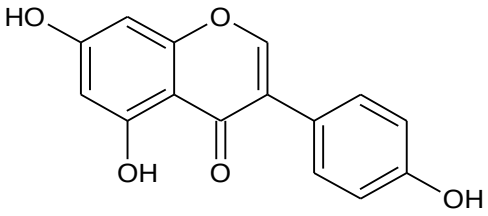
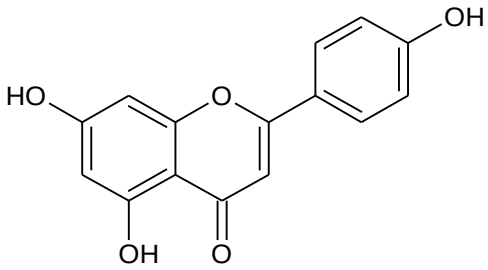


Figure 2.10: The investigations of the A ring using phloroglucinol, resorcinol, daidzein and genistein.

To investigate the effects on the B ring of MGO-trapping efficacy apigenin and luteolin, which possess the same A and C ring but comprise of different number of hydroxyl groups on the B-ring where compared against each other. It was found that the number of hydroxyl groups on the B-ring does not influence MGO-trapping efficacy where luteolin (with 2 hydroxyl groups at position C-2 and C-3) showed a slightly stronger trapping efficacy (Figure 2.11) (Shao *et al.*, 2014).

Apigenin



Luteolin

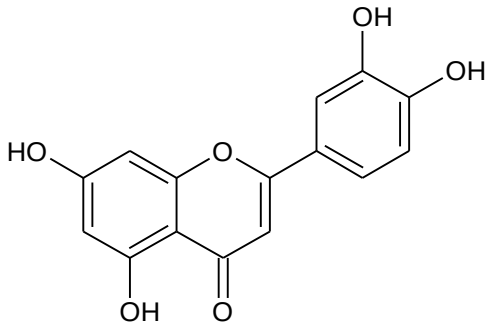


Figure 2.11: Structure of apigenin and luteolin with differences in hydroxyl substitution on the B ring.

To study the effects of the MGO-trapping efficacy of the C-ring, structures with identical A- and B-ring but different C-ring were investigated. Epicatechin, luteolin and quercetin were compared against each other. Epicatechin is a flavan-3-ol, consists of a hydroxyl group on C-3, a ketone group on ring C but no double bond. Quercetin and luteolin are flavones, consist of a double bond between C-2 and C-3 and a ketone group on C-4. The trapping efficacy was as follows: quercetin>luteolin>epicatechin. Quercetin has the best MGO trapping efficacy

followed by luteolin; they both trapped more than 90% of MGO within 8 h and epicatechin efficiently trapped more than 85% of MGO within 24 h (Figure 2.12).

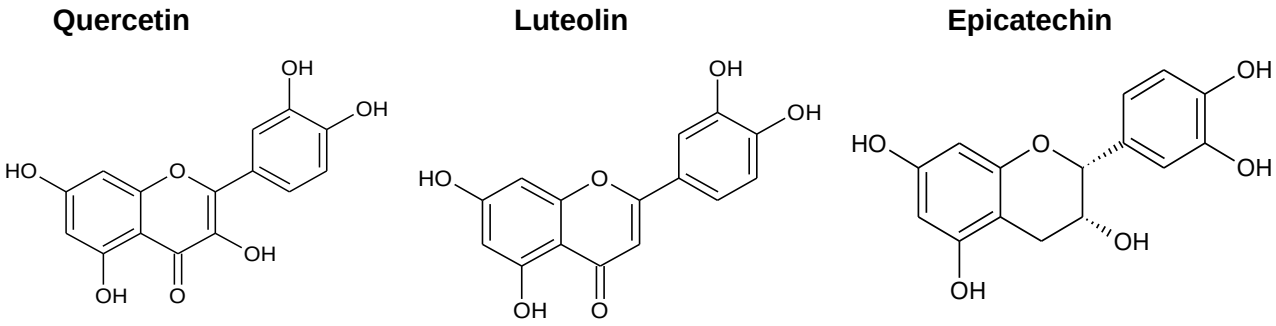


Figure 2.12: Differences in C ring structure of quercetin, luteolin and epicatechin with MGO trapping efficiency of quercetin>luteolin>epicatechin.

Little is known regarding the effect of trapping on the bioactivity of flavonoids and MGO. In this study, CAT, CHRY and NAR are used, to determine the effect of MGO and/or trapping on the antioxidant activity of flavonoids and the antibacterial activity of MGO in a physiological environment.

2.4 Aim and objectives

The aim of this study is to determine the effects of MGO on the antioxidant properties of flavonoids (CAT, CHRY and NAR) as well as the effect of flavonoids (CAT, CHRY and NAR) on the antibacterial effects of MGO.

The above aim will be achieved via the following objectives:

To determine if the presence of MGO or MGO trapping by flavonoids, CAT, CHRY and NAR:

1. Reduces the total polyphenol content (TPC), the total flavonoid content (TFC) and antioxidant activity measured with the Trolox equivalent antioxidant capacity (TEAC) and oxygen radical absorbance capacity (ORAC) assays.
2. Reduces flavonoid mediated inhibition of MGO induced bovine serum albumin (BSA) glycation.
3. Alters the antioxidant activity of flavonoids using the dichlorofluorescein diacetate (DCFH-DA) assay in the mouse fibroblast, L929 cell line.
4. Alters L929 cell viability and number evaluated with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium (MTT) and crystal violet (CV) assays respectively.
5. Reduces the antibacterial activity of MGO against Gram-positive (*B. subtilis*) and Gram-negative (*E. coli*) bacteria.

6. Alters MGO induced changes to bacteria structure evaluated with scanning electron microscopy (SEM).

Chapter 3: Effect of MGO binding with flavonoids on antioxidant activity and capacity

3.1 Introduction

Polyphenols are secondary metabolites found in plants, and can be divided into various subgroups due to the number of phenol rings and the structural elements that bind the rings together (Manach, 2004). Flavonoids are one of these subgroups, these polyphenols exhibit many biological activities, which includes antiviral, anti-inflammatory, antiallergenic, and vasodilating actions (Pietta, 2000). The antioxidant activity of flavonoids is due to the ability of these molecules, to scavenge ROS, bind metals that act as catalysts of the Fenton reaction, quench singlet oxygen, to donate an electron or hydrogen, to break chain propagation or suppress chain initiation or is a peroxide decomposer. In addition to these biological properties, flavonoids can trap MGO, a major precursor of AGEs (Shao *et al.*, 2014).

Methylglyoxal is a dicarbonyl compound present in cells under normal and pathological conditions as a by-product of cellular metabolism (Allaman *et al.*, 2015b). Under pathological conditions, MGO adversely affects cellular activity due to its high reactivity with basic residues of proteins, forming major precursors of AGEs (Maessen *et al.*, 2015) which is associated with pathologies such as T2DM, Alzheimer's disease, epilepsy, autism, anxiety, hyperalgesia and inflammation, among others (Dafre *et al.*, 2015).

Several flavonoids can inhibit AGE formation by trapping MGO to form mono- or di- MGO adducts (Liu *et al.*, 2017). The mechanism of trapping MGO is due to an increase in nucleophilicity of the unsubstituted carbons at the A ring, at a slightly alkaline pH, which results in the addition of MGO at positions C6 and C8 (Wang and Ho, 2012). MGO trapping has been described for CAT, genistein, quercetin, CHRY, luteolin, pyrogallol (PYR), daidzein, apigenin and phloretin (Shao *et al.*, 2014) however with increased trapping, it is unknown whether the ability of flavonoids to act as antioxidants to scavenge free radicals is compromised.

Therefore, the aim of the research in this chapter is to determine if the presence of MGO and/or MGO trapping alters the antioxidant content and properties of CAT, CHRY and NAR.

3.2 Materials

3.1.1 Chemicals, instruments and apparatus

Instruments, plastic disposables and glassware included: beakers, plastic pipettes, 15 mL centrifuge tubes, 96 well plates, pipette tips, spatulas and weighing boats were purchased from Greiner Bio-one supplied by LASEC, South Africa (SA).

The reagents and chemicals that were used in this study included: Folin-Ciolcateu (F-C) reagent, gallic acid, CAT, CHRY, NAR, sodium carbonate (Na_2CO_3), sodium nitrite (Na_2NO_2), aluminium chloride ($AlCl_3$), sodium hydroxide (NaOH), sodium chloride (NaCl), disodium hydrogen phosphate (Na_2HPO_4), sodium dihydrogen phosphate monohydrate ($NaH_2PO_4.H_2O$), hydrochloric acid (HCl), potassium peroxodisulfate ($K_2S_2O_8$), 2,2'-azo-bis(3 methylbenzothiazoline-6-sulfuricacid) diamonium salt (ABTS), Trolox, fluorescein, 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) and were obtained from Sigma Aldrich, SA.

Absorbance readings and fluorescence units were read using the EMax micro-plate reader supplied by Molecular devices, England, United Kingdom and Fluostar Omega fluorometer supplied by BMG LABTECH, Ortenberg, Germany. The equipment mentioned above was used in the Cell Biology and Histology Laboratory, Faculty of Health Sciences, University of Pretoria.

3.3 Solutions and buffers

3.3.1 Phosphate buffered saline solution

A 0.1 M phosphate buffered saline solution (PBS) was prepared by mixing Na_2HPO_4 (0.2 M, 28.39 g/L), $NaH_2PO_4.H_2O$ (0.2 M, 27.60 g/L) and NaCl (0.2 M, 10.88 g/L) in 1 L, with pH adjusted to 7.4 using NaOH.

3.3.2 Phosphate buffer

A 0.01 M phosphate buffer pH 7.00, was used specifically for the ORAC assay was prepared by mixing 1.099 g of Na_2HPO_4 with 1.689 g of $Na_2HPO_4.H_2O$ in 2 L ddH_2O and then adjusting the pH to 7.00 with NaOH.

3.4 Methods

Samples

Stock solutions of 100 mM of each flavonoid, (CAT, CHRY, NAR) and MGO were prepared and kept in the freezer. To prepare the working solutions, the stock solution was diluted to 2 mM of flavonoids and 2 mM and 4 mM MGO. Combinations of 1:1 and 1:2 mM of flavonoid:MGO were prepared.

3.4.1 Total polyphenol content assay

The F-C is an oxidation-reduction (redox) reaction where the phenolate ion is oxidized under alkaline conditions while reducing the phosphotungstic-phosphomolybdic complex in the reagent to a blue coloured solution (Figure 3.1). The maximum absorption is dependent on the alkaline solution and concentration of phenolic compounds (Duttagupta *et al.,* 2015). Although this assay is used to measure total polyphenol content (TPC), usually expressed as gallic acid equivalents (GAE) this reaction is essentially a redox reaction and therefore several researchers recommend that the F-C reaction should be considered as a measure of antioxidant capacity (Everette *et al.*, 2010).

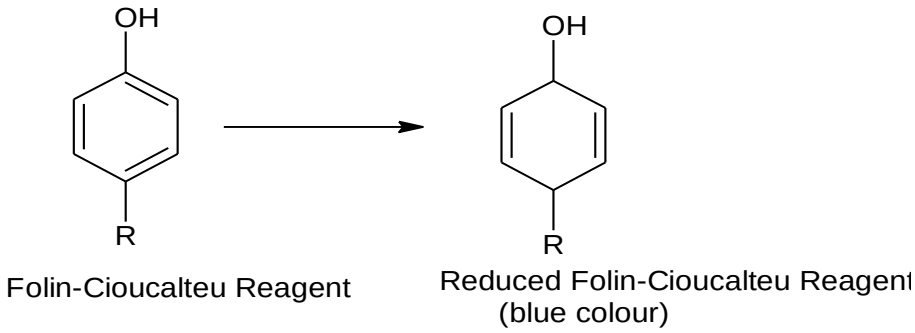


Figure 3.1: Reduction of F-C reagent forming a blue colour product.

Increasing volumes of MGO, flavonoid and the MGO: flavonoid mixtures (ratios 1:1 and 1:2) were added in triplicate to the wells of a 96 well plate. Double distilled water was added to make a final volume of 25 µL. A 50 µL volume of a 15x diluted F-C reagent (1 mL of the F-C reagent and 14 mL of distilled water) was added to all wells. Then, 50 µL of a 7.5% Na_2CO_3 solution was added to each sample. The samples were then mixed well, and the absorbance measured at 630 nm. The control consisted of double distilled water, Na_2CO_3 and F-C reagent. All data was expressed absorbance representing the polyphenol content.

3.4.2 Total flavonoid content assay

Aluminium chloride forms acid stable complexes with the C-4 keto group and either C-3 or C-5 hydroxyl groups of flavones and flavanols (Chang *et al.*, 2002) and acid labile complexes with the orthodihydroxyl groups in the A- or B-ring of flavonoids (Figure 3.2) (Kalita *et al.*, 2013).

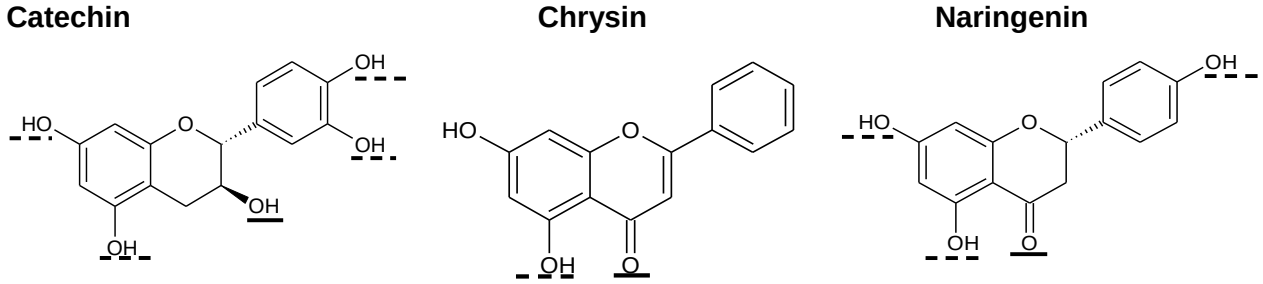


Figure 3.2: Reactive hydroxyl groups on flavonoids, CAT, CHRY and NAR that form acid labile complexes (broken line) and hydroxyl/ketone groups (underlined) that complex with aluminium chloride.

Increasing volumes of MGO, flavonoid and the MGO: flavonoid mixtures (ratios 1:1 and 1:2) were added in triplicate to the wells of a 96 well plate. Double distilled water was added to make a final volume of 25 µL. A volume of 30 µL of a 2.5% $NaNO_2$ solution was added to the plate with samples, followed by 30 µL of a 1.25% $AlCl_3$ solution. Then 100 µL of a 2% NaOH solution was added to each sample. The samples were then mixed well, and the absorbance measured at 450 nm. The control consisted of double distilled water, $NaNO_2$, $AlCl_3$ and NaOH. All data was expressed absorbance, representing the flavonoid content.

3.4.3 Trolox equivalent antioxidant capacity assay

The TEAC assay measures the quenching of the long –lived ABTS* radical cation. This ABTS radical cation is a blue/ green chromophore with a characteristic absorbance maximum at 734 nm (Fernandes de Oliveira *et al.*, 2012). This assay is an electron transfer based assay which involves the oxidation of 2,2' –azinobis(3-ethylbenzothiazoline-6-sulphonic acid) ($ABTS^{2-}$) to $ABTS^{\cdot-}$ by $K_2S_2O_8$, which is then reduced back to $ABTS^{2-}$ through its interaction with antioxidants, as shown in Figure 3.3.

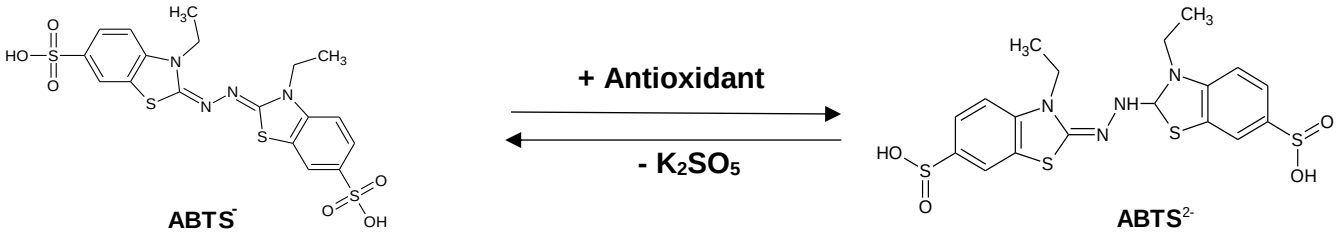


Figure 3.3: Reduction of ABTS radical by potassium persulfate through its interaction with an antioxidant compound during the Trolox equivalent antioxidant capacity assay (Zulueta *et al.*, 2009).

The $ABTS^{\cdot-}$ (2,2'-azo-bis(3 methylbenzothiazoline-6-sulfuric acid) diammonium salt) was generated by adding 3 mM of K_2S_2O solution to 8 mM of ABTS. The mixture was then left to react in the dark for about 12 h at room temperature. Then the working solution was prepared by diluting the $ABTS^{\cdot-}$ solution 30x with 0.1 M phosphate buffer, pH of 7.4.

Increasing volumes of MGO, flavonoid and the MGO: flavonoid mixtures were added in triplicate to the wells of a 96 well plate and then a volume of 290 µL of the working solution was added to each well. After an incubation time of 15 min for the Trolox standards and 30 minutes for the samples the absorbance of these samples was read at 734 nm. Trolox at a final concentration range of 0 – 0.033 mM was used, and all data was expressed as absorbance units. For the sample blanks each sample served as its own control there was no ABTS added, only buffer, whereas the vehicle control included reagent with only buffer.

3.4.4 Oxygen radical absorbance capacity assay

The ORAC assay is based on the oxidation of a fluorescent probe by peroxyl radicals, in a hydrogen atom transfer (HAT) process. Free radical initiators produce peroxyl radicals ($ROO^{\cdot}$) which quench the fluorescent probe over time. Antioxidants block the peroxyl radical oxidation of the fluorescent probe, until the antioxidant activity in the sample is depleted. The remaining peroxyl radicals destroy the fluorescence of the fluorescent probe, Figure 3.4. This assay continues until both antioxidant inhibition and inhibition percentage of the free radical damage is a single value (Ou *et al.*, 2002). In this assay AAPH was used as the peroxyl radical generator, fluorescein as a fluorescent probe and Trolox as standard.

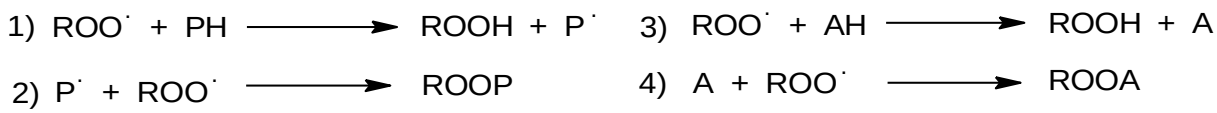


Figure 3.4: Schematic diagram of the ORAC assay principle (Huang et al., 2005). Key: PH= probe, $ROO^{\cdot}$= AAPH generated peroxyl radicals, AH= antioxidant.

A volume of 25 µL of a 1 mM flavonoid and the flavonoid:MGO mixtures were added to the well of an opaque 96 well plate. A volume of 150 µL of 0.139 mM fluorescein working solution was then added to each well followed by 25 µL of a 0.24 M AAPH solution. Fluorescence was measured every minute for 1 h 30 min at an excitation wavelength (Ex) of 485 nm and an emission wavelength (Em) of 520 nm. The net area under the decay curves (AUC) will be calculated using the following equation:

$$\mathbf{AUC_{net} = AUC_{sample} - AUC_{control}}$$

Trolox, at final concentrations of 0 – 0.05 mM was used to prepare a standard curve. All data was expressed as AUC. The blank contained buffer and fluorescein while the control contained AAPH, fluorescein and buffer.

3.4.5 Prevention of protein glycation

MGO induces protein glycation that leads to cellular dysfunction and death (Navarrete Santos *et al*., 2017). To evaluate whether flavonoid trapping reduces protein glycation, the MGO – albumin model was used. Serum albumin is the most abundant serum protein and readily undergoes glycation by MGO with the formation of Schiff base products. Flavonoids inhibit bovine serum albumin (BSA) glycation and theoretically, the trapping of MGO by flavonoids would reduce the MGO levels and consequently the extent of BSA glycation (Figure 3.5).

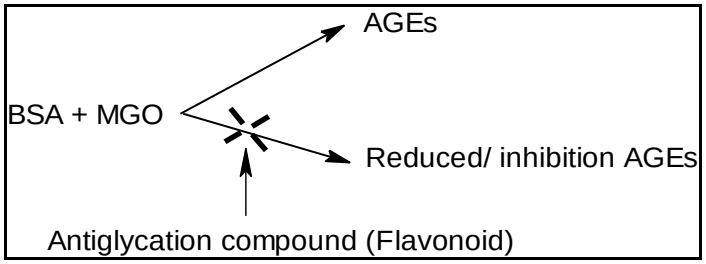


Figure 3.5: Schematic diagram of the interaction of BSA with MGO showing the formation of AGE, that can be inhibited with antiglycation compounds such as flavonoids.

Two different strategies were used. In a fluorescent 96 well plate:
A) A 50 µL volume of MGO or flavonoids, CAT, CHRY or NAR alone and in 1:1 and 1:2 ratios were added to 50 µL of a 40 mg/mL BSA solution prepared in 10 mM phosphate buffer pH 7.4. This mixture was made up to a final volume of 200 µL with buffer.

B) A 50 µL volume of MGO or flavonoids, CAT, CHRY or NAR alone and in 1:1 and 1:2 ratios were added to 50 µL of a 40 mg/mL BSA solution prepared in 10 mM phosphate buffer pH 7.4. In addition, MGO (50 µL, 56 mM, final concentration of 14 mM) was then added. This mixture was made up to a final volume of 200 µL with buffer.

The positive control (100% AGE formation) included BSA (50 µL, 40 mg/mL) and MGO (50 µL, 56 mM) only also made up to 200 µL with buffer.

A and B were incubated at 37˚C for seven days and then the fluorescence was measured at an Ex 330 nm and an Em 420 nm. The percentage AGE formation of extracts was calculated using the following equation:

$$\%\text{AGE formation} = \frac{\text{(sample - negative control)}}{\text{(positive control - negative control)}} \times 100$$

3.4.6 Data management and statistics

All experiments were done 3 times in triplicate, generating 9 data points. Data was reported as mean ± SEM. Data was tested for normality using the D'Agostino-Pearson and Shapiro-Wilk tests. Significant differences between groups were then analysed using either a Kruskal-Wallis test with Dunn's post hoc analysis or a one-way ANOVA with Tukey's post hoc analysis, $p < 0.05$.

3.5 Results

3.5.1 Total polyphenol content assay

The TPC was determined with the F-C assay. This assay was used to determine whether the interaction between the flavonoids and MGO, at phsyiological pH, reduces the ability of CAT, CHRY and NAR to participate in redox reaction either due to these flavonoids binding MGO or due to MGO interfering with the redox reaction. For CAT, from 0.04 mM to 0.23 mM a linear increase in absorbance was observed (Figure 3.6). MGO did not cause an increase or loss in absorbance and therefore did not interfere with the F-C assay or redox reaction. CAT:MGO and CAT:MGO2 combined at a 1:1 and 1:2 ratio showed a similar linear correlation in absorbance. There was no significant differences observed between CAT alone and its combinations with MGO (Table 3.1).

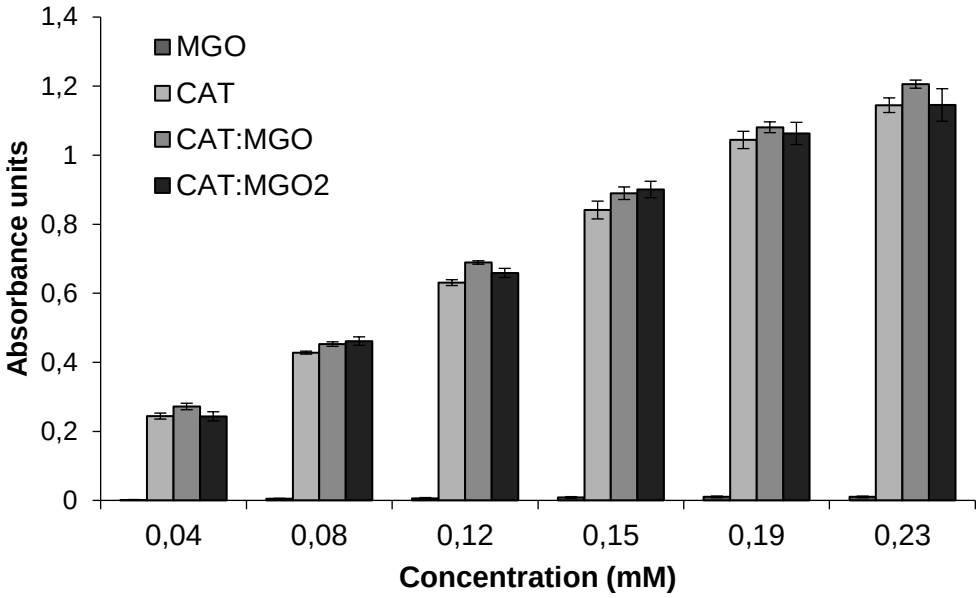


Figure 3.6: Effect of MGO and CAT in combination on CAT measured with the F-C assay. Data is expressed as mean ± SEM, with the asterisk (*) indicating significant difference between CAT and the combinations, (CAT:MGO (Ratio 1:1) and CAT:MGO2 (Ratio 1:2)) at $p < 0.05$.

CHRY showed a logarithmic increase rather than a linear increase, compared to CAT (Figure 3.7). Increased linearity was observed for the 1:1 and 1:2 ratio combinations (CHRY:MGO and CHRY:MGO2). Significant differences were observed in concentrations 0.04 - 0.12 mM compared to CHRY. Although differences were observed at specific concentrations, differences between 0.04-0.23 mM were not significant (Table 3.1).

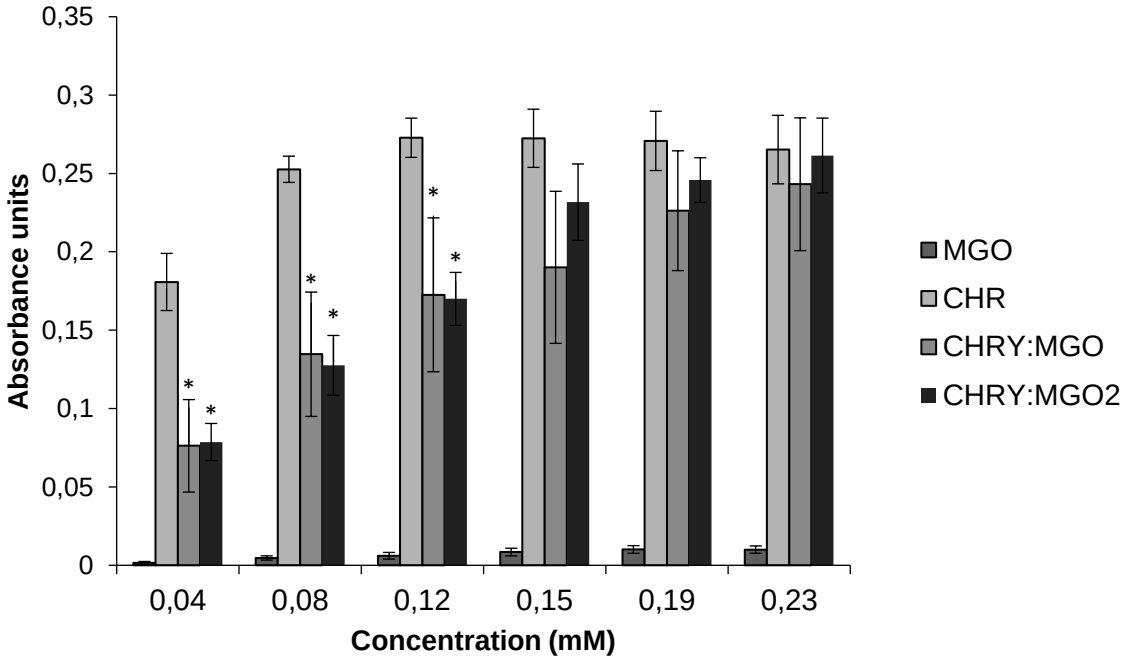


Figure 3.7: Effect of MGO and CHR in combination on CHR measured with the F-C assay. Data is expressed as mean ± SEM, with the asterisk (*) indicating significant difference between CHRY and the combinations (CHRY:MGO (Ratio 1:1) and CHRY:MGO2 (Ratio 1:2)) at $p < 0.05$.

NAR showed a somewhat similar logarithmic trend as CHRY (Figure 3.8). Like CAT, there were no significant differences between NAR and NAR in combination with MGO (Table 3.1).

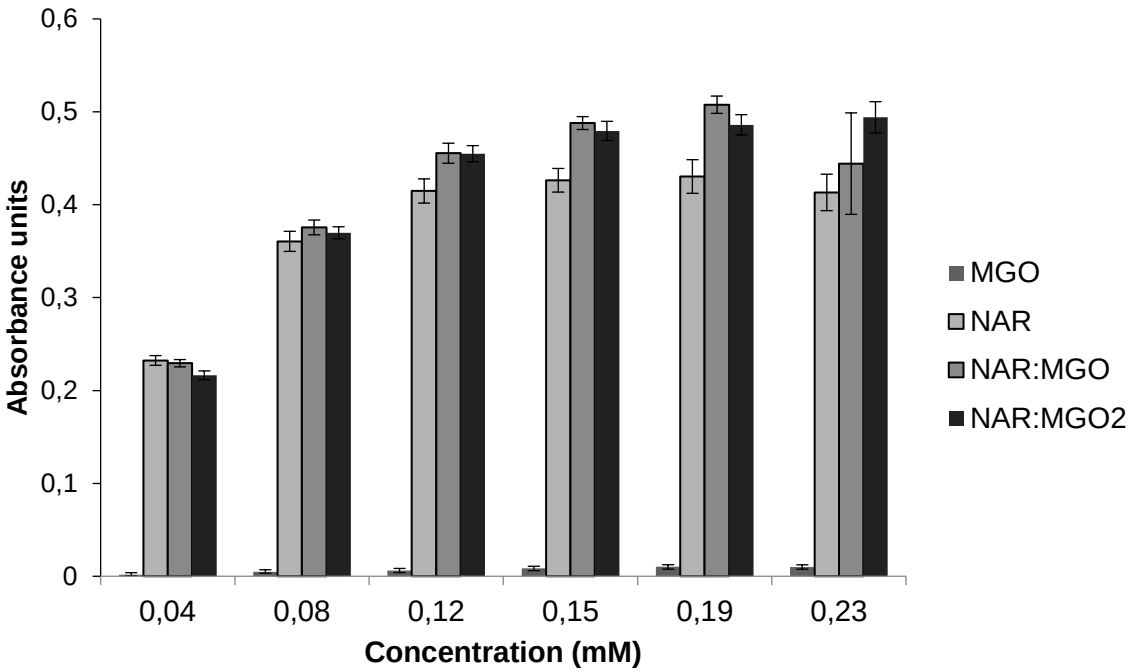


Figure 3.8: Effect of MGO and NAR in combination on NAR measured with the F-C assay. Data is expressed as mean ± SEM, with the asterisk (*) indicating significant difference between NAR and the combinations (NAR:MGO (Ratio 1:1) and NAR:MGO2 (Ratio 1:2)) at $p < 0.05$.

Table 3.1 represents the gradients of the phenolic content of the flavonoids (CAT, CHRY and NAR) and their combinations (CAT:MGO1, CAT:MGO2; CHRY:MGO1, CHRY:MGO2 and NAR:MGO1, NAR:MGO2). Of the flavonoids compared to CHRY and NAR, CAT had the greater antioxidant capacity while that of CHRY was the lowest and in the presence of MGO activity was unaltered.

Table 3.1: Summary of the TPC, gradients and statistics for each flavonoid in combination with MGO.

	CAT (a)	**CAT:MGO1 (b)**	**CAT:MGO2 (c)**	**a vs b**	**a vs c**	**b vs c**
Gradient	5,06	5,27	5,12	ns	ns	ns
r^2	0,99	0,99	0,98			
	CHRY (a)	**CHRY:MGO1 (b)**	**CHRY:MGO2 (c)**	**a vs b**	**a vs c**	**b vs c**
Gradient	0,92	1,01	1,14	ns	ns	ns
r^2	0,57	0,88	0,94			
	NAR (a)	**NAR:MGO1 (b)**	**NAR:MGO2 (c)**	**a vs b**	**a vs c**	**b vs c**
Gradient	1,58	1,86	1,96	ns	ns	ns
r^2	0,68	0,70	0,78			

3.5.2 Total flavonoid content (TFC) assay

Binding of MGO to flavonoids, via steric interactions (Figure 2.9) or reactions with the hydroxyl groups involved in the reaction with aluminium may reduce the measurement of TFC. MGO did not interfere with the aluminium chloride assay used to quantify flavonoids.

With increasing concentrations of CAT there is a linear increase in absorbance (Figure 3.9). For CAT in combination with 1 and 2 mM MGO there was also a linear increase in absorbance. A significant reduction was observed for CAT:MGO and CAT:MGO2 at all concentrations.

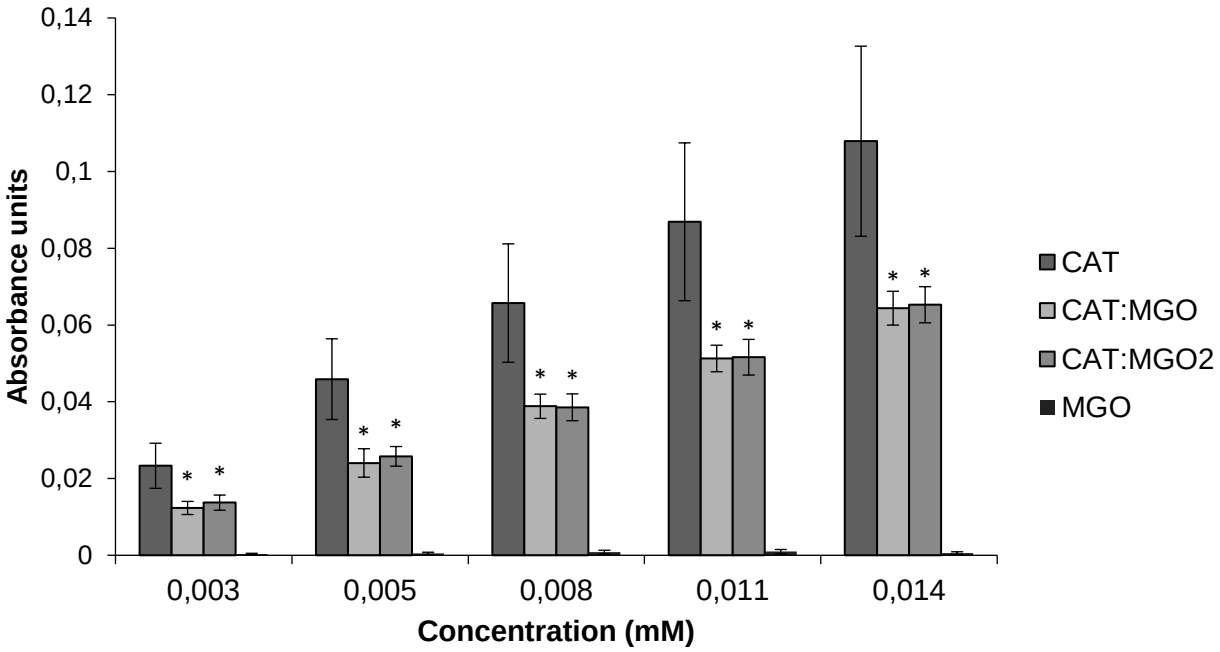


Figure 3.9: Effect of MGO and CAT interaction on the measurement of the on the total flavonoid content of CAT. Data is expressed as mean ± SEM, with * indicating significant differences between CAT and the combinations (CAT:MGO (Ratio 1:1) and CAT:MGO2 (Ratio 1:2)) at $p<0.05$.

For CHRY, the reaction of CHRY with aluminium was low compared to CAT. This reduction in reactivity compared to CAT may be due to the presence of a ketone instead of a hydroxyl group on position 4. A linear increase in absorbance was observed for CHRY:MGO and CHR:MGO2. Differences between groups was not significant, except for CHRY vs. CHRY:MGO1 (Table 3.2). High SEM bars may be related to the low absorbance readings or the poor aqueous solubility of CHRY, Figure 3.10 (Zhou *et al.*, 2014).

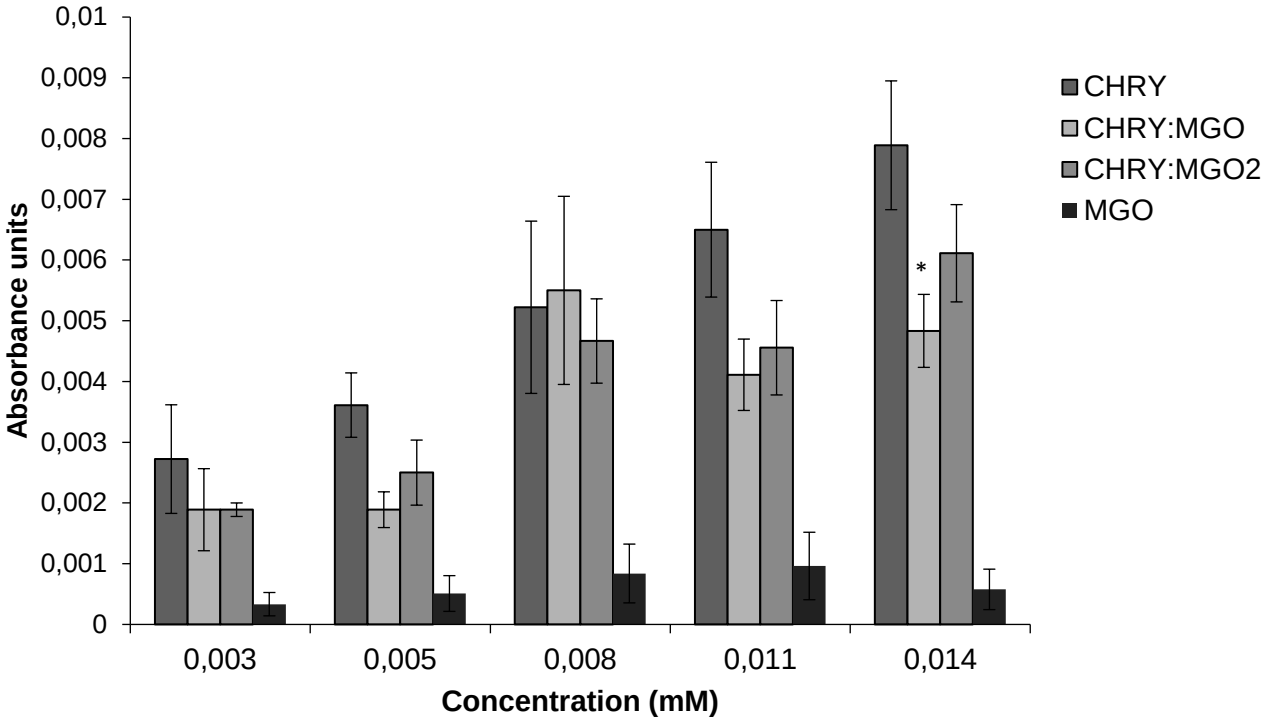


Figure 3.10: Effect of MGO and CHRY interaction on the measurement of the on the total flavonoid content of CHR. This data is expressed as mean ± SEM, with the asterisk (*) indicating significant difference between CHRY and the combinations (CHRY:MGO (Ratio 1:1) and CHRY:MGO2 (Ratio 1:2)) at $p < 0.05$.

NAR reacted with aluminium but not to the same degree as CAT. With increasing concentrations of NAR there is a linear increase in absorbance. Likewise, in combination with MGO there is an increase in absorbance. In combination with MGO, there is a significant reduction in the TFC of NAR from concentrations of 0.005 mM onwards (Figure 3.11).

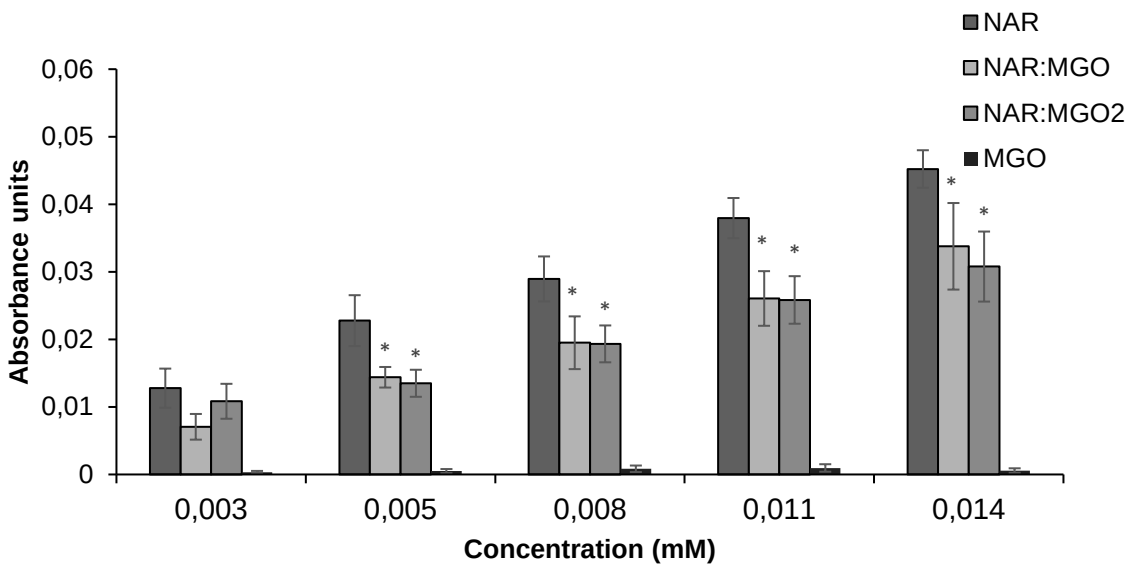


Figure 3.11: Effect of MGO and NAR interaction on the total flavonoid content of NAR. This data is expressed as mean ± SEM, with the asterisk (*) indicating significant difference between NAR and the combinations (NAR:MGO (Ratio 1:1) and NAR:MGO2 (Ratio 1:2)) at $p < 0.05$.

Table 3.2 represents the overall gradients of the flavonoid content of the flavonoids (CAT, CHRY and NAR) and their combinations (CAT:MGO1, CAT:MGO2; CHRY:MGO1, CHRY:MGO2 and NAR:MGO1, NAR:MGO2). CAT had the greatest TFC compared to CHRY and NAR. The presence of MGO did not alter the flavonoid content of CHRY but altered that of CAT and NAR.

Table 3.2: Summary of the TFC gradients and statistics for flavonoid alone and in combination with MGO.

	CAT (a)	**CAT:MGO1 (b)**	**CAT:MGO2 (c)**	**a vs b**	**a vs c**	**b vs c**
Gradient	7,69	4,63	4,75	**s**	**s**	ns
r^2	0,99	0,99	0,99			
	CHRY (a)	**CHRY:MGO1 (b)**	**CHRY:MGO2 (c)**	**a vs b**	**a vs c**	**b vs c**
Gradient	0,54	0,37	0,39	**s**	ns	ns
r^2	0,96	0,79	0,85			
	NAR (a)	**NAR:MGO1 (b)**	**NAR:MGO2 (c)**	**a vs b**	**a vs c**	**b vs c**
Gradient	3,17	2,40	2,18	**s**	**s**	ns
r^2	0,98	0,98	0,97			

3.5.3 Trolox equivalent antioxidant capacity assay

The TEAC assay involves a single electron transfer whereby the probe (oxidant, which is $ABTS^{\cdot-}$) abstracts an electron from the antioxidant resulting in a colour change of the probe ($ABTS^{2-}$); the degree of the colour change is thus proportional to the antioxidant concentration (Huang *et al.*, 2005). The ability of an antioxidant to scavenge ROS is evaluated with this assay.

The results show that MGO did not interfere in the TEAC assay i.e. there is no increase in absorbance. With increasing concentrations of CAT there is an increase in absorbance. CAT in combination with MGO did not alter the antioxidant capacity of CAT. There were no statistically significant differences between CAT and CAT in combination with MGO (Figure 3.12, Table 3.3).

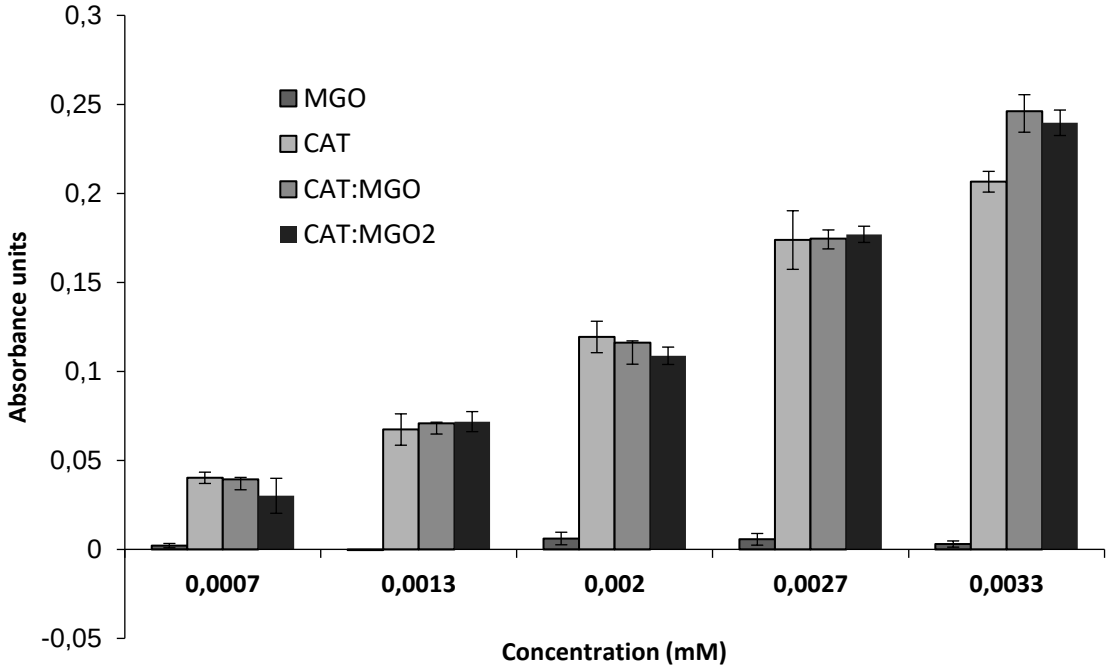


Figure 3.12: The effect of MGO on the antioxidant capacity measured with the TEAC assay of CAT. Data is expressed as mean ± SEM, with the asterisk (*) indicating significant difference between CAT and the combinations (CAT:MGO (Ratio 1:1) and CAT:MGO2 (Ratio 1:2)) at $p < 0.05$.

The interaction of CHRY and MGO influences the antioxidant capacity of CHRY. With increasing concentrations of CHRY there is an increase in absorbance (Figure 3.13). In a ratio of 1:1 with MGO there is a reduction in absorbance with a 2.4-fold reduction in the gradient. Statistical differences compared to CHRY are significant. Likewise, for CHRY in combination with MGO at 1:1 (CHRY:MGO1) and 1:2 ratio (CHRY:MGO2), there is a decrease in absorbance compared to CHRY, however differences between CHRY:MGO and CHRY:MGO2 are not statistically significant indicating that 0.1 mM MGO is sufficient to establish a reaction equilibrium.

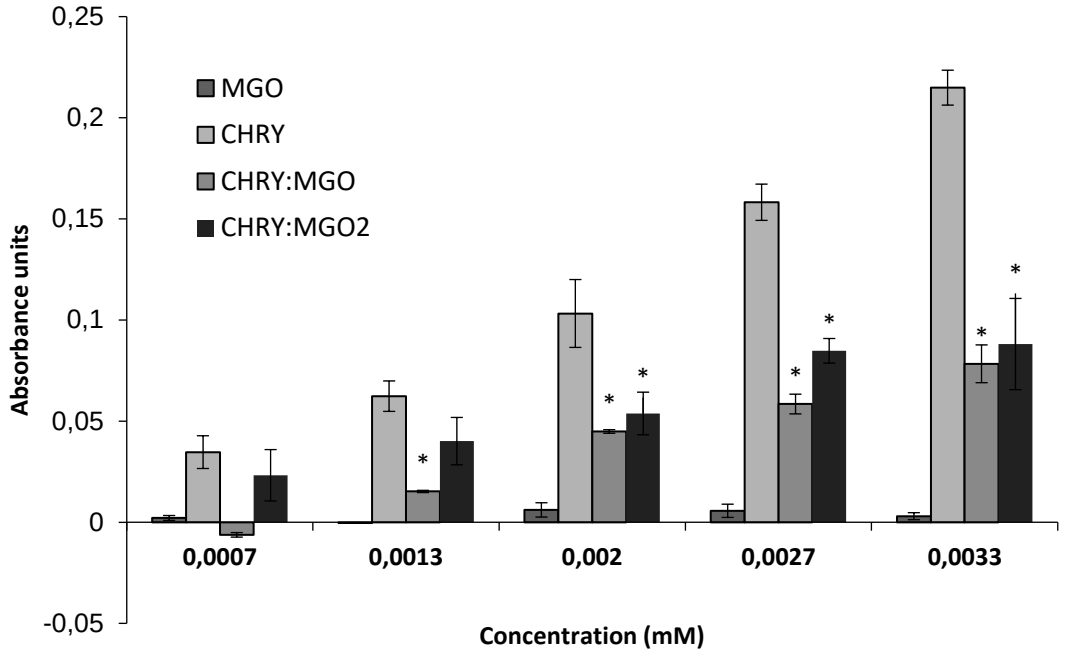


Figure 3.13: The effect of MGO on the antioxidant capacity measured with the TEAC assay of CHRY. This data is expressed as mean ± SEM, with the asterisk (*) indicating significant difference between CHRY and the combinations (CHRY:MGO (Ratio 1:1) and CHRY:MGO2 (Ratio 1:2) at $p < 0.05$.

As for CAT, with increasing concentrations of NAR there is an increase in absorbance. For the combinations of NAR:MGO and NAR:MGO differences are not statistically significant (Figure 3.14).

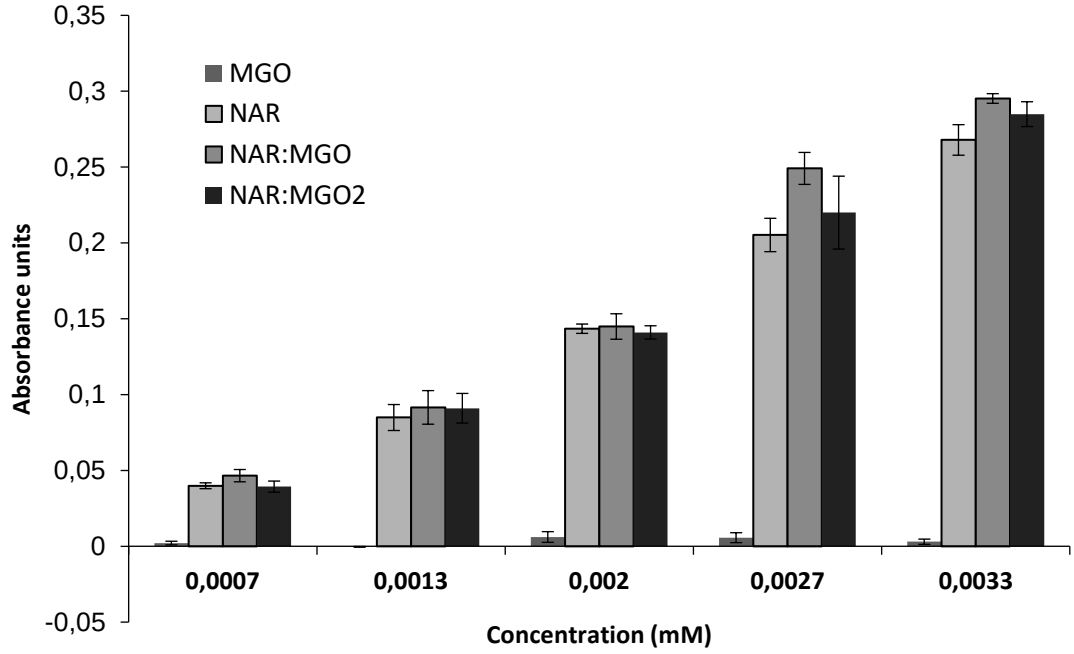


Figure 3.14: Effect of MGO on the antioxidant capacity measured with the TEAC assay of NAR. This data is expressed as mean ± SEM, with the asterisk (*) indicating significant difference between NAR and the combinations (NAR:MGO (Ratio 1:1) and NAR:MGO2 (Ratio 1:2)) at $p < 0.05$.

In summary, the antioxidant activity of CAT, CHRY and NAR is similar. In CHRY only there was a statistically significant loss of antioxidant activity observed in combination with MGO (Table 3.3). Table 3.3 below represents the overall antioxidant capacity of the flavonoids (CAT, CHRY and NAR) and their combinations (CAT:MGO1, CAT:MGO2; CHRY:MGO1, CHRY:MGO2 and NAR:MGO1, NAR:MGO2). The gradients for each graph was calculated to determine if there are significant differences between the respective flavonoid alone and in combination with MGO. Significant difference was observed for CHRY vs CHRY:MGO1 and CHRY vs CHR:MGO2, none of the other samples had significant difference at $p < 0.05$.

Table 3.3: Summary of the antioxidant (TEAC) gradients and statistics for flavonoid alone and in combination with MGO.

	CAT (a)	**CAT:MGO1 (b)**	**CAT:MGO2 (c)**	**a vs b**	**a vs c**	**b vs c**
Gradient	63,66[a]	71,42[b]	71,83[c]	ns	ns	ns
r^2	0,98	0,96	0,97			
	CHRY (a)	**CHRY:MGO1 (b)**	**CHRY:MGO2 (c)**	**a vs b**	**a vs c**	**b vs c**
Gradient	63,6[a]	26,37[b]	27,38[c]	**s**	**s**	ns
r^2	0,97	0,92	0,90			
	NAR (a)	**NAR:MGO1 (b)**	**NAR:MGO2 (c)**	**a vs b**	**a vs c**	**b vs c**
Gradient	81,17[a]	91,59[b]	86,42[c]	ns	ns	ns
r^2	0,98	0,98	0,97			

3.5.4 Oxygen radical absorbance capacity (ORAC) assay

The ORAC assay is based on the scavenging of peroxyl radicals generated by AAPH, by an antioxidant preventing the degradation of the fluorescein probe (Dudonné *et al.*, 2009). Figure 3.15a and b shows that although MGO is an oxidant, it does not interfere AAPH radical generation. Of the flavonoids evaluated, CAT had the lowest antioxidant capacity of 8.70 µM TE, and a decrease in antioxidant capacity for CAT:MGO1 and CAT:MGO2 at 0.93 and 2.94 µM TE, respectively. Both CHRY and NAR had similar antioxidant activity and for both flavonoids in the presence of MGO there was no significant difference in antioxidant activity.

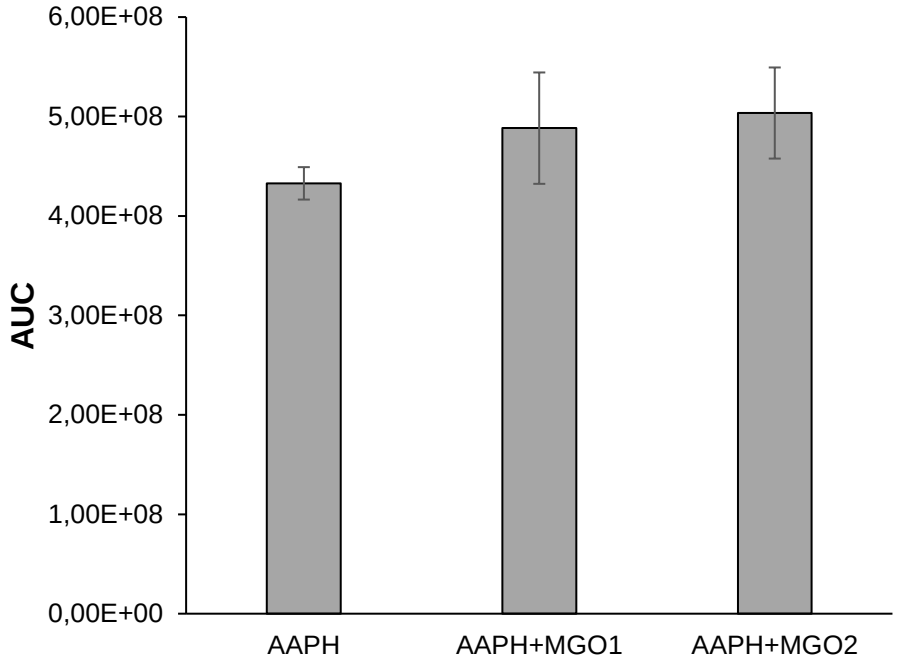


Figure 3.15a*:* The area under curve of AAPH alone and in combination with MGO1 and MGO2. Data expressed as an average of 4 experiment ± SD. No significant differences observed compared to AAPH alone.

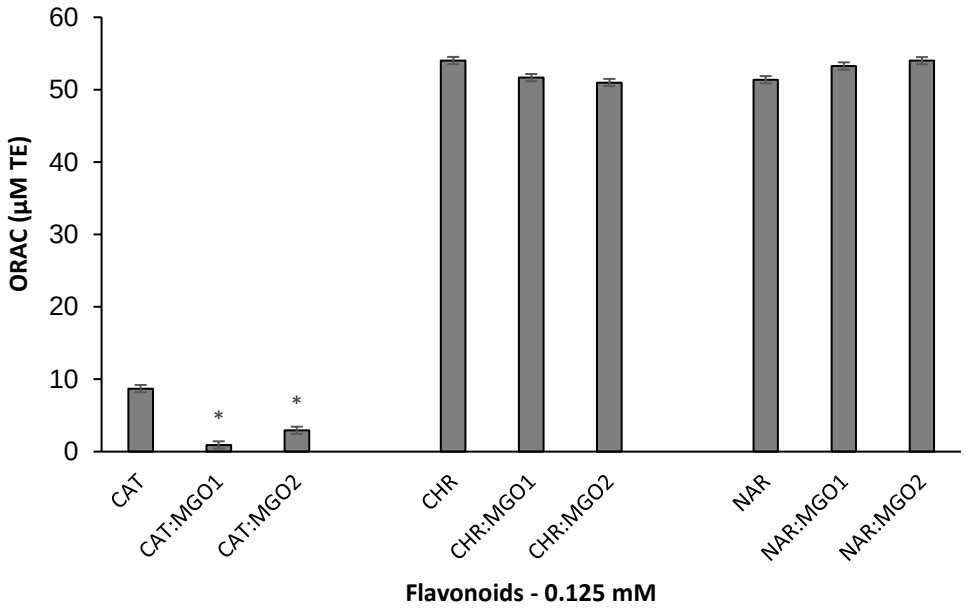


Figure 3.15b: Effect of MGO on antioxidant capacity of flavonoids CAT, CHRY and NAR measured with the ORAC assay. Data expressed as an average of 3 experiments ± SEM, with the asterisk (*) indicating significant difference between flavonoid and the flavonoid:MGO combinations, at $p < 0.05$. For these experiments, SEM is < 1%.

3.5.5 Anti-glycation activity

Figure 3.16a (experimental strategy A) and b (experimental strategy B) represents the anti-glycation properties of flavonoids, CAT, CHRY and NAR. MGO at 14mM (positive control) caused an increase in measured fluorescence and this was set at 100% AGE formation. Compared to the positive control, 0.25 mM MGO did not induce AGE formation while 0.5 mM MGO induced 31.12% AGE formation. The flavonoids (CAT, CHRY and NAR) alone and in combination with MGO (1:1 and 1:2 ratio) and BSA were not sufficient to induce significant levels of AGE.

In Figure 3.16b, the experimental sample contains 10 mg/mL BSA and 14 mM MGO. With the addition of flavonoids and MGO in combination, the MGO was increased to 14.25 and 14.50 mM, for MGO1 and MGO2, respectively. A flavonoid concentration of 0.25 mM CAT, CHRY and NAR was enough to reduce the %AGE formation from 100% to 85.36 ± 1.91% for CAT, 84.43 ± 1.96% for CHRY and 90.69 ± 1.95% for NAR. Equimolar combinations CAT:MGO and CHRY:MGO showed significant reduction in compared with the positive control of 85.93 ± 1.99% and 85.58 ± 2.58%, respectively. No significant differences were found for NAR:MGO compared with the positive control. At 1:2 ratio combination CAT:MGO2 and CHRY:MGO2 remained significantly different to the positive control with 88.90 ± 1.99% and 77.34 ± 4.17%, respectively, while NAR:MGO2 showed no significant difference compared with the postive control. In summary, all flavonoids and combinations of CAT and CHRY effectively inhibited MGO induced AGE formation while no effect was observed for NAR.

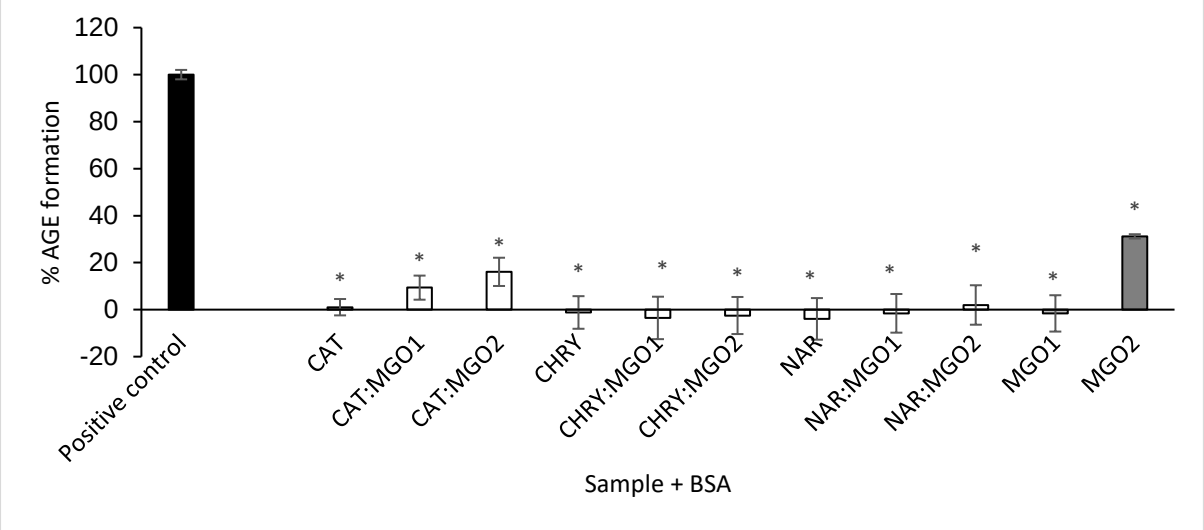


Figure 3.16a: Effects of flavonoids CAT, CHRY and NAR and the combination FLAV:MGO at 1:1 and 1:2 ratio against non-enzymatic glycation of BSA induced by MGO (Experimental stratergy A). Data is expressed as mean ± SEM, with the asterisk (*) indicating significant difference between positive control and samples with BSA only at $p < 0.05$.

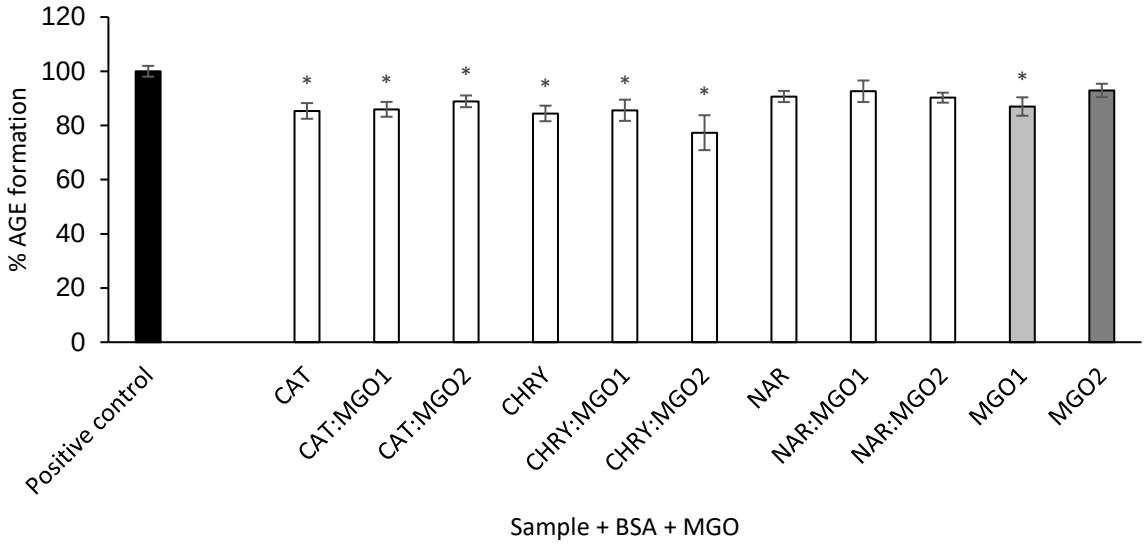


Figure 3.16b: Effects of flavonoids CAT, CHRY and NAR and the combination FLAV:MGO at 1:1 and 1:2 ratio against non-enzymatic glycation of BSA induced by MGO (Experimental stratery B). Data is expressed as mean ± SEM, with the asterisk (*) indicating significant difference between the positive control and samples exposed to BSA and MGO at $p < 0.05$.

3.6 Discussion

SAR plays an important role in determining the radical scavanging ability of compounds and for phenolic acids antioxidant activity is dependant on the number of hydroxyl groups in relation to the carboxyl functional groups (Balasundram *et al.*, 2006). In contrast, SAR are more complex in flavonoids compared to phenolic acids. The following conditions result in higher antioxidant activity: (i) the hydroxylation and position of the hydroxyl group (just as in phenolic acids), particularly the presence of an ortho-dihydroxyl on the ring B as it stabilises the aroxyl radical by electron delocalisation. The presence of hydroxyl groups on positions 3'- 4'- 5' of ring B, enhances antioxidant activity but may, in other conditions counteract the antioxidant effect and the compound acts as a prooxidant. Then, (ii) the radical scavenging capacity of flavonoids is enhanced by the double bond between C-2 and C-3, conjugated with the 4-oxo in the C-ring (Pietta, 2000). Another condition includes the (iii) the double bond between C-2 and C-3, conjugated with a 3-hydroxyl group in the C ring. On the contrary, the antioxidant activity may be reduced by: (i) substitution of the 3 hydroxyl group and substitution of the B-ring hydroxyl groups by methoxyl groups (Pietta, 2000; Seeram & Nair, 2002).

Flavonoids such as quercetin traps MGO with the formation of mono- and di-MGO adducts. Trapping as described by Zheng *et al.*, (2014) occurs *in vitro*, in a slightly alkaline environement, at pH 7.4. In these studies the change in MGO levels are measured and incubation of quercetin with MGO after 1 h results in more than 50.5% decrease in MGO levels. Although, the presence of the mono- and di-MGO derivatives have been identified the formation of these derivatives at physiological pH on the determination of antioxidant activity

is unknown. The aim of this current study was to determine the effects of MGO either as a consequence of binding or being freely in solution in combination with flavonoids.

Of the three flavonoids evaluated for TPC or reducing activity, CAT had the highest polyphenolic content, which was 0.4 and 0.06 fold greater that CHRY and NAR respectively. This may be related to the number of hydroxyl groups being present where CAT, NAR and CHRY have 5, 3 and 2 hydroxyl groups and the measured TPC gradients are 5.06, 1.86 and 0.92, respectively. In the presence of MGO, no changes were measured in antioxidant content with TPC and as adduct formation does not involve the hydroxyl groups (Figure 2.9) these functional groups can still undergo oxidation.

With flavonoids, aluminium chloride forms acid stable complexes with the C-4 keto group and either C-3 or C-5 hydroxyl groups of flavones and flavanols respectively (Chang *et al.*, 2002) and acid labile complexes with the ortho dihydroxyl groups in the A- or B-ring of flavonoids (Kalita *et al.*, 2013).

The TFC gradient measured for CAT was 7.69, for NAR was 3.17 and for CHRY was 0.54. This reactivity is related to the number of hydroxyl groups that can form labile complexes and the presence of a hydroxyl or ketone group on C3 that can form complexes with aluminium chloride. For CAT, NAR and CHRY, the number of hydroxyl groups that can form labile complexes are 4, 3 and 1 respectively (Figure 3.2). As these groups are not involved in adduct formation it is expected that mesurement of TFC will be unchanged. The presence of MGO did not alter TFC for CHRY, although differences were observed for CAT and NAR indicating possible steric effects.

The antiradical efficiency of antioxidant flavonoids is a function of the position and number of hydroxyl groups. Using the 2,2-diphenyl-1-picryl-hydrazyl-hydrat (DPPH) assay, a SET similar to the TEAC assay, Brand-Williams *et al.* (1995) observed that flavonoids had stoichiometries that correspond to the number of hydrogens available for donation on hydroxyl groups. In addition, the compounds whose second hydroxyl group is in the para or ortho positions have higher activities than those in the Meta position. Using the TEAC assay reported antioxidant activity was 2.4 mM, 1.53 mM and 1.43 mM for CAT, NAR and CHRY respectively (Heim *et al*., 2002); and may be related to the number of hydroxyl groups on the B ring. In the present study, CAT had the highest antioxidant activity, while the antioxidant activity of CHRY and NAR was similar, indicating that position rather than the number of hydroxyl groups is important for activity.

The TEAC assay measures the ability of antioxidants such as the flavonoids to inhibit reactive species by an electron transfer from antioxidant to the oxidant. The presence of MGO or the effect of MGO binding to CAT or NAR did not alter the antioxidant activity measured with the

TEAC assay. In contrast, the antioxidant activity of CHRY was reduced with the addition of MGO. In Hwang *et al*. (2018), trapping of MGO by CHRY was confirmed and di-MGO-conjugated adducts of CHRY were formed by classic aldol condensation and the reactions were at positions C6 or C6 and C8 in the A ring structure of CHRY. The CHRY MGO-conjugated adducts could not be confirmed by LC-MS, thus NMR was used (Hwang *et al.*, 2018). Villaño *et al.*, (2005) evalauted the antioxidant activity of wine phenolic compounds and the study was conducted using the ABTS, DPPH and ORAC assays respectively. The study reported TEAC to have a larger value for compounds with more hydroxyl groups in the B-ring; on the other hand, the antioxidant activity was higher for structures with two ortho-hydroxyl groups compared to those with a methoxy group adjacent to a hydroxy group (Villaño *et al.*, 2005). CAT and NAR has 2 and 1 hydroxyl group/s in the B ring respectively, whilst no hydroxyl groups are present in the B ring of CHR. Though the antioxidant activity of CAT was not affected, it cannot be concluded that trapping did not occur, It has been confirmed that MGO attaches to CAT at the C6 and or C8 position (ring A) by electrophilic substitution forming either a monocatechin-mono-MGO or a monocatechin-diMGO adduct (Yang *et al.*, 2018). It can be concluded that in spite of trapping, the hydroxyl groups in CAT are still functional and antioxidant activity is retained while for CHRY steric effects reduces activity.

The ORAC assay, which is considered to be a physiologically relevant assay for the measurement of antioxidant activity is widely used to measure the antioxidant activity in in foods relating to dietary antioxidants and physiological applications (Prior, 2015; Rodriguez-Amaya, 2015; Apak *et al*., 2016). This assay is based on the transfer of the hydrogen atom; whereby an antioxidant transferes a hydrogen atom to prevent the degradation of florescein probe by AAPH generated radicals. In this assay CAT showed the least antioxidant activity compared with CHRY and NAR, indicating that CAT is a strong antioxidant in electron transfer reactions, while CHRY and NAR are strong antixodants based on hydrogen atom transfer capacity, which may be due to the presence of the C4 ketone functional groups. The presence of MGO did not affect antioxidant activity of CHRY and NAR, but affected that of CAT, where a decrease in absorbance was observed for CAT in combination with MGO.

Ho *et al.* (2010) compared the anti-glycation capacities of several herbal infusions with that of green tea and found that herbal infusions which included flavonoids had anti-glycation capacities (Ho *et al.*, 2010). Likewise, CAT and CHRY used in the present study effectively reduced the %AGE formation. With the combinations with MGO, both CAT and CHRY effectively reduced %AGE formation. This effect may be due to direct trapping or prevention of AGE formation.

3.7 Variation in data for the different techniques was expected as different techniques employed detect different substrates (assay sensitivity and specificity). An independent, yet positive correlation was expected.

Conclusion

MGO in solution with CAT, CHRY and NAR under physiological conditions did not affect the TPC but affected TFC, the antioxidant activity of CHRY was reduced measured with the TEAC assay while the antioxidant activity of CAT was reduced measured with the ORAC assay. Overall the antioxidant activity of the flavonoids was not affected by the addition of MGO. The ability of 14 mM MGO to induce AGEs was reduced by CAT and CHRY. In the presence of added MGO, the combinations of CAT and CHRY still inhibited AGE formation. Therefore, changes in structure due to adduct formation may not necessarily interfere with the antioxidant and antiglycation activity. In conclusion, the trapping of methylglyoxal by flavonoids could possibly be an alternative method to effectively inhibit AGEs formation while retaining antioxidant activity.

Chapter 4: Effects of methylglyoxal in combination with flavonoids on L929 cell number, viability and cellular antioxidant activity

4.1 Introduction

Chemical assays such as the TEAC and ORAC assays measure the antioxidant activity without considering the physiological effects thereof. These chemical assays measure antioxidant activity in controlled systems and do not account for the relevant types of oxidants encountered, substrates to be protected or the partitioning of compounds (Frankel & Meyer, 2000). Cellular assays such as the DCFH-DA assay reflects biological activity by accounting for cellular uptake, partition of flavonoids between the different phases (liquid and solid or phase I and II metabolism) (Wolfe & Liu, 2008). Animal models and human studies offer the best measures for biological testing; however, these are time-consuming, expensive and are not ethically accepted for initial screening. Cell cultures address some biological issues i.e. cellular uptake, distribution and metabolism (Wolfe & Liu, 2007).

Flavonoids have well documented antioxidant activity and *in vitro* antioxidant activity studies have shown the antioxidant capacities of most flavonoids to be stronger than that of ascorbic acid and vitamin E (Prior & Cao, 2000). The *in vitro* flavonoid activity is reliant on SAR whereas flavonoid metabolites have greatly reduced *in vitro* antioxidant activity when compared to the parent compound, due to the blocking of free radical scavenging phenolic hydroxyl groups (Dueñas *et al.*, 2010; Procházková *et al.*, 2011). Besides acting as antioxidants and/or pro-oxidants, flavonoids also have diverse biological effects and these include the ability to inhibit lipoxygenase, cyclooxygenases, NADPH oxidases and metalloproteinases and this may affect cellular viability and cellular antioxidant activity.

MGO has been identified to be a major reactive carbonyl specie (RCS) in the human body and an AGEs precursor (Hu *et al.*, 2012). AGEs have been identified as age-related intracellular protein deposits of Parkinson and Alzheimer's disease (Webster *et al.*, 2005); this suggests a strong link between MGO, AGEs and aging. MGO is a potent glycating agent present in cells as a product of cellular metabolism (Allaman *et al.*, 2015). Different detoxifying mechanisms such as aldehyde dehydrogenase, aldose reductase, glyoxalase and carbonyl reductase pathways exist to circumvent MGO toxicity (Vander Jagt & Hunsaker, 2003). High MGO levels causes GSH trapping due to low glyoxalase-2 (Glo-2) activity, resulting in decreased GSH levels which is responsible for other cellular processes such as the defence against oxidative stress (Dringen, 2000).

A carbonyl scavenger may offer a therapeutic strategy to attenuate MGO toxicity. Polyphenols may be the leading scavengers in this regard as some polyphenols are able to trap MGO and

prevent AGE production. There is no information on the cellular effects of MGO and the consequence of trapping by flavonoids.

The aim of this chapter is to examine the cellular effects of MGO and flavonoids (CAT, CHRY and NAR) alone and in combination on cell number and viability as well as cellular antioxidant activity (CAA).

4.2 Materials

4.2.1 Chemicals, instruments and apparatus

All chemicals, instruments and apparatus used were the same as that used Chapter 3. In addition, potassium chloride (KCl), monosodium dihydrogen phosphate (NaH_2PO_4), crystal violet (CV), paraformaldehyde, acetic acid, Dulbecco's Modified Essential Medium (DMEM), trypsin, antibiotic solution (each mL containing 10 mg streptomycin, 10000 units penicillin and 25 µg amphotericin B), foetal bovine serum (FBS), obtained from Sigma Aldrich, SA were used.

4.3 Methods

4.3.1 Buffer and medium preparations

All buffers used were prepared similar to those in chapter 3, with the following additions:

4.3.1.1 Isotonic saline buffered PBS (isoPBS)

IsoPBS was prepared by mixing: NaCl (0.137M, 8g/L), KCl (3 mM, 0.22 g/L), NaH_2PO_4 (1.9 mM, 0.3 g/L), Na_2HPO_4 (8.1 mM, 1.15 g/L) in 1 litre, with pH adjusted to 7.4 using NaOH.

4.3.1.2 Supplemented Dulbecco's Modified Essential Medium (DMEM/FCS)

DMEM medium was prepared by mixing 13.55 g of DMEM powder with 3.77 g of $NaHCO_3$ in 1 litre of $dddH_2O$. The pH was adjusted to 7.4, followed by sterile filtration, using 0.2 µm sterile filters. To the medium, 1% of antibiotics and 10% FCS was added. The DMEM medium containing FCS (DMEM/FCS) was then kept at 4˚C when not in use.

4.3.2 Sample preparations

All flavonoid and MGO as well as flavonoid: MGO combinations were prepared as described in Chapter 3.

4.3.3 Mouse fibroblast (L929) cell line

The L929 cell (doubling time = approx. 30 h), is an adherent cell line widely used to evaluate toxicity (Eldeniz *et al.*, 2007).

Vials containing the cells were thawed rapidly in warm water at 37°C and the cells were then suspended in 5 mL DMEM/FCS before being collected by centrifugation. The supernatant was removed, and the cells were suspended in fresh medium. The L929 cells were plated at $4x10^4$ cells per mL in 25 cm^2 or 75 cm^2 cell culture flasks and were maintained until confluency at 37°C at 5% CO_2.

The L929 cells were passaged by removing the medium from the confluent monolayer before adding 1 mL of a 5% trypsin in PBS (T-PBS) and the flask was then placed at 37°C for 1 – 2 min. A 5 mL volume of DMEM/FCS was then added to the T-PBS containing the detached cells, before the solution was transferred to a 15 mL centrifuge tube. The L9292 cells were collected by centrifugation at 800 xg for 2 min. The supernatant was removed, and the cells were re-suspended in 5 mL DMEM/FCS. The number of cells was determined by counting a 10 µL aliquot of cells using a hemocytometer and was then diluted to the desired concentration in DMEM/FCS.

A 90 µL volume of L929 cells were plated at a concentration of 4 x 10^4 cells/mL and were left overnight to attach and then were used to determine the effect of 1 mM 10 µL CAT, CHRY and NAR alone (final concentration 0.1 mM), MGO (1 and 2 mM, final concentration 0.1 and 0.2 mM) alone and in combination (ratio 1:1 and a 1:2) on CAA, cell number and viability.

4.3.4 DCFH-DA assay - Cellular antioxidant activity

DCFH-DA is a probe that is often used to detect cellular peroxides. The probe enters cells and accumulates in the cytosol where it is deacetylated by esterases to 2',7'- dichlorofluorescin (DCFH). This hydrophobic, non-fluorescent product is converted by reactive species (oxidized) into 2',7'dichlorofluorescin (DCF) (Figure 4.1) that fluoresces strongly at an Ex of 525 nm and an Em of 488 nm (Applerot *et al*., 2012).

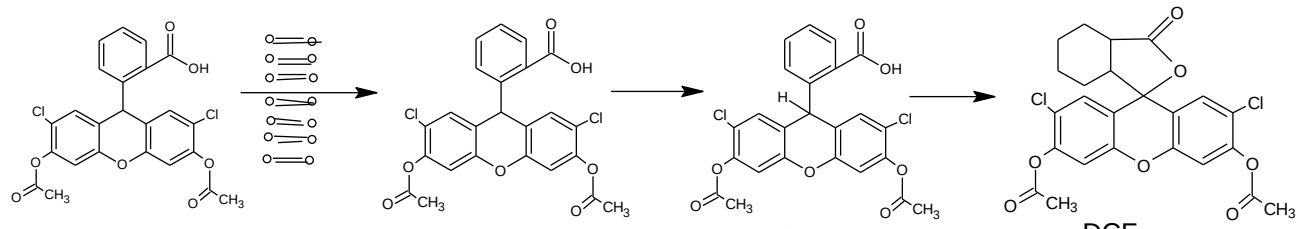


Figure 4.1: Conversion of non-fluorescent DCFH-DA to fluorescent DCF by viable cells.

The ability of MGO, flavonoids and combination to induce A) oxidative damage and B) to protect L929 cells against AAPH induced oxidative damage was determined.

For A, 50 µL of a 75 µM dichlorofluorescein (DCFH-DA) solution (final concentration, 25 µM) was added to each well of a 96 well plate. After 1 h incubation at 37˚C, the medium was removed, and cells were washed once with PBS. A 50 µL volume of each of sample, MGO, flavonoid, MGO: flavonoid (ratio 1:1 and 1:2) was added to each well (no AAPH added).

For the determination of B), 50 µL of a 75 µM dichlorofluorescein (DCFH-DA) solution was also added to each well and then after 1 h incubation at 37˚C, the medium was removed, and cells were washed. A 50 µL volume of each of sample, MGO, flavonoid, MGO: flavonoid (ratio 1:1 and 1:2) was added to each well and then 50 µL of a 7.5 mM AAPH solution was added.

For every 2 min within 60 min, the change in fluorescence was measured at an Ex 485 nm and an Em 520 nm. Two controls were included the vehicle control (VC), cells with buffer only and a positive control (PC), L929 cells with AAPH only. The change in gradient was measured, and the percentage oxidative damage was calculated as follows.

% Oxidative damage (%OD) = Gradient $_{Sample}$/Gradient $_{VC}$ *100

% Cellular antioxidant activity (%CAA) = 100 – [(Gradient$_{Sample + AAPH}$)/ (Gradient$_{PC}$)*100]

4.3.5 Crystal Violet assay – Cell number

Crystal Violet (CV) is a basic dye (high pH) that ionizes in water and binds via ionic interactions with the negatively charged backbone of DNA and negatively charged protein rich in acidic residues such as glutamic and aspartic acid. Due to the ionic interactions, the bound dye can be solubilized at low pH and quantified and the degree of staining correlates with cell number (Figure 4.2) (Bonnekoh *et al.*, 1989; Vega-Avila & Pugsley, 2011).

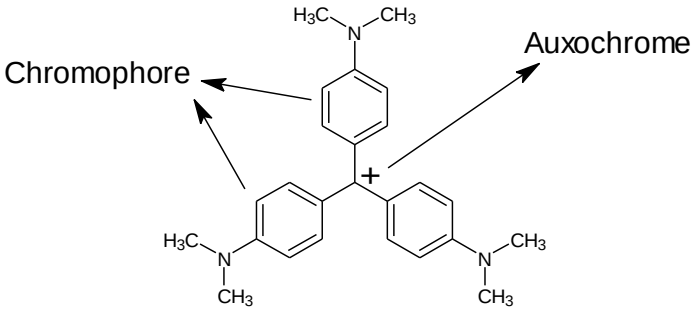


Figure 4.2: Chemical structure of Crystal Violet after deprotonation in water. The positively charged auxochromic group binds negatively charged DNA and protein with negative amino acids such as aspartic acid.

A volume of 90 µL L929 cells plated at a concentration of 4 x 10^4 cells per mL in a 96 well plate was allowed to attach and grow for 24 h at 37˚C at 5% CO_2. Following exposure to 10 µL of the flavonoids, MGO alone and in combination (ratio 1:1) for 24 h; a 10 µL volume of a 20% of a paraformaldehyde (final concentration, 2%) was added for 30 min. Following fixation, the medium containing fixative was removed, the plate was rinsed well with water and then left to dry. The attached L929 cells were stained by adding 100 µL of a 0.1% CV (prepared in 200 mM formic acid) solution for 30 min at room temperature. The dye solution was then removed, and the plates were washed with water and left to dry. The bound dye was solubilized in 100 µL of a 10% acetic acid solution and then 100 µL of the extracted dye was transferred to a 96 well plate and then the absorbance of this solution was measured at 630 nm. The control consisted of non-exposed L929 cells and the data was expressed as % cell number compared to control (100%).

4.3.6 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay – Cell viability

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay is a reduction assay, where the quantity of formazan that is formed is assumed to be directly proportional to the number of viable cells (Vega-Avila & Pugsley, 2011). The yellow water soluble, MTT is converted by mitochondrial succinate dehydrogenase into an insoluble purple coloured formazan product as shown in Figure 4.3. However, research has shown that MTT is also reduced in other cellular compartments in the cytoplasm and plasma membrane (Bernas & Dobrucki, 2002). The purple formazan product, which can be solubilized by various organic solvents (Vega-Avila & Pugsley, 2011) and can be quantified as a measure of cell viability.

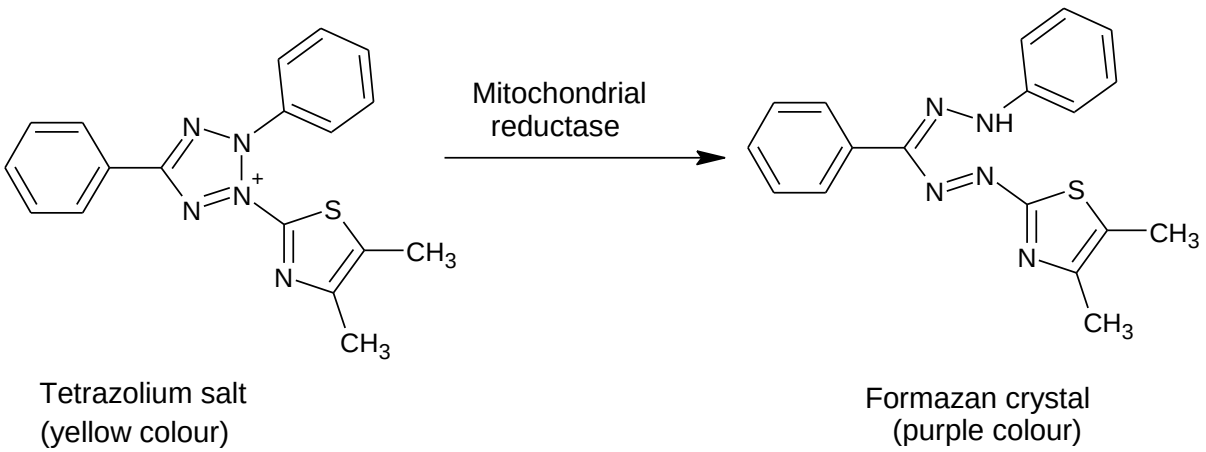


Figure 4.3: Conversion of tetrazolium salt to purple coloured formazan crystals by mitochondrial succinate reductase as an indicator of cell viability.

To the L929 cells exposed to MGO, flavonoid, MGO: flavonoid (ratio 1:1 and 1:2) for 24 h, 10 µL of a 1 mg/mL MTT solution was added to each well and the plate was incubated for 3 h at 37°C. The medium was then removed, and plates were then carefully blotted dry. The MTT formazan crystals were solubilized with 100 µL of a 25% DMSO in ethanol solution with shaking. The solubilized dye was then transferred to a 96 well plate and the absorbance was measured at 570 nm. The control contained non-exposed L929 cells and the data was expressed as % cell number compared to control (100%).

4.3.7 Data management and statistics

The same methods as described in Chapter 3 were used for data and statistical analysis.

4.4 Results

4.4.1 Cellular oxidative/antioxidant effects - DCFH-DA assay

In the absence of AAPH, CHRY and NAR did not cause cellular oxidative damage although CAT caused an 18.84% increase in %OD. Both MGO1 and MGO2 did not cause oxidative damage (Table 4.1a). In combination CAT:MGO in a ratio of 1:1 and 1:2 caused 19.57 ± 8.73% and 15.85 ± 6.23% oxidative damage although differences compared with CAT were not statistically significant. The presence of MGO did not increase the %OD of CHRY and NAR and differences compared to the flavonoids alone was also not statistically different.

The oxidative effect of AAPH was increased from 100% to 226.57 ± 89.90% and reduced to 84.30 ± 45.75% for 1 and 2 mM (final concentration 0.5 and 1mM) MGO, respectively. Of the flavonoids: only CAT effectively reduced AAPH induced oxidative damage while a strong pro-oxidant effect was observed for CHRY and NAR. For CAT:MGO ratio of 1:1 and 1:2, CAA was retained and was not significantly different from CAT alone. Similarly, the CAA of CHRY and NAR was also unaltered in the presence of MGO (Table 4.1b). These results indicate that although in the absence of AAPH the oxidative effects of CAT and combinations is slightly increased, CAT and CAT:MGO combinations can still effectively scavenge AAPH. In contrast, CHRY and NAR alone and in combination with AAPH have strong pro-oxidant effects.

Table 4.1a: % Cellular oxidative damage caused by MGO, CAT, CHRY and NAR alone and in combination with on L929 cells.

Oxidative effects			
	0 mM MGO	**0.1 mM MGO (1:1 ratio)**	**0.2 mM MGO (1:2 ratio)**
		0.90 ± 1.07%	1.05 ± 6.23%
0.1 mM CAT	18.84 ± 7.11%	19.57 ± 8.73%	15.85 ± 6.23%
0.1 mM CHRY	-2.07 ± 0.66%	-4.07 ± 1.17%	-0.11 ± 0.87%
0.1 mM NAR	-1.93 ± 0.25%	0.43 ± 1.04%	-0.47 ± 0.35%

No significant difference observed between control and flavonoids as well as between flavonoids and 1:1 and 1:2 combinations at p<0.05.

Table 4.1b: % Cellular antioxidant activity of MGO, CAT, CHRY and NAR alone (AAPH added) and in combination with MGO on L929 cells.

CAA (AAPH added)			
	0 mM MGO	**0.1 mM MGO (1:1 ratio)**	**0.2 mM MGO (1:2 ratio)**
		226,57 ± 89,90%	84,30 ± 45,75%
0.1 mM CAT	38,64 ± 9,11%	38,31 ± 9,25%	34,38 ± 7,18%
0.1 mM CHRY	208,06 ± 110,77%	183,31 ± 125,43%	248,07 ± 107,01%
0.1 mM NAR	171,55 ± 24,92%	155,57 ± 87,93%	159,18 ± 133,68%

No significant difference observed between control and flavonoids as well as between flavonoids and 1:1 and 1:2 combinations at p<0.05

4.4.2 Cell number and viability

No change in cell number was observed for L929 cells exposed for 24 h to 0.1 and 0.2 mM MGO. CAT alone did cause a slight increase and CHRY and NAR a decrease in cell number (Table 4.2), although not statistically significant. In the presence of MGO, compared to CAT, CHRY and NAR alone, there was no significant change in cell number.

Table 4.2: Effect of MGO, CAT, CHRY and NAR alone and in combination with MGO on L929 number following 24 h exposure.

Cell number (CV assay)			
	0 mM MGO	**0.1 mM MGO (1:1 ratio)**	**0.2 mM MGO (1:2 ratio)**
	100 ± 1,00%	101,10 ± 0,26%	98,60 ± 0,50%
0.1 mM CAT	104,50 ± 0,19%	109,39 ± 0,23%	106,29 ± 0,22%
0.1 mM CHRY	90,91 ± 0,32%	85,71 ± 0,44%	85,91 ± 0,51%
0.1 mM NAR	86,71 ± 0,42%	88,91 ± 0,51%	89,51 ± 0,31%

No significant differences observed compared with the control (No flavonoid, no MGO added). No significant differences observed between flavonoid only, and flavonoid with added MGO.

The measurement of cell viability is a more sensitive indicator of toxicity. CHRY and NAR caused a significant decrease in cell viability, compared to the control and CAT. Compared to

0.1 and 0.2 mM MGO, increased toxicity was observed for MGO:CHRY (1:1) and MGO:CHRY (1:2) as well as MGO:NAR (1:1) and MGO:NAR (1:2) (Table 4.3). This loss of viability reflects the effects observed for the CAA for CHRY and NAR alone and in combination with MGO in the presence of AAPH (Table 4.1).

Table 4.3: Effect of MGO, CAT, CHRY and NAR alone and in combination with MGO on L929 viability following 24 h exposure.

Cell viability (MTT assay)			
	0 mM MGO	**0.1 mM MGO (1:1 ratio)**	**0.2 mM MGO (1:2 ratio)**
	100,00 ± 0,57%	117,54 ± 0,16%	107,53 ± 0,73%
0.1 mM CAT	99,72 ± 0,42%	108,88 ± 0,02%	105,68 ± 1,00%
0.1 mM CHRY	74,20 ± 0,41% *	68,01 ± 0,35%*	72,45 ± 0,04%*
0.1 mM NAR	76,06 ± 0,66%*	74,30 ± 0,07%*	81,63 ± 0,41%

Significant difference against the control indicated by an asterisk (*) at $p < 0.05$. *No significant differences observed between flavonoid only, and flavonoid with added MGO.*

4.5 Discussion

Flavonoids are antioxidants and can also trap MGO reducing MGO levels. Epidemiological studies have reported correlation between the consumption of diet derived flavonoids and the prevention of age-related diseases such as CVD, cancer and neurodegenerative diseases, however, the effects of cellular uptake of flavonoids need to be considered (Panich *et al.*, 2017).

Three main routes of MGO toxicity have been described and these are the direct inhibition of enzyme activity, the development genotoxicity that leads to carcinogenicity and an indirect effect via the depletion of GSH, that diminishes cellular antioxidant activity (Kalapos, 2008). At higher concentration MGO can trigger cell death via apoptosis (Chan *et al.*, 2005).

The ability of MGO and flavonoids, alone and in combination, to induce oxidative damage and to protect L929 cells against AAPH induced damage was determined. CAT caused an increase in oxidative damage, alone and in the presence of MGO. CAT has been identified as a pro-oxidant which is dependent on concentration as well as the presence of oxygen (Labuda *et al.,* 2003). The pro-oxidant property of flavonoids is a function of the reduction strength of flavonoids in the order quercetin > rutin> epigallocatechin gallate > CAT. Oxidation of CAT can result in an increase in fluorescence at an Ex 314 nm and Em at 470 nm (Bark *et al.*, 2011) however, as the fluorescence of DCFH DA is measured at Ex 485 nm and an Em 520 nm this fluorescence will not cause interference. In this study, CAT caused oxidation even if trapping had occurred.

In the presence of AAPH, the %OD is increased for 0.1 mM MGO but not for 0.2 mM MGO. In a similar study Legnanga (2017), reported that 0.5 and 1 mM MGO caused significant increase

in oxidative damage from 100% to 358.86 ± 38.51% and 298.83 ± 49.04% for 0.5 and 1 mM respectively in SC-1 cells. Legnanga (2017) conducted a similar study using phenolic acids. Increased fluorescence may be due to the effects on MGO, which depletes GSH and inhibits antioxidant enzymes. Consequently, the antioxidant capacity of a cell is reduced and is therefore more sensitive to the effects of oxidants such as AAPH.

In the DCFH DA assay, the SD values were large, and this may be due to the sensitivity of the assay or other assay related factors such as variability in the number of cells plated. Low SD values for the CV and MTT assays indicates that this is not the result of poor plating but rather assay related factors which must be addressed in future studies.

Interestingly although a slight pro-oxidant effect was observed for CAT alone and in combination with MGO without AAPH, an antioxidant effect was observed, where CAT, CAT:MGO (1:1) and CAT:MGO (1:2) effectively scavenged AAPH generated radicals. Both CHRY and NAR had a pro-oxidant effect in the presence of AAPH, although this cannot be concluded due to high variation between experiments.

Exposure to MGO alone and in combination with flavonoids did not cause a significant decrease in cell number. MGO at the concentrations evaluated was not cytotoxic. Some reduction although statistically not significant compared to the control was observed for CHRY and NAR alone and in combination with MGO. In a study in which the cytotoxicity of pyrogallol (PYR), caffeic acid (CA) and gallic acid (GA) alone and in combination with MGO was determined in the SC-1 (mouse fibroblast) and the Caco-2 (human colon adenocarcinoma, epithelial) cell lines, a slight increase in cell number, although not significantly different to the control, was reported (Legnanga, 2017), these results concur with the current study. The cytotoxicity of MGO has also been evaluated by Tu *et al*., (2013) in ECV304 (*Cellosaurus*, endothelial) cells, which were treated with 0.5 mM MGO for 2 h and the cell number reduced to 50% relative to the control and was reduced to less than 10% at an MGO dose of higher than 0.5 mM (Tu *et al.*, 2013), these results reflect a dose response. A similar effect was reported by Rabie *et al*., (2016); where the authors tested for toxicity using fibroblast (SC-1), mouse macrophage (RAW 264.7) and colon carcinoma (Caco-2) cell lines at a 48 h MGO exposure. A hormetic effect (low-dose stimulation and high-dose inhibition) was observed at low concentrations of < 3.2 mM whilst at higher concentrations of > 32.47 mM toxicity was observed, these results concur with a dose response and concur with the results of the current study.

The MTT assay is more sensitive assay than the CV assay as it is dependent on cellular activity rather than DNA/protein content. Chrysin, NAR and MGO alone and in combination were cytotoxic. No significant differences were observed between the individual flavonoids and combinations. In another study the phenolic acids, PYR, CA and GA alone and in

combination at 0.1 and 0.2 mM no cytotoxicity with the MTT assay was observed in the SC-1 and Caco-2 cell lines (Legnanga, 2017).

Tu *et al*., (2013) evaluated the effects of MGO on ECV304 cells on cell viability and reported that cell viability was reduced by 10 – 15% at concentration between 0.5 mM and 5 mM and was further reduced to <50% viability after exposure to 10 mM MGO. In another study HepG2 (human hepatoma) cells were treated with 1, 3 or 10 mM MGO for 36 h. A concentration of 1 mM MGO caused a small but not significant increase in cell viability while 3 and 10 mM MGO caused a significant loss in cell viability (Seo *et al.*, 2014). Therefore, at low concentrations MGO is not cytotoxic but can deplete antioxidant pathways increasing sensitivity to oxidants such as AAPH as was observed in the present study.

Antioxidants, such as curcumin, can prevent MGO-induced cell death and apoptotic biochemical changes such as mitochondrial release of cytochrome c, cleavage of poly [ADP-ribose] polymerase (PARP), and caspace-3 activation (Chan *et al.*, 2005). Measurement of these markers of toxicity may provide a more sensitive indication of toxicity. Under various conditions, flavonoids may act as pro-oxidants by promoting the oxidation of other compounds (Procházková *et al.*, 2011). Pro-oxidative effects of flavonoids are often a result of auto-oxidation of the flavonoid resulting in semiquinones, quinones, H_2O_2, $O_2^{\cdot-}$production and interaction with transition metal ions (Halliwell, 2008; Lu *et al*., 2011; Andueza *et al*., 2015). NAR has been reported to exhibit pro-oxidative effects. The cell viability of human lymphocytes evaluated using the Trypan blue exclusion assay was reduced to 90% after exposure to 200 µM NAR. This concentration of NAR also accelerated the oxidation of deoxyribose induced by Fe^{3+}/H_2O_2 and induced lipid hydroperoxide levels of 0.44 nmol/mg of protein (Yen *et al.*, 2003). Although CAT is a good antioxidant, it exhibits pro-oxidative effects as it rapidly triggers haemoglobin oxidative degradation and free iron redox reaction at low concentrations of 0.05-0.1 mM (Lu *et al.*, 2011). CHRY is the main phenolic compound detected in propolis with a content of 9.8 µmol/g of propolis. Pro-oxidative effects have been observed in propolis and with CHRY being one of the main components in propolis it may contribute to this effect (Sobočanec *et al.*, 2006). In combination it appears that the flavonoids rather than the MGO affects cellular viability, however via glycation MGO may increase cellular aging making the cell more sensitive or susceptible to the effects of oxidants.

4.6 Conclusion

CAT alone and in combination with MGO, in the absence of AAPH caused slight oxidative damage but was able to significantly reduce the oxidative effects of AAPH. Conversely, CHRY and NAR alone and in combination with MGO, did not cause oxidative damage in the absence

of AAPH, but also did not protect the L929 against AAPH induced oxidative damage. No changes in cell number were observed for MGO and flavonoids alone and in combination after 24 h exposure. With cell viability, both CHRY and NAR, alone and in combination with MGO, had reduced the viability of L929 cells. Effects were not related to MGO toxicity but rather due to a possible pro-oxidant effect of CHRY and NAR.

Chapter 5: Effect of flavonoids on the antibacterial activity of MGO

5.1 Introduction

The antibacterial activity of Manuka honey is due to the presence of leptosperin, dihydroxyacetone and MGO (Stewart *et al.*, 2014). In *P. aeruginosa,* Manuka honey caused de-flagellation of the bacteria associated with a decrease in the expression of the major structural flagellin protein, FliC, and responsible flagellin-associated genes, including fliA, fliC, flhF, fleN, fleQ and fleR (Roberts *et al.*, 2015). Rabie *et al*. (2016) attributed these effects to the presence of MGO and reported that MGO was found to inhibit Gram-positive, *B. subtilis* and *S. aureus* at an MIC of 0.8 mM and 1.2 mM, respectively, while the MIC for Gram-negative, *P. aeruginosa and E. coli was* 1.0 mM and 1.2 mM, respectively. In an ultrastructural study by Rabie *et al.* (2016) the effects of 0.5, 1.0 and 2 mM MGO on *B. subtilis* and *E. coli* morphology was investigated with SEM. At 0.5 mM no changes in bacteria morphology was observed. At 1 mM MGO fewer fimbriae were present and the flagella were less or absent while remaining structures appeared stunted and fragile. At a higher concentration of 2 mM MGO, fimbriae and flagella were absent, the bacteria were rounded with shrinkage and membrane integrity was compromised. As flagella play an important role in adhesion, infection and biofilm formation and swarming, these effects would reduce virulence. In addition, the ability of MGO to compromise membrane integrity at high concentrations can cause direct killing of bacteria.

Flavonoids found in honey can also kill bacteria via the inhibition of enzyme activity (Cushnie & Lamb, 2005) or a pro-oxidant mechanism, via the formation of H_2O_2 (Cushnie & Lamb, 2005; Sirk *et al.*, 2008). CAT has low antibacterial activity (Veluri *et al.,* 2004) while CHRY has antibacterial activity against several Gram-positive and -negative bacteria (Liu *et al.*, 2010). At 0.1 mM NAR strongly inhibits Gram-negative bacterium *V. harveyi* but the effect *on E. coli and B. subtilis* was not significant *(*Ulanowska *et al*., 2006)

The aim of this chapter is to determine whether flavonoids CAT, CHRY and NAR alters the antibacterial activity of MGO as well as MGO induced changes in *E. coli* and *B. subtilis* morphology.

5.2 Materials

5.2.1 Chemicals, instruments, reagents, apparatus and bacteria

Instruments, plastic disposables and glassware were the same as used in Chapter 3 and 4.

The reagents and equipment: tryptone, yeast extract, agar, poly-L-lysine, petri dishes, glutaraldehyde and ethanol were obtained from Department of Biochemistry, Genetics and Microbiology, Faculty of Natural and Agricultural Sciences, University of Pretoria, supplied by Sigma-Aldrich, SA.

The Zeiss Ultra Plus FEG scanning electron microscope was used for morphological analysis of bacteria in the Laboratory for Microscopy and Microanalysis, University of Pretoria.

The bacteria used included: *B. subtilis* (13933) and *E. coli* (700928) and were obtained from the Department of Biochemistry, Genetics and Microbiology, Faculty of Natural and Agricultural Sciences, University of Pretoria, supplied to them by the American Type Culture Collection (ATCC), USA.

5.2.2 Buffers and/or medium and coverslips

5.2.2.1 Lysogeny broth

Lysogeny broth was prepared by mixing 10 g of tryptone, 5 g of yeast extract and 10 g of NaCl in 1 L of ddH_2O. The pH of the solution was adjusted to 7.5, then autoclaved to achieve sterility. Broth was kept at 4˚C when not in use.

5.2.2.2 PBS

PBS was prepared as described in chapter 3.

5.3 Methods

5.3.1 Establishment of bacteria cultures

Bacteria *E. coli* and *B. subtilis* were used as representative Gram-negative and -positive bacteria as they are model organisms and can be cultured in a level 1 biosafety laboratory. The major differences between these bacteria are related to cell wall and this represents differences in sensitivity to antibacterial agents such as MGO. In Gram-negative bacteria the cell wall is 70-120 Å thick with a high lipid content of 20-30% and a low murein content. It consists of a thin single layer of peptidoglycan, a periplasmic space and an outer membrane. The Gram-positive bacteria cell wall is 100-120 Å thick, with a low lipid content and a high murein content of 70-80%. It has a thick multilayer of peptidoglycan with many teichoic acids and has no outer membrane and periplasmic space (Figure 5.1).

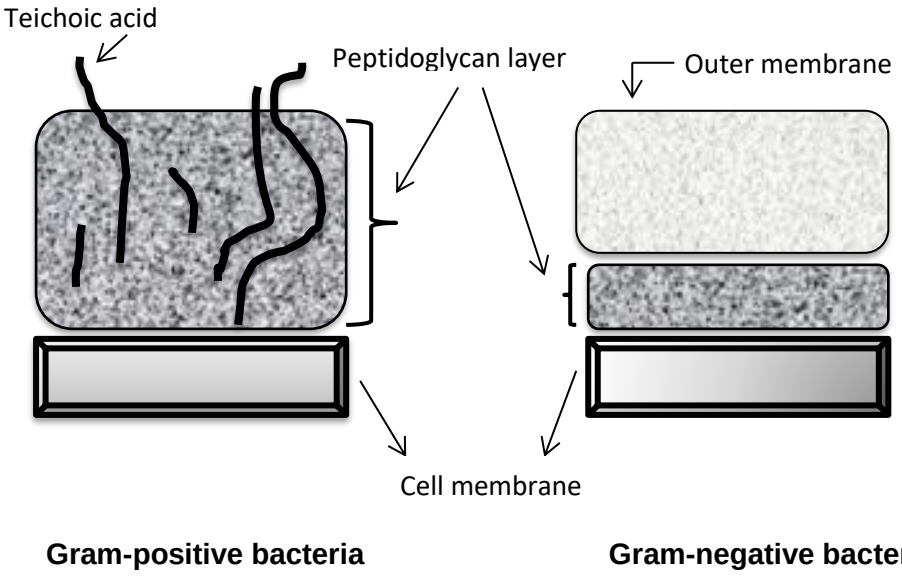


Figure 5.1: Structural difference between Gram-positive and Gram-negative bacteria.

Cultures of both bacteria were prepared as presented in Figure 5.2. Both *E. coli* and *B. subtilis* stock cultures were removed from the -70˚C freezer and the bacteria were streaked on separate Lysogeny agar plates (1% tryptone, 0.5% yeast extract, 1% sodium chloride, pH 7.5, 2% (w/v) bacteriological agar) using a sterile streaker and were grown overnight at 37˚C to establish single colonies of bacteria. Once the colonies were established three to five colonies of each bacterium was selected and placed in a flask containing 25 mL Lysogeny broth. The flasks were then placed in an incubator at 37˚C on a shaker set at 150 rpm and were left to grow overnight. After the incubation period the bacteria was then diluted 100x using 10 mL Lysogeny broth and were left to grow for another 3 h in the incubator at 37˚C on a shaker set at 150 rpm. At this point the optical density of the bacteria was read at 600 nm. The bacteria were then diluted further using Lysogeny broth to an optical density (OD) of 0.011 as summarized (Figure 5.2).

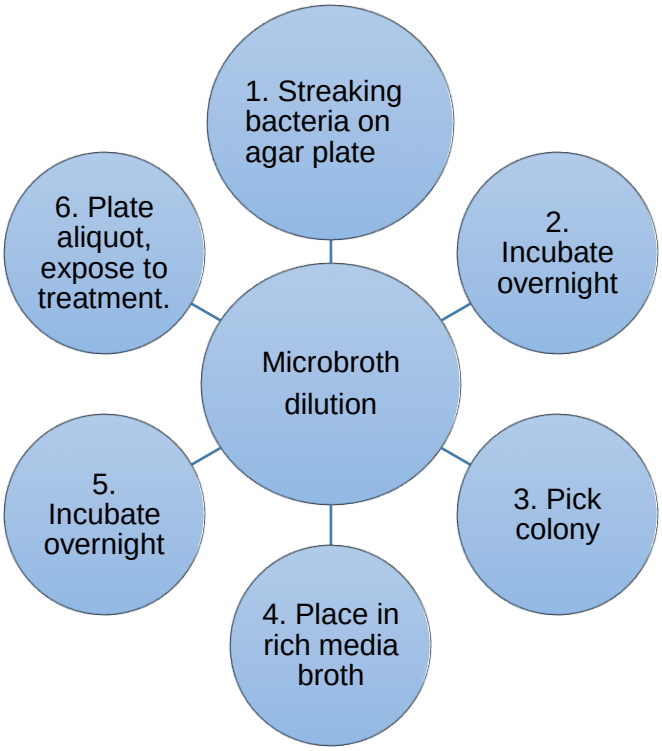


Figure 5.2: Schematic diagram of steps required for establishing bacteria cultures for the microbroth dilution assay.

Microbroth dilution assay

The microbroth dilution assay, is a rapid method to determine the effect of drugs on the growth of bacteria (Rani *et al.*, 2017). In this study, this assay was used to determine the effect of growth of the flavonoids, MGO and the flavonoid: MGO combinations.

A volume of 90 μL of Lysogeny bacterial culture with an absorbance of 0.011, was added to three rows in a sterile 96 well polypropylene microtitre plate, and then 10 μL of sample (MGO (final concentration, 0.1 and 0.2 mM), flavonoids (final concentration, 0.1 mM) and 0.1 of the flavonoid:MGO (ratio 1:1 and 1:2) combinations) was added in triplicate. These concentrations were selected to obtain loss in antibacterial activity but not total eradication. The sterile control consisting of 100 μL Lysogeny broth, and a growth control consisting of 90 μL of Lysogeny bacterial culture (absorbance 0.011) and 10 μL broth were added. A targeted final OD reading in every well was 0.01. Absorbance was measured immediately after plating (T0) and after 24-hour incubation (T24) at 600 nm. Percentage inhibition of bacteria was calculated by using this formula:

$$\textbf{Percent inhibition} = 100-[((T24-T0)/G100)*100]$$

Where G100 is the growth control.

5.3.2 Bacteria morphology - Scanning electron microscopy

Scanning electron microscopy provides a detailed image of the external characteristics of bacteria and is widely used to evaluate the effects of various drugs on the surface topography and composition; generally cell wall integrity and structure (Goldstein *et al.*, 2017). Carbon, oxygen and hydrogen has poor scattering properties, therefore to evaluate external cell morphology biological tissue and cells must be coated with a thin layer of carbon to make a non-conductive sample, conductive. The ultrastructure of the bacteria is then determined with a scanning electron microscope and the principles of microscopy is presented in Figure 5.3 (Zhu *et al.*, 2013).

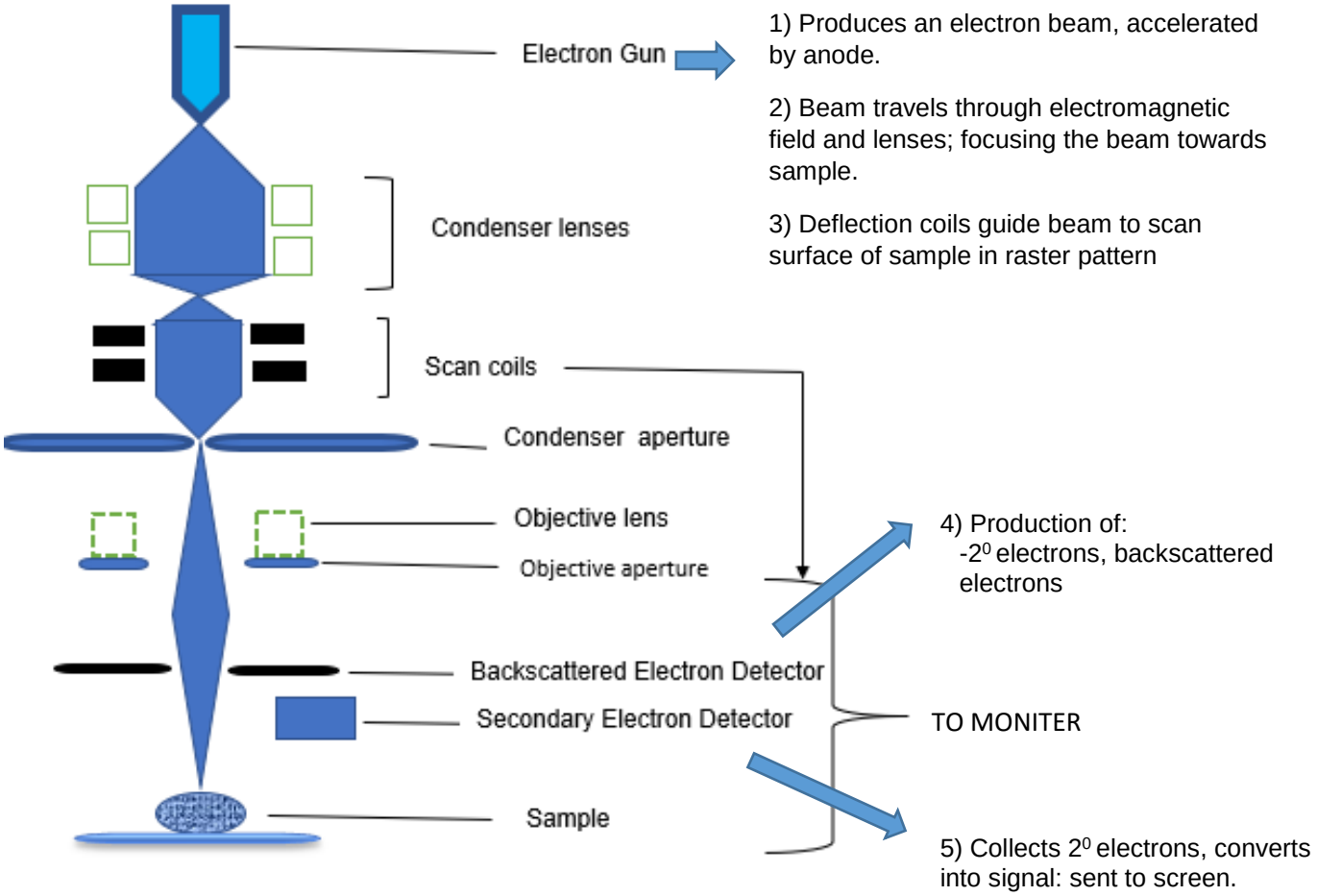


Figure 5.3: Schematic diagram of a scanning electron microscope.

5.3.2.1 Poly-L-lysine coating of cover slips

The charge of bacteria cell wall is negative and therefore to ensure effective attachment of the bacteria to glass coverslips the glass coverslips must be coated with positively charged poly-L-lysine.

Poly-L-lysine coated glass cover slips were prepared by washing clean glass cover slips with an alkaline solution of 10% NaOH in 60% ethanol (EtOH) for 2 h. The cover slips were then

rinsed 5x with ddH_2O. In a sterile environment, the cover slips were completely covered with 100% EtOH, for 30 min. After drying they were transferred into Petri dishes and then each was coated with poly-L-lysine solution, incubated for 2 h before washing the poly-L-lysine coated cover slips approximately ten times with sterile water. The cover slips were then left to dry at room temperature for 3 days before use.

The effects of MGO and flavonoids, alone as well as in combinations, on the morphological changes of bacteria were investigated using the SEM. The bacteria was exposed to 0.1 and 0.2 mM of MGO alone, 0.1 mM flavonoids (CAT, CHRY and NAR) alone as well as the 1:1 and 1:2 mM MGO:flavonoid combinations for 24 h. Exposed bacteria was then spread on round glass cover slips. The cover slips were then dried for 10 minutes and washed in PBS for 20 minutes. The samples were then fixed in a 2.5 % glutaraldehyde and 2.5% formaldehyde in 0.075 M sodium phosphate (NaP) buffer of pH 7.4 for 30 minutes and then washed three times in the same buffer. The samples then underwent secondary fixation in 1% osmium tetraoxide for 30 minutes, before being washed again as described above. The samples were dehydrated by using an increasing serial dehydration step with 30 %, 50 %, 70 % and 90 % EtOH, followed by three changes of absolute EtOH. The 100% EtOH was removed and then a 100% hexamethyldisilazane (HMDS) was then added, for 30 minutes. Approximately 2 drops of HMDS was placed on the cover slips and the samples were left to dry. Once the samples are dried, the cover slips were mounted on aluminium stubs, coated with carbon and then viewed with the SEM.

5.3.3 Data management and statistics

The same methods as described in Chapter 3 were used for data and statistical analysis.

5.4 Results

5.4.1 Effect of flavonoids on the antibacterial activity of MGO

Against *B. subtilis*, 0.1 mM MGO inhibited growth by 30.38% while CAT at the same concentration did not inhibit bacterial growth. In the CAT:MGO combinations at a 1:1 ratio with a final concentration of 0.1 mM MGO and CAT, 13.39% inhibition of growth (2.27-fold reduction) was observed. The inhibition of 0.2 mM MGO was 36.52% and for the combination of 1:2 was reduced to 11.66% (3.13-fold reduction). Differences between MGO and CAT:MGO were significant indicating that the presence of CAT reduces the antibacterial activity of MGO against *B. subtilis* (Figure 5.4).

Against *E. coli*, 0.1 mM MGO inhibited growth by 26.90% while no inhibition was found for CAT. For CAT:MGO, 1:1 ratio, inhibition was reduced to 5.5% (4.98 fold reduction). Likewise,

0.2 mM MGO inhibited growth by 33.20% and in combination with CAT, inhibition was 12,82% (2.6-fold reduction). Differences between MGO and the combinations were statistically significant. Although for both bacterial there is several fold loss of activity between MGO and the combinations, the measured activity of CAT:MGO (1:1) and MGO:CAT (1:2) is similar for *B. subtilis* but greater for CAT:MGO (1:2) against *E. coli* (Figure 5.4).

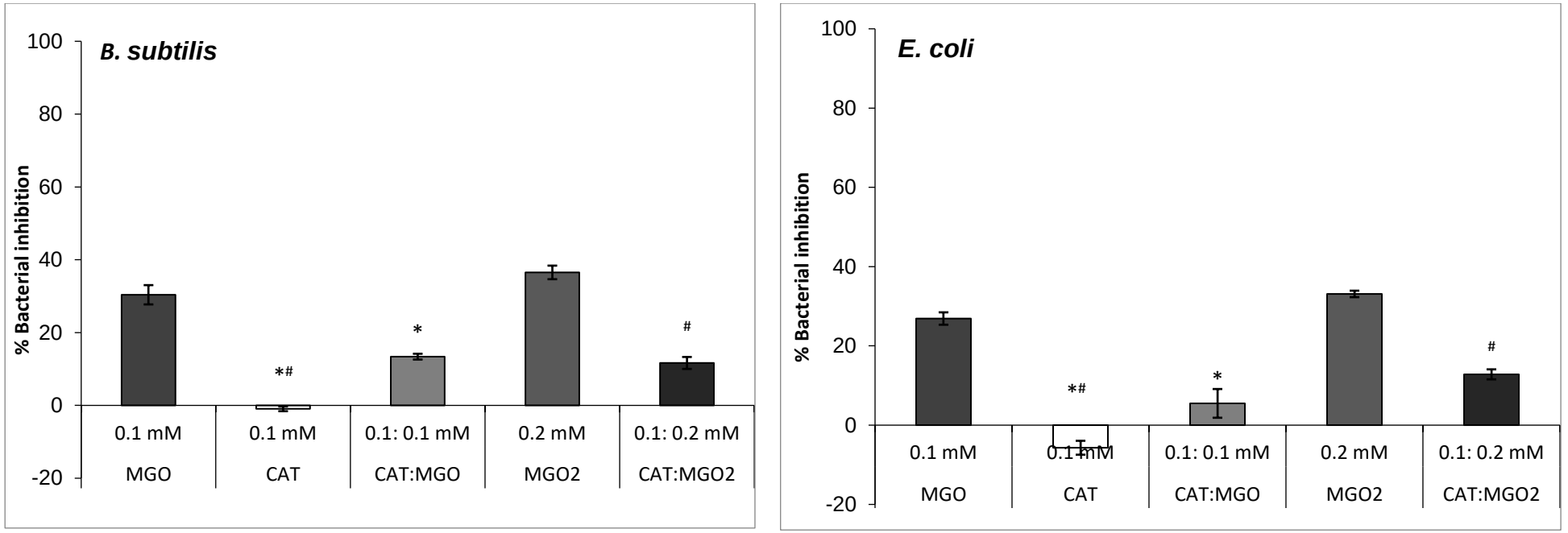


Figure 5.4: Antibacterial activity of CAT and MGO alone as well as in combination at a 1:1 and 1:2 ratio on a) *B. subtilis* and b) *E. coli.* The data is presented as mean ± SEM; with *representing significant difference in comparison to MGO and # indicating significant difference in comparison to MGO2.

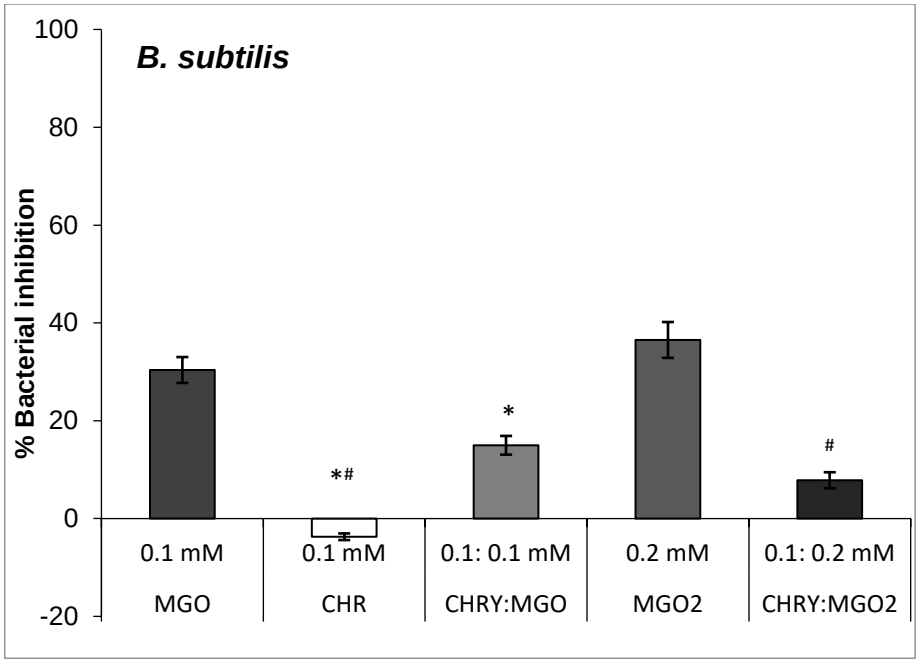
In Figure 5.5, 0.1 mM MGO inhibits the growth of *B. subtilis* by 30.38% while 0.1 mM CHRY had no inhibitory activity. At a ratio of 1:1, a statistically significant reduction in antibacterial activity to 14.98% was observed (2.02-fold reduction). MGO, 0.2 mM inhibited growth by 36.52% and the 1:2 ratio of CHRY:MGO by 7.84% (4.65-fold reduction). Differences were statistically significant.

Against *E. coli*, the inhibition of 0.1 mM MGO was 26.90% and no inhibition was observed for NAR. For the CHRY:MGO, 1:1 ratio, the antibacterial activity was 2.47% (11-fold reduction). MGO at 0.2 mM inhibited growth by 33.10% and for the combination CHRY was 9.55% indicating a 3.5-fold reduction in the measured activity of MGO. Differences between, MGO and CHRY:MGO were statistically significant.

The inhibition of *B. subtilis* (Figure 5.6), by 0.1 mM MGO was 30.38% and 0.1 mM NAR was 8.80%. In the For NAR:MGO, 1:1 ratio, inhibition was 12.84%, and was a statistically significant 2.37-fold reduction in antibacterial activity. Inhibition of 0.2 mM MGO was 36.52% and in combination with NAR, inhibition was reduced to 7.73% (4.73-fold reduction).

E. coli, inhibition by 0.1 mM MGO was 26.90% while no inhibition was observed for NAR. The 1:1, NAR:MGO ratio reduced bacterial growth to 2.05% (13.1-fold reduction). A concentration of 0.2 mM MGO inhibited growth by 33.10% and for the combination by 13.9% causing a 2.4-fold loss in the activity of MGO. Differences for MGO vs MGO:NAR was statistically significant.

In summary, the presence of flavonoids reduced the antibacterial activity of MGO against *B. subtilis* and *E. coli*.



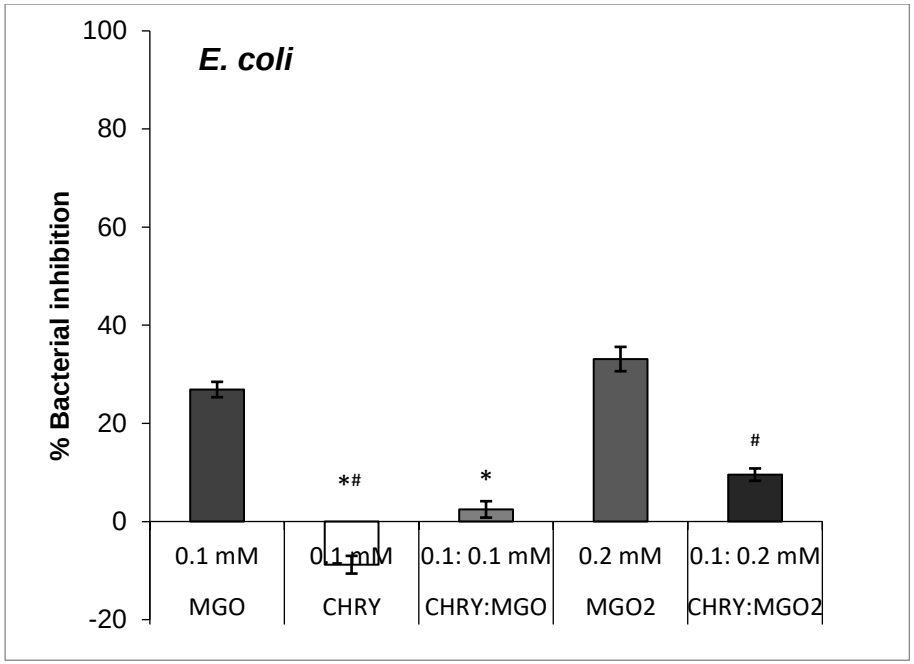
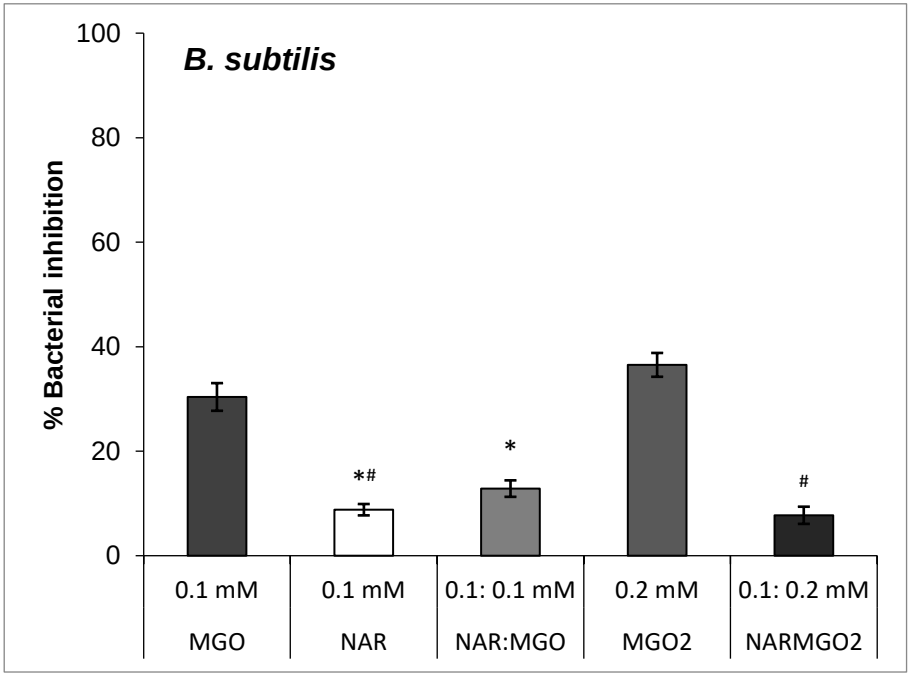


Figure 5.5: Antibacterial activity of CHRY and MGO alone as well as in combination at a 1:1 and 1:2 ratio on c) *B. subtilis* and d) *E. coli*. The data is presented as mean ± SEM; with *representing significant difference in comparison to MGO and # indicating significant difference in comparison to MGO2.



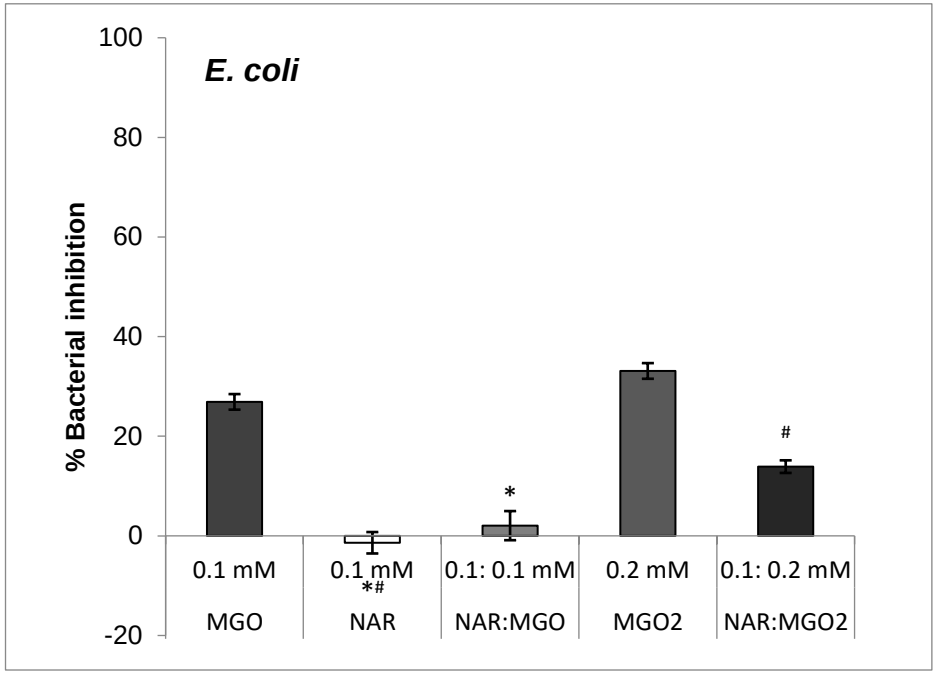


Figure 5.6: Antibacterial activity of NAR and MGO alone as well as in combination at a 1:1 and 1:2 ratio on e) *B. subtilis* and f) *E. coli*. The data is presented as mean ± SEM; with *representing significant difference in comparison to MGO and # indicating significant difference in comparison to MGO2.

5.4.2 Morphological effects of MGO, flavonoids alone and in combination on *B. subtilis*

The effects of MGO and each flavonoid as well as each combination on the morphology of the bacteria was determined. Figure 5.7 shows the control *B.subtilis* (A) with presence of flagella (grey arrow) and an intact cell wall. In the presence of 0.1 mM MGO (B) fimbriae still can be observed, however, the bacterial cell wall is disrupted, and cells are clumped. In the presence of 0.2 mM MGO (C) the bacterial membrane is disintegrated and there is a loss of cell content. The CAT treated bacteria (D) appears to have a smooth membrane although some cell wall disruption and loss of content is observed. The CAT:MGO (1:1) (E) treated bacteria shows the presence of holes (white arrows) resulting in leakage of intracellular content. The CAT:MGO (1:2) (F) treated bacteria shows a loss of cell wall integrity and the morphology is similar to MGO2 (B).

The CHRY treated bacteria (G) appears to be normal, fimbriae can be seen, and the cell wall is intact, and morphology is similar to the control. With the CHRY:MGO (1:1) treated bacteria (H), leakage of intracellular contents is observed due to loss of cell wall integrity and the CHRY:MGO (1:2) treated bacteria (I) there is loss of cellular content, disintegrated membrane and shrinkage similar to that observed for 0.2 mM MGO (C).

The cell membrane of the NAR treated bacteria (J) appears to be completely disrupted giving the bacterium an irregular shape, the same is also observed with NAR:MGO (1:1) (K). The NAR:MGO (1:2) (K) shows complete disruption of the cell wall.

The observed morphology for *B. subtilis* exposed to CAT, CHRY and NAR, is similar to the findings in Figures 5.4, 5.5 and 5.6, where it was found that CAT and CHRY did not inhibit bacterial growth while NAR caused inhibition. Also, an increase in MGO from 0.1 mM to 0.2 mM alone showed an increased toxicity (Figure 5.7) these correlated with the results of Figure 5.4, 5.5 and 5.6.

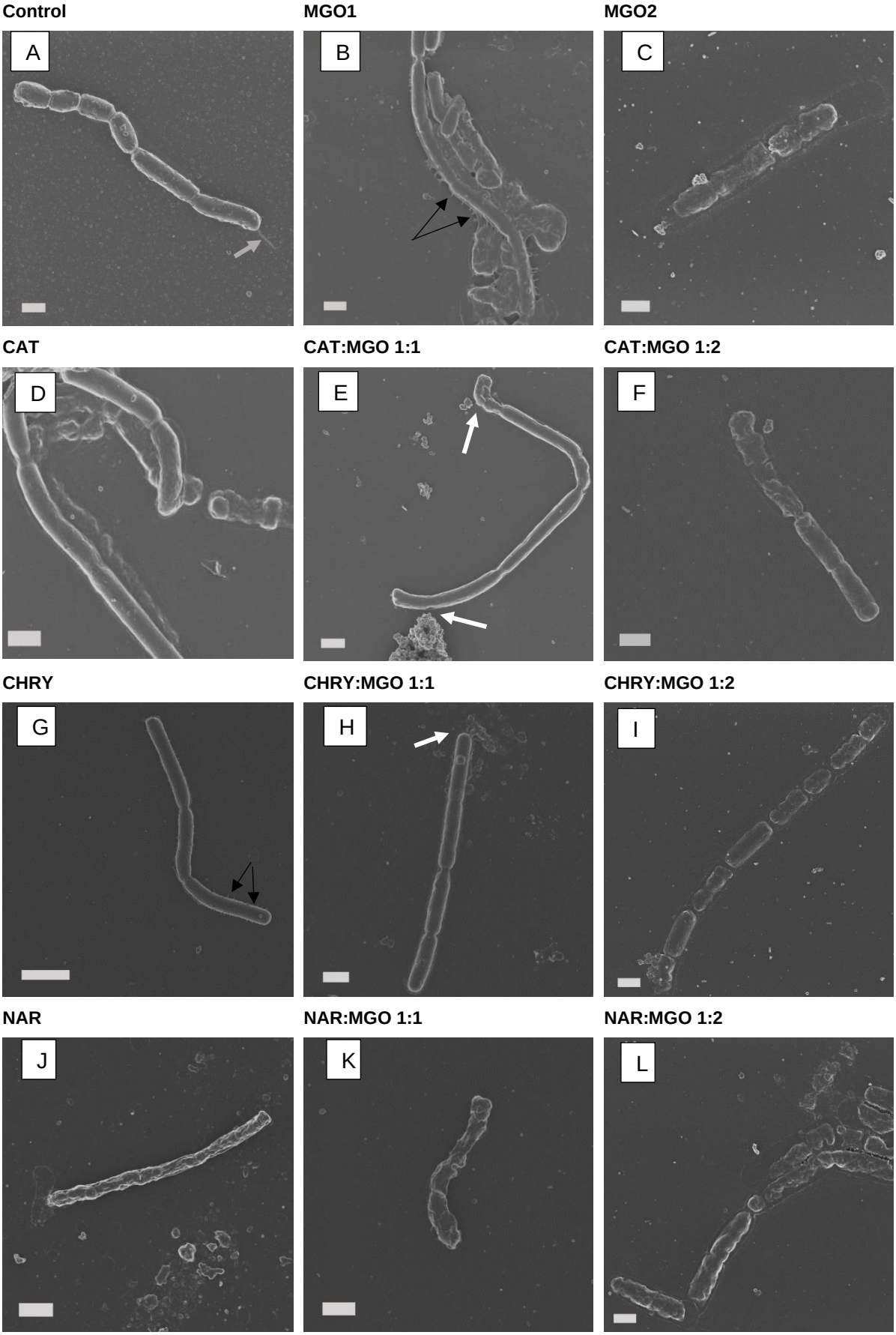


Figure 5.7: Scanning electron micrographs of *B. subtilis* exposed to 0.1 mM flavonoid alone and in combination with 0.1 mM and 0.2 mM MGO respectively. Micrographs taken at 15.00 Kx magnification, all scale bars 1µm except CAT is 2 µm. Black arrows indicate fimbriae, white arrows blebbing and the grey arrow indicates the flagella.

5.4.3 Morphological effects of MGO, flavonoids alone and in combination on *E. coli*

In Figure 5.8, the control (A) shows a rough membrane with fine fimbriae and pilli (black arrows). The MGO treated bacteria (B) appears flattened with indentations, some bacteria became 'rounder' or rather cocci shaped. The MGO2 treated bacteria (C) appears in numerous aggregates that are tightly packed; a few indentations are observed, and the shape of some bacteria also appear to be cocci-like rather than rod-shaped.

At 0.1 mM CAT, CHRY and NAR caused, morphological changes associated with toxicity. CAT (D) treated *E. coli* had minor indentations, but generally were quite rounded and appeared similar to the control with very few fimbriae. The CHRY (G) treated bacteria had a rough membrane similar to the control, although in some bacteria the membrane appears to be disrupted with membrane wrinkling and loss of cell wall integrity (J). NAR was the most toxic with damage to the cell wall and membrane and exposure of the intracellular content.

The CAT:MGO 1:1 (E) exposed bacteria are flat with indentations. The CAT:MGO 1:2 (F) appears to be similar to CAT:M.GO 1:1 although more rounded with some clumping. The CHRY:MGO 1:1 (H) treated bacteria were flattened with indentations in the centre of the bacteria. The CHRY:MGO 1:2 (I) treated bacteria were fewer, and those present were rounded with pits and indentation. The NAR:MGO 1:1 (K) were also fewer, and bacteria that were present had indentations and the NAR:MGO2 1:2 (L) treated bacteria were clumped cocci-shaped cells with indentations similar to that observed for *E. coli* exposed to 0.2 mM MGO.

This morphological study identifies that NAR has strong antibacterial activity and if flavonoids at 0.1 mM bind MGO with a loss of antibacterial activity, MGO at 0.1 and 0.2 mM alone and in combination with flavonoids retains the ability to kill Gram-positive and -negative bacteria. In addition, *E. coli* appears to be the more sensitive bacteria to the effects of the flavonoids and MGO alone and in combination.

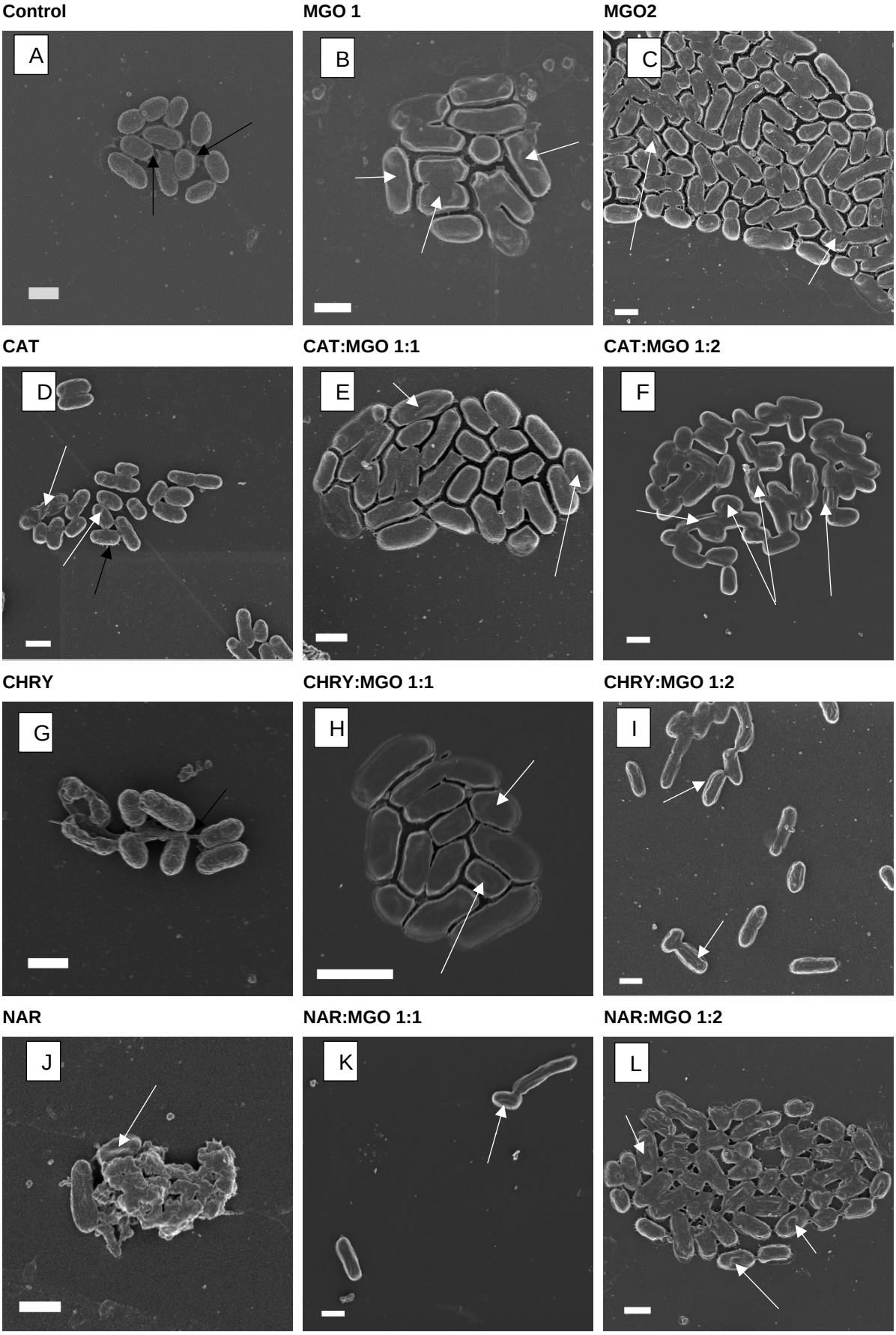


Figure 5.8: Scanning electron micrographs of *E. coli* exposed to 0.1 mM flavonoid alone and in combination with 0.1 mM and 0.2 mM MGO respectively. Micrographs taken at 15.00 Kx magnification, all scale bars are 1 µm except CHRY is 2 µm. The white arrows represent membrane damage the presence of pits and indentations. Black arrows indicate fimbriae.

In table 5.1 the observed effect on the ultrastructural changes on *B. subtilis* and *E. coli* is summarised.

Table 5.1: Effects of MGO, CAT, CHRY and NAR alone and in combination with on *B. subtilis* and *E. coli*.

	B. subtilis	***E. coli***
Control	Rod shaped, short chains, smooth cell wall fimbriae, and flagella.	Rod shaped, smooth cell wall fimbriae, and flagella.
MGO1	Rod shaped, clumped, cell wall is disrupted, and fimbriae are present.	Flattened forming aggregates with indentations, presence of cocci shaped.
MGO2	Cell wall has disintegrated. Loss of cell content. No fimbriae and flagella.	Tightly packed aggregates with indentations, presence of cocci-shaped cells.
CAT	Smooth cell wall with some damage and loss of content. No fimbriae and flagella.	Fimbriae, aggregates with indentations. Fimbriae are present.
CAT:MGO 1:1	Bacteria are flattened with indentations. No fimbriae and flagella.	Flattened, tightly packed aggregates with indentations. No fimbriae present.
CAT:MGO 1:2	Bacteria are flattened with indentations, and cell wall disruption. No fimbriae and flagella.	Tightly packed aggregates with indentations. No fimbriae present.
CHRY	Rod shaped, short chains, smooth cell wall fimbriae and flagella.	Disrupted membrane, rough surface as control. Presence of pili.
CHRY:MGO 1:1	Blebbing and leakage of intracellular content. No fimbriae and flagella.	Aggregates with indentations.
CHRY:MGO 1:2	Loss of cellular content, disintegrated cell wall and shrinkage. No fimbriae and flagella.	Few or less aggregation with indentations or pits.
NAR	The cell wall completely disrupted, bacterium with an irregular shape. No fimbriae and flagella.	Membrane wrinkling and surface disruption. Indentation observed.
NAR:MGO 1:1	Cell wall completely disrupted, bacterium with an irregular shape. No fimbriae and flagella.	Few or less clumping, presence of indentations.
NAR:MGO 1:2	Complete disruption and disintegration of the cell wall. No fimbriae and flagella.	Cocci-shaped aggregates and indentations.

5.5 Discussion

Due to the emergence of multi-drug resistant bacterial strains being a global problem, there is an increasing need for new antimicrobial agents (Daglia, 2012). MGO has been found to be a major bactericidal agent in Manuka honey (Kwakman *et al*., 2011) and although MGO has well-documented antibacterial activity, it is highly reactive (Ghosh *et al.,* 2014) and the formation of AGE can adversely affect protein structure and function.

MGO has been identified as the bioactive compound responsible for the antibacterial activity of Manuka honey. Mavric *et al*., (2008) identified and quantified MGO as the dominant antibacterial constituent of *Leptospermum scoparium* honeys from New Zealand and reported the MICs needed for inhibition of bacterial growth was 1.1 mM for *E. coli* and *S. aureus.* Antibacterial compounds found in honey including MGO, target the cell wall of bacteria and cause structural changes resulting in cell wall damage (Brudzynski & Sjaarda, 2014). Though MGO is bactericidal at high concentrations, *E. coli* may be stimulated to produce MGO through normal glycolysis (Tötemeyer *et al.*, 1998). Detoxification occurs via the reaction with GSH which is followed by the reaction of glyoxalase I and II to produce free GSH and D-lactate as well as the GSH-gated K^+ channels protect *E. coli* against MGO. However, should MGO concentration increase, bacterial inhibition is observed which at a later stage leads to bacterial death (Tötemeyer *et al.*, 1998). Detoxification of MGO is essential for Gram-negative bacteria. Little to no substantial information available on the protective mechanism in Gram-positive bacteria as these bacteria lack GSH, KefB and KefC activities (Liyanage *et al.*, 2001) and may be more sensitive to the effects of MGO.

The ability of 0.1 and 0.2 mM MGO to inhibit growth by 30.38% and 36.52% respectively against *B. subtilis* and 26.90 and 33.10% respectively against *E. coli* confirms the inhibitory effect of MGO. Changes to bacteria morphology associated with toxicity after exposure to 0.1 and 0.2 mM MGO confirms toxicity and findings were similar to that reported by Rabie *et al*., (2016) where exposure to 2 mM MGO, revealed the absence of fimbriae and loss of membrane integrity.

At a concentration of 0.1 mM CAT was not toxic (0.1 mM, microbroth dilution assay, Figure 5.4) but showed minor changes to the morphology of bacteria associated with toxicity (SEM) as seen in Table 5.1. As observed in the present study, the evaluation of a Japanese green tea extract against *Streptococcus mutan*s found that the antibacterial activity of tea catechins or rather catechins without a galloyl moiety, such as found in EGCG, is very weak (Sakanaka *et al.*, 1989). The suggested mechanism of action are associated with membrane injury, including effects on membrane fluidity (Kajiya *et al.*, 2004). CAT can also inhibit ATPase activity by inhibiting bacterial DNA gyrase through binding to the ATP binding site of the gyrase B subunit; which is considered as target for antibacterial drugs (Gradišar *et al.*, 2007). The MIC_{90} of CAT was quantified to be 5050 µg/mL (17.4 mM) against *E. coli*. ATCC 25922 (He *et*

al., 2014) and in another study, the MIC for CAT was quantified to be 600 µg/mL (2.07 mM) against *E. coli*. ATCC 25922 (Ajiboye *et al.*, 2016). Therefore, findings of the present study, confirm the lack of toxicity at 0.1 mM, although morphological features of toxicity were observed at the same concentration.

Similar to CAT, CHRY at the concentrations evaluated had limited toxicity. In a study by Wu *et al.*, 2013, the MIC_{50} of CHRY was 36.72 µg/mL (0.141 mM) determined with the microbroth dilution assay. In another study whereby the synthesis and biological evaluation of 7-O-alkyl modified CHRY analogues as antibacterial agents was observed, CHRY had an MIC_{50} of 50 µg/mL (0.197 mM) against *B. subtilis* and 50 µg/mL (0.197 mM) against *Klebsiella aerogenes* (a Gram-negative bacterium) whereas its analogues had a lower MIC_{50} (Babu *et al.*, 2006). The seven flavonoids isolated from *Halostachys capsica* included CHRY and was reported to have a MIC of 100 µg/mL (0.394 mM) against *E. coli* and 200 µg/mL (0.783 mM) against *B. subtilis* (Liu *et al.*, 2010). Differences between reports can be attributed to the use of different assays and bacterial strains (Cushnie & Lamb, 2005b). In the present study exposure to 0.1 mM CHRY using the microbroth dilution assay, showed no antibacterial effect, although with SEM, ultrastructural changes associated with toxicity against *E. coli* was observed. This confirms the findings of (Liu *et al.*, 2010) that showed that *E. coli* was more sensitive to the effects of CHRY and that the antibacterial activity of CHRY is greater than CAT.

NAR is amongst one of the few flavonoids that have been isolated and identified to possess antibacterial activity (Cushnie & Lamb, 2005b). A study on the antimicrobial effects of Finnish plant extracts containing flavonoids and other phenolic compounds found that NAR exhibited antibacterial activity against all microbes used in the study (including *B. subtilis* and *E. coli*), except *Aspergillus niger* and *Candida albicans* (Rauha *et al.,* 2000). The antibacterial activity of 10 mg/mL (36.7 mM) NAR found in extracts of some Asian edible plants inhibited *E. coli*, *Salmonella infantis*, *S. aureus* and *B. cereus* (Alzoreky & Nakahara, 2003). The MIC value for NAR is reported to be 1 mg/mL (3.64 mM) against *E. coli* 25922 (Wang *et al.*, 2018). SEM evaluation of morphology in the current study shows that at 0.1 mM NAR induces morphological changes associated with toxicity.

The mechanism of antibacterial activity of flavonoids is unknown, but generally they affect cytoplasmic membrane function, nucleic acid synthesis and energy metabolism (Lee *et al.*, 2010). Effects on cellular morphology indicate that adverse effects on the cell wall structure is a major event and may be secondary to changes in nucleic acid synthesis and/or energy metabolism. Differences in the reported MIC values as seem for CAT, CHRY and NAR is related to the different assays used to determine MIC and the strain of bacteria (Cushnie & Lamb, 2005b), although in the present study toxicity is confirmed.

Polyphenols can trap MGO possibly protecting the human body against AGE formation. This trapping effect can either reduce the activity of MGO and/or flavonoids. Therefore, this study also provides information whether polyphenols in honey types will compromise the antibacterial activity of honey. This may not necessary occur in honey which has an acidic pH, but may occur with dilution and digestion, especially duodenal digestion.

In a flavonoid MGO ratio of 1:1 ratio, the antibacterial activity of MGO on B. subtilis was reduced by CAT, CHRY and NAR by 2.27, 2.02 and 2.37-fold. Inhibition by 0.2 mM MGO was 36.52% and was reduced by 3.13, 4.65 and 4.73 -fold for CAT, CHRY and NAR respectively. Against *E. coli,* 0.1 mM MGO inhibited bacterial activity while CAT, CHRY and NAR showed no inhibition. At a flavonoid :MGO ratio of 1:1 ratio the inhibition of MGO by CAT, CHRY and NAR was 4.98, 11.00 and 13.1 -fold reduced. At a ratio of 1:2, the inhibition of bacterial activity by MGO in the presence of CAT, CHRY and NAR was reduced 2.6, 3.5 and 2.4 -fold respectively. This part of the study shows that the presence of flavonoids at the concentrations evaluated reduces the antibacterial activity of MGO.

Structural changes to flavonoids have been shown to alter the antibacterial activity of flavonoids. Inhibition of ATPase activity (related to DNA gyrase) is associated with antibacterial activity. Wu *et al.,* (2013) evaluated the SAR of flavonoids' inhibition of *E. coli* by evaluating effects on DNA gyrase activity. For bacterial inhibition, authors found that (i) the presence of a methoxyl group substitution at the C-3 and C-8 in the A ring and (ii) hydroxyl group substitution at the C-5 in the A ring and C-4' in the B ring ensue. However, inhibition was reduced in the event of: (i) presence of methoxyl group at C-3' in the B ring and (ii) the presence of the hydroxyl group at C-6 in the A ring, C-3' and C-5' in the B ring as well as C-3 in the C ring (Wu *et al*., 2013). Likewise, trapping of MGO and the associated formation of mono and di -MGO adducts may increase antibacterial activity or alternatively the reduction of MGO levels can compromise antibacterial activity. Legnanga, (2017) found that at 1:1 ratio combination of PYR and MGO (1 mM) caused a significant decrease in the antibacterial activity of MGO from 86.76 ± 10.17% to 71.72 ± 11.16% against *B. subtilis*. For antibacterial activity against *E. coli*; no significant decrease due to MGO trapping by phenolic acids was observed. A limitation of the study by Legnanga was that it did not evaluate a dose-response and only evaluated the effect of a single concentration.

The results of this present study showed that some of the flavonoid- and MGO- and flavonoid:MGO- treated bacteria lost fimbriae, pilli and/or flagella (Table 5.1). The bacterial membrane lost its integrity and was deformed with the presence of indentations, pits or holes. This could be as a result of interaction of the MGO, flavonoids or of any adducts that form with the cell wall and/or inhibition of the cellular pathways. Other physical attributes seen in *B. subtilis* included blebbing (Figure 5.7 H), and may be due to the sensitization of the phospholipid bilayer of the cytoplasmic membrane of the cell wall (Samarakoon *et al.*, 2012).

MGO has been reported to inhibit protein synthesis and *E. coli* treated with MGO loses its shape from being rod-shaped to cocci, and the cells adhere to each other (Chaki *et al.*, 2010). A similar effect was observed in the current study for *E. coli* exposed to MGO (Figure 5.8).

A similar study was conducted and the morphology of the antibacterial effects of MGO trapping by phenolic acids were captured; the findings correlated with the findings of this study whereby the MGO-treated *B. subtilis* showed few or an absence of pilli and flagella, damaged cell walls and the presence of holes on the cell walls (Legnanga, 2017).

5.6 Conclusion

In conclusion, the flavonoids CAT, CHRY and NAR at 0.1 mM had no antibacterial activity against *E. coli* while inhibition was observed only with NAR against *B. subtilis*. MGO at 0.1 and 0.2 mM inhibited bacterial growth. In combination at a ratio of 1:1 and 1:2 the antibacterial activity was significantly reduced. MGO as well as NAR caused major changes to bacteria morphology. In combination (MGO: flavonoid), although the antibacterial activity of MGO was reduced, ultrastructure toxicity associated effects were also observed for bacteria.

Chapter 6: Concluding discussion

In recent decades the relationship between many pathological conditions; atherosclerosis, T2DM, aging and carbonyl stress has been the focus of much research interest (Ulrich & Cerami, 2001; Wondrak *et al.*, 2006). Many studies have shown that hyperglycaemia is the greatest factor in diabetic complications, especially T2DM (Hu *et al.*, 2012). Studies have also shown that hyperglycaemia-induced formation of AGEs promote diabetes related complications such as retinopathy, cataract generation, neuropathy and atherosclerosis (Li *et al.*, 2014). Reactive carbonyl compounds, GO and MGO are strong glycation agents *in vivo* and *in vitro* (Lo *et al.*, 2006b). The effect of MGO is not entirely disadvantageous as honey such as manuka honey containing high MGO has strong antibacterial activity (Latrot and Henley, 2009). However, MGO remains a potent protein-glycating agent and an important precursor of AGEs (Majtan, 2011) and consequently concern has been expressed in the use of manuka honey for the treatment of wounds in patients with T2DM.

AGEs have drawn great attention for their micro- and macrovascular effects, therefore the prevention of RCS and AGEs formation and accumulation is a focus of research (Lo *et al.*, 2011). Pharmaceutical agents that have been developed to scavenge these reactive intermediates, have side effects and an example is the toxicity of aminoguanidine used in diabetic patients (Li *et al.*, 2014). Likewise, metformin, an AGE inhibitor both *in vivo* and *in vitro*, also causes adverse side effects in patients with diabetes (Thomas *et al.*, 2005). Therefore, it is critical to develop effective and safe agents to scavenge AGEs (Lishuang, 2011).

Polyphenols are attractive therapeutic molecules as research has shown that polyphenols can scavenge ROS and RNS (Hensley *et al.*, 2000), suppress nuclear factor -kB activation which controls a variety of gene expression related to inflammation and carcinogenesis (Fresco et al., 2006) and inhibit AGEs by trapping MGO (Wu & Yen, 2005).

Little is known regarding the consequence of MGO trapping on the antioxidant activity of flavonoids and the contrary is unknown, whether with trapping is the antibacterial activity of MGO reduced in a physiological environment. Therefore, the aim of this study was to determine whether flavonoids, CAT, NAR and CHRY were able to retain their antioxidant properties even if trapping of MGO has occurred and to what extent is the antibacterial activity of MGO is reduced after trapping.

The TPC determined using the F-C assay showed that polyphenol content was in the order CAT > NAR > CHRY while MGO did not show to have any polyphenolic content or interference. In combination with MGO, no significant changes were observed. The total flavonoid content (TFC) was then determined using the aluminium chloride method. The TFC was in the order CAT> NAR> CHRY, with MGO having no effect. Combined with MGO, the

TFC of the flavonoids was significantly reduced for CAT and NAR, but not for CHRY. No significant differences were observed between MGO alone and flavonoid:MGO1 or flavonoid:MGO2. Differences in TPC and TFC may be due to MGO reacting to the C groups C6 and C8 and not to the hydroxyl and ketone groups that are involved in these reactions although some steric effects may occur.

Antioxidant activity measured with the TEAC assay showed that the antioxidant activity of CAT and NAR was greater than CHRY. In combination with MGO no changes in antioxidant activity was observed for CAT and NAR. Significant differences was only observed for CHRY combinations; at 1:1 ratio from 0.001 mM to 0.003 mM and for the 1:2 ratio was observed from 0.002 to 0.003 mM, CHRY, with a 2.75 fold loss of activity for the 1:1 combination and a 2.44 fold loss of activity for the 1:2 combination. These results indicate that trapping or the presence of MGO does not alter the electron transfer antioxidant activity of CAT and NAR but that of CHRY is affected.

In the ORAC assay, NAR appeared to have the greatest antioxidant capacity related to hydrogen atom transfer while CHRY and CAT had the least. MGO had no antioxidant activity and did not interfere with the ORAC assay. With the flavonoid:MGO combinations, antioxidant activity was unaltered for all flavonoids except CAT. Using the BSA-MGO glycation assay, MGO induced AGE formation and all flavonoids except NAR and NAR:MGO combinations could still significantly reduce AGE formation.

In the mouse fibroblast (L929) cell line, the ability of MGO, CAT, CHRY and NAR and combinations to induce oxidative damage and reduce AAPH oxidative stress was determined, using the DCFH-DA assay. The ability of MGO, CAT, CHRY and NAR and combinations alone (no AAPH was added) to induce oxidative damage was evalauted. Only CAT induced oxidative stress in L929 cells, at the concentrations evalauted showing a slight pro-oxidant effect. The same assy, with AAPH added following exposure, revealed an antioxidant effect for CAT alone and in combination with MGO. A pro-oxidant effect was observed for CHRY and NAR alone and in combination with MGO. It can be concluded that the observed effects was due to the pro-oxidant effects of CHRY and NAR. However, large variability in the data makes some of these effects questionable.

The effects of 24 h exposure to MGO, the flavonoids and MGO:flavonoid combinations on cell viability and cell number was determined with the CV and MTT assays respectively. No changes in cell number was observed after exposure, however cell viability was reduced for CHRY and NAR alone and in combination with MGO, indicating reduced viability is due to the pro-oxidant effects observed at 0.1 mM. This confirms the possible pro-oxidant rather than an antioxidant effect observed for CHRY and NAR evaluated with the DCFH-DA assay.

The consequence of possible trapping on MGO antibacterial activity was determined at 0.1 mM in the microbroth assay and the effect of 0.1 and 0.2 mM concentrations on bacterial morphology was determined after 24 h exposure. Flavonoids CAT, CHRY and NAR at 0.1 mM did not inhibit the growth of *E. coli.* Inhibition was observed only with NAR against *B. subtilis*. MGO effectively killed both bacteria, but in combination with all flavonoids at a ratio of 1:1 and 1:2 the antibacterial activity was significantly reduced indicating MGO trapping. At 0.1 mM based on the effect on *B. subtilis* morphology, NAR was the most toxic while, CAT and CHRY only had antibacterial activity against *E.coli*. Morphological features of toxicity included loss of fimbriae and flagella, membrane damage and lysis.

Limitations and recommendations

This study was an exploratory study and was limited to one flavonoid from each flavonoid group. A future study should involve the effects of at least 2-3 flavonoids from an individual flavonoid group, since structural characteristics may play an important role in trapping and subsequent measurement of bioactivity.

A limitation is that in the present study, the flavonoids and MGO were mixed together and based on previous studies (Wang & Ho, 2012; Hwang *et al.*, 2018) the assumption was made that adduct formation had occurred. Also several studies have shown that the extent of adduct formation is dependent on structure and concentrations used (Jung *et al.*, 2008; Wang & Ho, 2012; Casassa, 2017; Hwang *et al.*, 2018). Using high performance liquid chromatography coupled to mass spectometry (HPLC/MS) it is possible to quantify the extent of adduct formation as well as the strcuture of derivatives that formed (Cheng *et al.*, 2009; Sharp, 2009). This information can then be used to determine if flavonoid adducts are present in Manuka honey and other honey types. Honey has an acidic pH, therefore based on other studies, trapping may not occur but once diluted and especially in the oral and duodenal phases of digestion as well as in wounds adduct formation can occur.

Antioxidant, cellular effects and antibacterial activity are concentration and time dependant. By doing dosage and time based studies a better understanding of these effects can be established. For example at low concetrations depending on the flavonoid, in cellular models an antioxidant effect can occur whereas at high concetrations a pro-oxidant effect can occur. These effects have been described for some polyphenolic compounds i.e. green tea catechins (Lambert & Elias, 2010) and epigallocatechin gallate in animals, where at high concentrations cysteine conjugates formed (Halliwell, 2008).

In the present study, changes to cell number and vaibility was determined. MGO has been reported to bind and deplete GSH (Dafre *et al.*, 2015) and inhibit antioxidant pathways by binding to the thiol groups of enzymes and via AGE formation. The effects on GSH levels and

enzyme levels can be determined with the fluoresent dye, o-phtalaldehyde (Senft *et al.*, 2000) and GPx, SOD and catalase levels can be determined with Western blotting (Lim *et al.*, 2010).

A dosage effect can also be used to determine antibacterial activity. The effects of MGO trapping by flavonoids on the effects of MGO on FliC, and responsible flagellin-associated genes, including fliA, fliC, flhF, fleN, fleQ and fleR (Roberts *et al.*, 2015) can then be determined, also with Western blotting.

In conclusion, trapping of MGO by flavonoids can alter antioxidant effects as well as antibacterial activity. A future detailed study of the degree of trapping and the effect of pH on biochemical mechanisms involved and mechanisms in cellular models should be undertaken.

References

Ajiboye, T., Aliyu, M., Isiaka, I., Haliru, F., Ibitoye, O., Uwazie, J., Muritala, H., Bello, S., Yusuf, I. & Mohammed, A. 2016. Contribution of reactive oxygen species to (+)-catechin-mediated bacterial lethality. *Chemico-Biological Interactions,* 258: 276-287.

Akram, M. 2011. Flavonoids and phenolic acids: Role and biochemical activity in plants and human. *Journal of Medicinal Plants Research,* 5(32): 6697-6703.

Al-Adham, I., Haddadin, R. & Collier, P. eds. 2013. *Types of Microbicidal and Microbistatic Agents.* 5 ed.: John Wiley & Sons. p. 5-70.

Allaman, I., Belanger, M. & Magistretti, P.J. 2015. Methylglyoxal, the dark side of glycolysis. *Frontiers in Neuroscience,* 9(23): 1-12.

Alzoreky, N. & Nakahara, K. 2003. Antibacterial activity of extracts from some edible plants commonly consumed in Asia. *International Journal of Food Microbiology,* 80(3): 223-230.

Ammon, H.P. & Wahl, M.A. 1991. Pharmacology of *Curcuma longa*. *Planta Medica,* 57(1): 1-7.

Andueza, A., García-Garzón, A., de Galarreta, M.R., Ansorena, E., Iraburu, M.J., López-Zabalza, M.J. & Martínez-Irujo, J.J. 2015. Oxidation pathways underlying the pro-oxidant effects of apigenin. *Free Radical Biology & Medicine,* 87: 169-180.

Apak, R.A., Özyürek, M., Güçlü, K. & Çapanoğlu, E. 2016. Antioxidant activity/capacity measurement. 2. Hydrogen atom transfer (HAT)-based, mixed-mode (electron transfer (ET)/HAT), and lipid peroxidation assays. *Journal of Agricultural & Food Chemistry,* 64(5): 1028-1045.

Araujo, C.C. & Leon, L.L. 2001. Biological activities of *Curcuma longa L*. *Memorias do Instuto Oswaldo Cruz,* 96(5): 723-728.

Ávila, H.P., Smânia, E.d.F.A., Delle Monache, F. & Júnior, A.S. 2008. Structure–activity relationship of antibacterial chalcones. *Bioorganic & Medicinal Chemistry,* 16(22): 9790-9794.

Babu, K.S., Babu, T.H., Srinivas, P., Kishore, K.H., Murthy, U. & Rao, J.M. 2006. Synthesis and biological evaluation of novel C (7) modified chrysin analogues as antibacterial agents. *Bioorganic & Medicinal Chemistry Letters,* 16(1): 221-224.

Bachi, K., Sierra, S., Volkow, N.D., Goldstein, R.Z. & Alia-Klein, N. 2017. Is biological aging accelerated in drug addiction? *Current Opinion in Behavioral Sciences,* 13: 34-39.

Balasundram, N., Sundram, K. & Samman, S. 2006. Phenolic compounds in plants and agri-industrial by-products: Antioxidant activity, occurrence, and potential uses. *Food Chemistry,* 99(1): 191-203.

Bark, K.-M., Yeom, J.-E., Yang, J.-I., Yang, I.-J., Park, C.-H. & Park, H.-R. 2011. Spectroscopic studies on the oxidation of catechin in aqueous solution. *Bullitin of the Korean Chemical Society,* 32(9): 3443-3447.

Beatty, S., Koh, H.-H., Phil, M., Henson, D. & Boulton, M. 2000. The role of oxidative stress in the pathogenesis of age-related macular degeneration. *Survey of Opthamology,* 45(2): 115-134.

Bernard, F.-X., Sable, S., Cameron, B., Provost, J., Desnottes, J.-F., Crouzet, J. & Blanche, F. 1997. Glycosylated flavones as selective inhibitors of topoisomerase IV. *Antimicrobial Agents & Chemotherapy,* 41(5): 992-998.

Bernas, T. & Dobrucki, J. 2002. Mitochondrial and nonmitochondrial reduction of MTT: interaction of MTT with TMRE, JC-1, and NAO mitochondrial fluorescent probes. *Cytometry,* 47(4): 236-242.

Bhuiyan, M.N.I., Mitsuhashi, S., Sigetomi, K. & Ubukata, M. 2017. Quercetin inhibits advanced glycation end product formation via chelating metal ions, trapping methylglyoxal, and trapping reactive oxygen species. *Bioscience, Biotechnology, & Biochemistry,* 81(5): 882-890.

Bonnekoh, B., Wevers, A., Jugert, F., Merk, H. & Mahrle, G. 1989. Colorimetric growth assay for epidermal cell cultures by their crystal violet binding capacity. *Archives of Dermatological Research,* 281(7): 487-490.

Booth, I.R., Ferguson, G., Miller, S., Li, C., Gunasekera, B. & Kinghorn, S. 2003. Bacterial production of methylglyoxal: a survival strategy or death by misadventure? *Biochemical Society Transactions,* 31(6): 1406-1408.

Brudzynski, K. & Sjaarda, C. 2014. Antibacterial compounds of Canadian honeys target bacterial cell wall inducing phenotype changes, growth inhibition and cell lysis that resemble action of β-lactam antibiotics. *Public Library of Science One,* 9(9): e106967.

Casassa, L.F. 2017. Flavonoid Phenolics in Red Winemaking. *Phenolic Compounds-Natural Sources, Importance and Applications*: InTech. [Online] Available from: https://www.intechopen.com/books/phenolic-compounds-natural-sources-importance-and-applications/flavonoid-phenolics-in-red-winemaking [Accessed: 6 January]

Chaki, S., Mukherjee, S., Das, S., Mookerjee, M. & Dastidar, S.G. 2010. Evaluation of bactericidal action of methylglyoxal and its further potentiation in the presence of honey. *International Journal of Biomedical & Pharmaceutical Sciences,* 4(1): 66-69

Chan, W.H., Wu, H.J. & Hsuuw, Y.D. 2005. Curcumin inhibits ROS formation and apoptosis in methylglyoxal-treated human hepatoma G2 cells. *Annals of the New York Academy of Sciences,* 1042(1): 372-378.

Chang, C.-C., Yang, M.-H., Wen, H.-M. & Chern, J.-C. 2002. Estimation of total flavonoid content in propolis by two complementary colorimetric methods. *Journal of Food & Drug Analysis,* 10(3): 178-182.

Cheng, K.-W., Zeng, X., Tang, Y.S., Wu, J.-J., Liu, Z., Sze, K.-H., Chu, I.K., Chen, F. & Wang, M. 2009. Inhibitory mechanism of naringenin against carcinogenic acrylamide formation and nonenzymatic browning in Maillard model reactions. *Chemical Research in Toxicology,* 22(8): 1483-1489.

Choi, E.M., Suh, K.S., Kim, Y.J., Hong, S.M., Park, S.Y. & Chon, S. 2016. Glabridin alleviates the toxic effects of methylglyoxal on osteoblastic MC3T3-E1 cells by increasing expression of the glyoxalase system and Nrf2/HO-1 signaling and protecting mitochondrial function. *Journal of Agricultural & Food Chemistry,* 64(1): 226-235.

Cushnie, T.P.T. & Lamb, A.J. 2005a. Antimicrobial activity of flavonoids. *International Journal of Antimicrobial Agents,* 26(5): 343-356.

Cushnie, T.T. & Lamb, A.J. 2005b. Detection of galangin-induced cytoplasmic membrane damage in *Staphylococcus aureus* by measuring potassium loss. *Journal of Ethnopharmacology,* 101(1-3): 243-248.

Dafre, A., Goldberg, J., Wang, T., Spiegel, D. & Maher, P. 2015. Methylglyoxal, the foe and friend of glyoxalase and Trx/TrxR systems in HT22 nerve cells. *Free Radical Biology & Medicine,* 89: 8-19.

Dhananjayan, K., Gunawardena, D., Hearn, N., Sonntag, T., Moran, C., Gyengesi, E., Srikanth, V. & Muench, G. 2017. Activation of macrophages and Mmcroglia by interferon–γ and lipopolysaccharide increases methylglyoxal production: A new mechanism in the development of vascular complications and cognitive decline in type 2 diabetes mellitus? *Journal of Alzheimer's Disease,* 59(2): 467-479.

Dringen, R. 2000. Metabolism and functions of glutathione in brain. *Progress in Neurobiology,* 62(6): 649-671.

Dudonné, S., Vitrac, X., Coutiere, P., Woillez, M. & Mérillon, J.-M. 2009. Comparative study of antioxidant properties and total phenolic content of 30 plant extracts of industrial interest using DPPH, ABTS, FRAP, SOD, and ORAC assays. *Journal of Agricultural & Food Chemistry,* 57(5): 1768-1774.

Dueñas, M., González-Manzano, S., González-Paramás, A. & Santos-Buelga, C. 2010. Antioxidant evaluation of O-methylated metabolites of catechin, epicatechin and quercetin. *Journal of Pharmaceutical & Biomedical Analysis,* 51(2): 443-449.

Eldeniz, A., Mustafa, K., Ørstavik, D. & Dahl, J. 2007. Cytotoxicity of new resin-, calcium hydroxide-and silicone-based root canal sealers on fibroblasts derived from human gingiva and L929 cell lines. *International Endodontic Journal,* 40(5): 329-337.

Everette, J.D., Bryant, Q.M., Green, A.M., Abbey, Y.A., Wangila, G.W. & Walker, R.B. 2010. Thorough study of reactivity of various compound classes toward the Folin− Ciocalteu reagent. *Journal of Agricultural & Food Chemistry,* 58(14): 8139-8144.

Felgines, C., Texier, O., Morand, C., Manach, C., Re, C., Scalbert, A., Coise, F., Texier, O., Mo-, C., Manach, C., Scalbert, A. & Re, C. 2000. Bioavailability of the flavanone naringenin and its glycosides in rats. *American Journal of Physiology Gastrointestinal & Liver Physiology,* 279(6): 1148-1154.

Fernandes de Oliveira, A.M., Sousa Pinheiro, L., Souto Pereira, C.K., Neves Matias, W., Albuquerque Gomes, R., Souza Chaves, O., Vanderlei de Souza, M.d.F., Nóbrega de

Almeida, R. & Simões de Assis, T. 2012. Total phenolic content and antioxidant activity of some Malvaceae family species. *Antioxidants,* 1(1): 33-43.

Frankel, E.N. & Meyer, A.S. 2000. The problems of using one-dimensional methods to evaluate multifunctional food and biological antioxidants. *Journal of the Science of Food & Agriculture,* 80(13): 1925-1941.

Fulle, S., Protasi, F., Di, G., Pietrangelo, T., Beltramin, A., Boncompagni, S., Vecchiet, L. & Fano, G. 2004. The contribution of reactive oxygen species to sarcopenia and muscle ageing. *Experimental Gerontology,* 39(1): 17-24.

Ghasemzadeh, A. & Ghasemzadeh, N. 2011. Flavonoids and phenolic acids: Role and biochemical activity in plants and human. *Journal of Medicinal Plants Research,* 5(31): 6697-6703.

Goldstein, J.I., Newbury, D.E., Michael, J.R., Ritchie, N.W., Scott, J.H.J. & Joy, D.C. 2017. *Scanning Electron Microscopy and X-Ray Microanalysis.4th ed.* Springer. p.147-161.

Gradišar, H., Pristovšek, P., Plaper, A. & Jerala, R. 2007. Green tea catechins inhibit bacterial DNA gyrase by interaction with its ATP binding site. *Journal of Medicinal Chemistry,* 50(2): 264-271.

Granvogl, M., Beksan, E. & Schieberle, P. 2012. New insights into the formation of aroma-active Strecker aldehydes from 3-oxazolines as transient intermediates. *Journal of Agricultural & Food Chemistry,* 60(25): 6312-6322.

Halliwell, B. 2008. Are polyphenols antioxidants or pro-oxidants? What do we learn from cell culture and in vivo studies? *Archives of Biochemistry & Biophysics,* 476(2): 107-112.

Halliwell, B. & Gutteridge, J.M. 5th ed. 2015. *Free Radicals in Biology and Medicine.* Oxford University Press. p.77-151.

Han, Y., Randell, E., Vasdev, S., Gill, V., Gadag, V., Newhook, L.A., Grant, M. & Hagerty, D. 2007. Plasma methylglyoxal and glyoxal are elevated and related to early membrane alteration in young, complication-free patients with type 1 diabetes. *Molecular & Cellular Biochemistry,* 305(1-2): 123-131.

Haraguchi, H., Tanimoto, K., Tamura, Y., Mizutani, K. & Kinoshita, T. 1998. Mode of antibacterial action of retrochalcones from *Glycyrrhiza inflata*. *Phytochemistry,* 48(1): 125-129.

Harman, D. 1981. The aging process. *Proceedings of the National Academy of Sciences of the United States of America,* 78(11): 7124-7128.

He, M., Wu, T., Pan, S. & Xu, X. 2014. Antimicrobial mechanism of flavonoids against *Escherichia coli* ATCC 25922 by model membrane study. *Applied Surface Science,* 305: 515-521.

Heim, K.E., Tagliaferro, A.R. & Bobilya, D.J. 2002. Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships. *The Journal of Nutritional Biochemistry,* 13(10): 572-584.

Hensley, K., Robinson, K.A., Gabbita, S.P., Salsman, S. & Floyd, R.A. 2000. Reactive oxygen species, cell signaling, and cell injury. *Free Radical Biology & Medicine,* 28(10): 1456-1462.

Ho, S.-C., Wu, S.-P., Lin, S.-M. & Tang, Y.-L. 2010. Comparison of anti-glycation capacities of several herbal infusions with that of green tea. *Food Chemistry,* 122(3): 768-774.

Hu, T.Y., Liu, C.L., Chyau, C.C. & Hu, M.L. 2012. Trapping of methylglyoxal by curcumin in cell-free systems and in human umbilical vein endothelial cells. *Journal of Agricultural Food Chemistry,* 60(33): 8190-8196.

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

Hwang, S., Kim, H., Zuo, G., Wang, Z., Lee, J.-Y. & Lim, S. 2018. Anti-glycation, carbonyl trapping and anti-inflammatory activities of chrysin derivatives. *Molecules,* 23(7): 1752-1766.

Ikigai, H., Nakae, T., Hara, Y. & Shimamura, T. 1993. Bactericidal catechins damage the lipid bilayer. *Biochimica et Biophysica Acta (BBA)-Biomembranes,* 1147(1): 132-136.

Islam, M.T. 2017. Oxidative stress and mitochondrial dysfunction-linked neurodegenerative disorders. *Neurological Research,* 39(1): 73-82.

Jenkins, R., Roberts, A. & Brown, H.L. 2015. On the antibacterial effects of manuka honey: mechanistic insights. *Research & Reports in Biology,* 6: 215-224.

Jensen, T.M., Vistisen, D., Fleming, T., Nawroth, P.P., Jørgensen, M.E., Lauritzen, T., Sandbæk, A. & Witte, D.R. 2015. Impact of intensive treatment on serum methylglyoxal levels among individuals with screen-detected type 2 diabetes: the ADDITION-Denmark study. *Acta Diabetologica,* 52(5): 929-936.

Jensen, T.M., Vistisen, D., Fleming, T., Nawroth, P.P., Rossing, P., Jorgensen, M.E., Lauritzen, T., Sandbaek, A. & Witte, D.R. 2016. Methylglyoxal is associated with changes in kidney function among individuals with screen-detected type 2 diabetes mellitus. *Diabetic Medicine,* 33(12): 1625-1631.

Joshi, H., Hegde, A.R., Shetty, P.K., Gollavilli, H., Managuli, R.S., Kalthur, G. & Mutalik, S. 2018. Sunscreen creams containing naringenin nanoparticles: Formulation development and i*n vitro* and i*n vivo* evaluations. *Photodermatology Photoimmunology & Photomedicine,* 34(1): 69-81.

Jung, H.A., Jung, Y.J., Yoon, N.Y., Jeong, D.M., Bae, H.J., Kim, D.-W., Na, D.H. & Choi, J.S. 2008. Inhibitory effects of *Nelumbo nucifera* leaves on rat lens aldose reductase, advanced glycation endproducts formation, and oxidative stress. *Food & Chemical Toxicology,* 46(12): 3818-3826.

Jung, J. 2016. Emerging utilization of chrysin using nanoscale modification. *Journal of Nanomaterials,* 2016: 1-7.

Kajiya, K., Hojo, H., Suzuki, M., Nanjo, F., Kumazawa, S. & Nakayama, T. 2004. Relationship between antibacterial activity of (+)-catechin derivatives and their interaction with a model membrane. *Journal of Agricultural & Food Chemistry,* 52(6): 1514-1519.

Kalapos, M.P. 2008. The tandem of free radicals and methylglyoxal. *Chemico-Biological Interactions,* 171(3): 251-271.

Kalapos, M.P. 2013. Where does plasma methylglyoxal originate from? *Diabetes Research & Clinical Practice,* 99(3): 260-271.

Kalita, P., Tapan, B.K., Pal, T.K. & Kalita, R. 2013. Estimation of total flavonoids content (TFC) and anti oxidant activities of methanolic whole plant extract of *Biophytum sensitivum* Linn. *Journal of Drug Delivery & Therapeutics,* 3(4): 33-37.

Kamaratos, A.V., Tzirogiannis, K.N., Iraklianou, S.A., Panoutsopoulos, G.I., Kanellos, I.E. & Melidonis, A.I. 2014. Manuka honey-impregnated dressings in the treatment of neuropathic diabetic foot ulcers. *International Wound Journal,* 11(3): 259-263.

Ke, J.Y., Banh, T., Hsiao, Y.H., Cole, R.M., Straka, S.R., Yee, L.D. & Belury, M.A. 2017. Citrus flavonoid naringenin reduces mammary tumor cell viability, adipose mass, and adipose inflammation in obese ovariectomized mice. *Molecular Nutrition and Food Research,* 61(9): 1-12.

Lambert, J.D. & Elias, R.J. 2010. The antioxidant and pro-oxidant activities of green tea polyphenols: a role in cancer prevention. *Archives of Biochemistry & Biophysics,* 501(1): 65-72.

Lazarević, M., Mazzon, E., Momčilović, M., Basile, M., Colletti, G., Petralia, M., Bramanti, P., Nicoletti, F. & Miljković, Đ. 2018. The H2S donor GYY4137 stimulates reactive oxygen species generation in BV2 cells while suppressing the secretion of TNF and nitric oxide. *Molecules,* 23(11): 2966-2979.

Lee, K.-A., Moon, S.H., Kim, K.-T., Mendonca, A.F. & Paik, H.-D. 2010. Antimicrobial effects of various flavonoids on *Escherichia coli* O157: H7 cell growth and lipopolysaccharide production. *Food Science & Biotechnology,* 19(1): 257-261.

Legnanga, M. 2017. *The trapping of methylglyoxal by phenolic acids: effect on antioxidant and antibacterial activity.* University of Pretoria [dissertation].

Li, X., Zheng, T., Sang, S. & Lv, L. 2014. Quercetin inhibits advanced glycation end product formation by trapping methylglyoxal and glyoxal. *Jornal of Agricultural & Food Chemistry,* 62(50): 12152-12158.

Lim, J., Sanders, R.A., Snyder, A.C., Eells, J.T., Henshel, D.S. & Watkins III, J.B. 2010. Effects of low-level light therapy on streptozotocin-induced diabetic kidney. *Journal of Photochemistry & Photobiology B: Biology,* 99(2): 105-110.

Lin, J.A., Wu, C.H., Lu, C.C., Hsia, S.M. & Yen, G.C. 2016. Glycative stress from advanced glycation end products (AGEs) and dicarbonyls: An emerging biological factor in cancer onset and progression. *Molecular Nutrition & Food Research,* 60(8): 1850-1864.

Lin, J.K., Chen, P.C., Ho, C.T. & Lin-Shiau, S.Y. 2000. Inhibition of xanthine oxidase and suppression of intracellular reactive oxygen species in HL-60 cells by theaflavin-3,3'-digallate,

(-)- epigallocatechin-3-gallate, and propyl gallate. *Journal of Agricultural and Food Chemistry,* 48(7): 2736-2743.

Liu, G., Xia, Q., Lu, Y., Zheng, T., Sang, S. & Lv, L. 2017. Influence of quercetin and its methylglyoxal adducts on the formation of alpha-dicarbonyl compounds in a lysine/glucose model system. *Journal of Agricultural Food Chemistry,* 65(10): 2233-2239.

Liu, H., Mou, Y., Zhao, J., Wang, J., Zhou, L., Wang, M., Wang, D., Han, J., Yu, Z. & Yang, F. 2010. Flavonoids from *Halostachys caspica* and their antimicrobial and antioxidant activities. *Molecules,* 15(11): 7933-7945.

Liyanage, H., Kashket, S., Young, M. & Kashket, E.R. 2001. *Clostridium beijerinckii* and *Clostridium difficile* detoxify methylglyoxal by a novel mechanism involving glycerol dehydrogenase. *Applied Environmental Microbiology,* 67(5): 2004-2010.

Lo, C.Y., Hsiao, W.T. & Chen, X.Y. 2011. Efficiency of trapping methylglyoxal by phenols and phenolic acids. *Journal of Food Science,* 76(3): H90-H96.

Lo, C.Y., Li, S., Tan, D., Pan, M.H., Sang, S. & Ho, C.T. 2006. Trapping reactions of reactive carbonyl species with tea polyphenols in simulated physiological conditions. *Molecular Nutrition & Food Research,* 50(12): 1118-1128.

Loeffler, K.W., Koehler, C.A., Paul, N.M. & De Haan, D.O. 2006. Oligomer formation in evaporating aqueous glyoxal and methyl glyoxal solutions. *Environmental Science & Technology,* 40(20): 6318-6323.

Lu, N., Chen, P., Yang, Q. & Peng, Y.-Y. 2011. Anti-and pro-oxidant effects of (+)-catechin on hemoglobin-induced protein oxidative damage. *Toxicology in Vitro,* 25(4): 833-838.

Maessen, D.E., Stehouwer, C.D. & Schalkwijk, C.G. 2015. The role of methylglyoxal and the glyoxalase system in diabetes and other age-related diseases. *Clinical Science,* 128(12): 839-861.

Majtan, J. 2011. Methylglyoxal-a potential risk factor of manuka honey in healing of diabetic ulcers. *Evidence Based Complementary & Alternative Medicine,* 2011: 1-5.

Manach, C. 2004. Polyphenols : food sources and bioavailability . *American Journal of Clinical Nutrition,* 79(5): 727-747.

Mandal, M.D. & Mandal, S. 2011. Honey: its medicinal property and antibacterial activity. *Asian Pacific Journal of Tropical Biomedicine,* 1(2): 154-160.

Matsuda, H., Morikawa, T., Ando, S., Toguchida, I. & Yoshikawa, M. 2003. Structural requirements of flavonoids for nitric oxide production inhibitory activity and mechanism of action. *Bioorganic & Medicinal Chemistry,* 11(9): 1995-2000.

Matumoto-Pintro, P.T., Murakami, A.E., Vital, A.C., Croge, C., da Silva, D.F., Ospina-Roja, I.C. & Guerra, A.F. 2017. Effects of storage time and temperature on lipid oxidation of egg powders enriched with natural antioxidants. *Food Chemistry,* 228: 463-468.

Mavric, E., Wittmann, S., Barth, G. & Henle, T. 2008. Identification and quantification of methylglyoxal as the dominant antibacterial constituent of Manuka (*Leptospermum scoparium)* honeys from New Zealand. *Molecular Nutrition & Food Research,* 52(4): 483-489.

Monika, P., Basavaraj, B., Murthy, K., Ahalya, N. & Gurudev, K. 2017. Nanocapsules of catechin rich extract for enhanced antioxidant potential and *in vitro* bioavailability. *Journal of Applied Pharmaceutical Science,* 7(1): 184-188.

Mori, A., Nishino, C., Enoki, N. & Tawata, S. 1987. Antibacterial activity and mode of action of plant flavonoids against *Proteus vulgaris* and *Staphylococcus aureus*. *Phytochemistry,* 26(8): 2231-2234.

Morrell, C.N. 2008. Reactive oxygen species: finding the right balance. *Circulation Research,* 103(6): 571-572.

Murakami, N., Tamura, S., Koyama, K., Sugimoto, M., Maekawa, R. & Kobayashi, M. 2004. New analogue of arenastatin A, a potent cytotoxic spongean depsipeptide, with anti-tumor activity. *Bioorganic & Medicinal Chemistry Letters,* 14(10): 2597-2601.

Nemet, I., Varga-Defterdarović, L. & Turk, Z. 2006. Methylglyoxal in food and living organisms. *Molecular Nutrition & Food Research,* 50(12): 1105-1117.

Orhan, D.D., Özçelik, B., Özgen, S. & Ergun, F. 2010. Antibacterial, antifungal, and antiviral activities of some flavonoids. *Microbiological Research,* 165(6): 496-504.

Ota, N., Soga, S. & Shimotoyodome, A. 2016. Daily consumption of tea catechins improves aerobic capacity in healthy male adults: a randomized double-blind, placebo-controlled, crossover trial. *Bioscience, Biotechnolology & Biochemistry,* 80(12): 2412-2417.

Ou, B., Huang, D., Hampsch-Woodill, M., Flanagan, J.A. & Deemer, E.K. 2002. Analysis of antioxidant activities of common vegetables employing oxygen radical absorbance capacity (ORAC) and ferric reducing antioxidant power (FRAP) assays: a comparative study. *Journal of Agricultural & Food Chemistry,* 50(11): 3122-3128.

Panich, U., Ananta, W., Onkoksoong, T. & Jaemsak, K. 2017. Effects of cellular uptake of flavonoids against peroxynitrite-mediated cell cytotoxicity. *Siriraj Medical Journal,* 59(5): 237-241.

Pereira, R.M.S., López, B.G.-C., Diniz, S.N., Antunes, A.A., Moreno Garcia, D., Rocha Oliveira, C. & Marcucci, M.C. 2017. Quantification of flavonoids in Brazilian orange peels and industrial orange juice processing wastes. *Agricultural Sciences,* 8(7): 631-644.

Pietta, P.-G. 2000. Flavonoids as antioxidants. *Journal of Natural Products,* 63(7): 1035-1042.

Plaper, A., Golob, M., Hafner, I., Oblak, M., Šolmajer, T. & Jerala, R. 2003. Characterization of quercetin binding site on DNA gyrase. *Biochemical & Biophysical Research Communications,* 306(2): 530-536.

Poprac, P., Jomova, K., Simunkova, M., Kollar, V., Rhodes, C.J. & Valko, M. 2017. Targeting free radicals in oxidative stress-related human diseases. *Trends Pharmacological Sciences,* 38(7): 592-607.

Prasad, S., Gupta, S.C. & Tyagi, A.K. 2017. Reactive oxygen species (ROS) and cancer: role of antioxidative nutraceuticals. *Cancer Letters,* 387: 95-105.

Prior, R.L. 2015. Oxygen radical absorbance capacity (ORAC): New horizons in relating dietary antioxidants/bioactives and health benefits. *Journal of Functional Foods,* 18:797-810.

Prior, R.L. & Cao, G. 2000. Antioxidant phytochemicals in fruits and vegetables: diet and health implications. *HortScience,* 35(4): 588-592.

Procházková, D., Boušová, I. & Wilhelmová, N. 2011. Antioxidant and prooxidant properties of flavonoids. *Fitoterapia,* 82(4): 513-523.

Quideau, S. 2006. Why bother with polyphenols? *Polyphénols Actualités,* 24(1): 10-14.

Rabie, E., Serem, J.C., Oberholzer, H.M., Gaspar, A.R. & Bester, M.J. 2016. How methylglyoxal kills bacteria: An ultrastructural study. *Ultrastructural Pathology,* 40(2): 107-111.

Raffa, D., Maggio, B., Raimondi, M.V., Plescia, F. & Daidone, G. 2017. Recent discoveries of anticancer flavonoids. *European Journal of Medicinal Chemistry,* 142: 213-228.

Rani, R., Sharma, D., Chaturvedi, M. & Yadav, J. 2017. Antibacterial activity of twenty different endophytic fungi isolated from *Calotropis procera* and time kill assay. *Clinical Microbiology,* 6(3): 280-285.

Rauha, J.-P., Remes, S., Heinonen, M., Hopia, A., Kähkönen, M., Kujala, T., Pihlaja, K., Vuorela, H. & Vuorela, P. 2000. Antimicrobial effects of Finnish plant extracts containing flavonoids and other phenolic compounds. *International Journal of Food Microbiology,* 56(1): 3-12.

Roberts, A.E., Maddocks, S.E. & Cooper, R.A. 2015. Manuka honey reduces the motility of *Pseudomonas aeruginosa* by suppression of flagella-associated genes. *Journal of Antimicrobial Chemotherapy,* 70(3): 716-725.

Rodriguez-Amaya, D.B. 2015. Status of carotenoid analytical methods and *in vitro* assays for the assessment of food quality and health effects. *Current Opinion in Food Science,* 1: 56-63.

Rondeau, P. & Bourdon, E. 2011. The glycation of albumin : Structural and functional impacts. *Biochimie,* 93(4): 645-658.

Sakanaka, S., Kim, M., Taniguchi, M. & Yamamoto, T. 1989. Antibacterial substances in Japanese green tea extract against *Streptococcus mutans*, a cariogenic bacterium. *Agricultural & Biological Chemistry,* 53(9): 2307-2311.

Samarakoon, K., Senevirathne, M., Lee, W.-W., Kim, Y.-T., Kim, J.-I., Oh, M.-C. & Jeon, Y.-J. 2012. Antibacterial effect of citrus press-cakes dried by high speed and far-infrared radiation drying methods. *Nutrition Research & Practice,* 6(3): 187-194.

Seeram, N.P. & Nair, M.G. 2002. Inhibition of lipid peroxidation and structure− activity-related studies of the dietary constituents anthocyanins, anthocyanidins, and catechins. *Journal of Agricultural & Food Chemistry,* 50(19): 5308-5312.

Seidler, N.W. 2013. Basic biology of GAPDH. *Advances in Experimental Medicine & Biology,* 985: 61-101.

Senft, A.P., Dalton, T.P. & Shertzer, H.G. 2000. Determining glutathione and glutathione disulfide using the fluorescence probe o-phthalaldehyde. *Analytical Biochemistry,* 280(1): 80-86.

Seo, K., Ki, S.H. & Shin, S.M. 2014. Methylglyoxal induces mitochondrial dysfunction and cell death in liver. *Toxicological Research,* 30(3): 193-198.

Shalev, I. & Belsky, J. 2016. Early-life stress and reproductive cost: A two-hit developmental model of accelerated aging? *Medical Hypotheses,* 90: 41-47.

Shao, X., Chen, H., Zhu, Y., Sedighi, R., Ho, C.-T. & Sang, S. 2014. Essential structural requirements and additive effects for flavonoids to scavenge methylglyoxal. *Journal of Agricultural & Food Chemistry,* 62(14): 3202-3210.

Sharp, A. 2009. Beneficial effects of honey dressings in wound management. *Nursing Standard,* 24(7): 66-72.

Singh, R., Barden, A., Mori, T. & Beilin, L. 2001. Advanced glycation end-products : a review. *Diabetologia,* 44(2): 129-146.

Sirk, T.W., Brown, E.F., Sum, A.K. & Friedman, M. 2008. Molecular dynamics study on the biophysical interactions of seven green tea catechins with lipid bilayers of cell membranes. *Journal of Agricultural & Food Chemistry,* 56(17): 7750-7758.

Sobočanec, S., Šverko, V., Balog, T., Šarić, A., Rusak, G., Likić, S., Kušić, B., Katalinić, V., Radić, S. & Marotti, T. 2006. Oxidant/antioxidant properties of Croatian native propolis. *Journal of Agricultural & Food Chemistry,* 54(21): 8018-8026.

Stefek, M. 2011. Natural flavonoids as potential multifunctional agents in prevention of diabetic cataract. *Interdisciplinary Toxicology,* 4(2): 69-77.

Stewart, J.A., McGrane, O.L. & Wedmore, I.S. 2014. Wound care in the wilderness: is there evidence for honey? *Wilderness & Environmental Medicine,* 25(1): 103-110.

Sultanbawa, Y., Cozzolino, D., Fuller, S., Cusack, A., Currie, M. & Smyth, H. 2015. Infrared spectroscopy as a rapid tool to detect methylglyoxal and antibacterial activity in Australian honeys. *Food Chemistry,* 172: 207-212.

Szwergold, B. 2017. Reactions between methylglyoxal and its scavengers in-vivo appear to be catalyzed enzymatically. *Medical Hypotheses,* 109: 153-155.

Tötemeyer, S., Booth, N., Nichols, W., Dunbar, B. & Booth, I. 1998. From famine to feast: the role of methylglyoxal production in *Escherichia coli*. *Molecular Microbiology,* 27(3): 553-562.

Tsuchiya, H. & Iinuma, M. 2000. Reduction of membrane fluidity by antibacterial sophoraflavanone G isolated from *Sophora exigua*. *Phytomedicine,* 7(2): 161-165.

Tu, C.-Y., Chen, Y.-F., Lii, C.-K. & Wang, T.-S. 2013. Methylglyoxal induces DNA crosslinks in ECV304 cells via a reactive oxygen species-independent protein carbonylation pathway. *Toxicology in Vitro,* 27(4): 1211-1219.

Ulanowska, K., Tkaczyk, A., Konopa, G. & Węgrzyn, G. 2006. Differential antibacterial activity of genistein arising from global inhibition of DNA, RNA and protein synthesis in some bacterial strains. *Archives of Microbiology,* 184(5): 271-278.

Ulrich, P. & Cerami, A. 2001. Protein glycation, diabetes, and aging. *Recent Progress in Hormone Research,* 56(1): 1-22.

UMFHA. 2019. *Unique Manuka Factor Honey Association*. [Online] Available from: http://www.umf.org.nz/grading-systems-explained/ [Accessed: 3 March].

Valko, M., Leibfritz, D., Moncol, J., Cronin, M.T., Mazur, M. & Telser, J. 2007. Free radicals and antioxidants in normal physiological functions and human disease. *The International Journal of Biochemistry & Cell Biology,* 39(1): 44-84.

Vander Jagt, D.L. & Hunsaker, L.A. 2003. Methylglyoxal metabolism and diabetic complications: roles of aldose reductase, glyoxalase-I, betaine aldehyde dehydrogenase and 2-oxoaldehyde dehydrogenase. *Chemico-Biological Interactions,* 143: 341-351.

Vega-Avila, E. & Pugsley, M.K. 2011. An overview of colorimetric assay methods used to assess survival or proliferation of mammalian cells. *Proceedings of the Western Pharmacology Society,* 54(10): 10-14.

Veluri, R., Weir, T.L., Bais, H.P., Stermitz, F.R. & Vivanco, J.M. 2004. Phytotoxic and antimicrobial activities of catechin derivatives. *Journal of Agricultural & Food Chemistry,* 52(5): 1077-1082.

Verma, N. & Manna, S.K. 2016. Advanced glycation end products (AGE) potently induce autophagy through activation of RAF protein kinase and nuclear factor kappaB (NF-kappaB). *The Journal of Biological Chemistry,* 291(3): 1481-1491.

Villaño, D., Fernández-Pachón, M.S., Troncoso, A.M. & García-Parrilla, M.C. 2005. Comparison of antioxidant activity of wine phenolic compounds and metabolites *in vitro*. *Analytica Chimica Acta,* 538(1-2): 391-398.

Walle, T., Otake, Y., Brubaker, J., Walle, U. & Halushka, P. 2001. Disposition and metabolism of the flavonoid chrysin in normal volunteers. *British Journal of Clinical Pharmacology,* 51(2): 143-146.

Wang, L.-H., Zeng, X.-A., Wang, M.-S., Brennan, C.S. & Gong, D. 2018. Modification of membrane properties and fatty acids biosynthesis-related genes in *Escherichia coli* and *Staphylococcus aureus*: Implications for the antibacterial mechanism of naringenin. *Biochimica et Biophysica Acta (BBA)-Biomembranes,* 1860(2): 481-490.

Wang, Q., Wang, H. & Xie, M. 2010. Antibacterial mechanism of soybean isoflavone on *Staphylococcus aureus*. *Archives of Microbiology,* 192(11): 893-898.

Wang, W., Fu, X.W., Dai, X.L., Hua, F., Chu, G.X., Chu, M.J., Hu, F.L., Ling, T.J., Gao, L.P., Xie, Z.W., Wan, X.C. & Bao, G.H. 2017. Novel acetylcholinesterase inhibitors from Zijuan tea and biosynthetic pathway of caffeoylated catechin in tea plant. *Food Chemistry,* 237: 1172-1178.

Wang, Y. & Ho, C.-T. 2012. Flavour chemistry of methylglyoxal and glyoxal. *Chemical Society Reviews,* 41(11): 4140-4149.

Webster, J., Urban, C., Berbaum, K., Loske, C., Alpar, A., GÄrtner, U., De Arriba, S.G., Arendt, T. & Münch, G. 2005. The carbonyl scavengers aminoguanidine and tenilsetam protect against the neurotoxic effects of methylglyoxal. *Neurotoxicity Research,* 7(1-2): 95-101.

Wolfe, K.L. & Liu, R.H. 2007. Cellular antioxidant activity (CAA) assay for assessing antioxidants, foods, and dietary supplements. *Journal of Agricultural & Food Chemistry,* 55(22): 8896-8907.

Wolfe, K.L. & Liu, R.H. 2008. Structure− activity relationships of flavonoids in the cellular antioxidant activity assay. *Journal of Agricultural & Food Chemistry,* 56(18): 8404-8411.

Wondrak, G.T., Jacobson, M.K. & Jacobson, E.L. 2006. Antimelanoma activity of apoptogenic carbonyl scavengers. *Journal of Pharmacology & Experimental Therapeutics,* 316(2): 805-814.

Wu, C.-H. & Yen, G.-C. 2005. Inhibitory effect of naturally occurring flavonoids on the formation of advanced glycation endproducts. *Journal of Agricultural & Food Chemistry,* 53(8): 3167-3173.

Wu, D., Kong, Y., Han, C., Chen, J., Hu, L., Jiang, H. & Shen, X. 2008. D-Alanine: D-alanine ligase as a new target for the flavonoids quercetin and apigenin. *International Journal of Antimicrobial Agents,* 32(5): 421-426.

Wu, T., Zang, X., He, M., Pan, S. & Xu, X. 2013. Structure–activity relationship of flavonoids on their anti-*Escherichia coli* activity and inhibition of DNA gyrase. *Journal of Agricultural & Food Chemistry,* 61(34): 8185-8190.

Yang, B.-N., Choi, E.-H. & Shim, S.-M. 2017. Inhibitory activities of kaempferol against methylglyoxal formation, intermediate of advanced glycation end products. *Applied Biological Chemistry,* 60(1): 57-62.

Yang, L., Li, X., Wu, Z., Feng, C., Zhang, T., Dai, S. & Dong, Q. 2018. The inhibition of methylglyoxal-induced histone H1 Nε-carboxymethyllysine formation by (+)-catechin. *Journal of Agricultural & Food Chemistry*, 66(23): 5812-5820.

Yen, G.-C., Duh, P.-D., Tsai, H.-L. & Huang, S.-L. 2003. Pro-oxidative properties of flavonoids in human lymphocytes. *Bioscience, Biotechnology, & Biochemistry,* 67(6): 1215-1222.

Yu, Z., Kong, L. & Chen, Y. 2002. Antidepressant activity of aqueous extracts of *Curcuma longa* in mice. *Journal of Ethnopharmacology,* 83(1): 161-165.

Zhang, Y.-M. & Rock, C.O. 2004. Evaluation of epigallocatechin gallate and related plant polyphenols as inhibitors of the FabG and FabI reductases of bacterial type II fatty-acid synthase. *Journal of Biological Chemistry,* 279(30): 30994-31001.

Zheng, X., Meng, W.D., Xu, Y.Y., Cao, J.G. & Qing, F.L. 2003. Synthesis and anticancer effect of chrysin derivatives. *Bioorganic & Medicinal Chemistry Letters,* 13(5): 881-8844.

Zhou, L., Zhang, P., Yang, G., Lin, R., Wang, W., Liu, T., Zhang, L. & Zhang, J. 2014. Solubility of chrysin in ethanol and water mixtures. *Journal of Chemical & Engineering Data,* 59(7): 2215-2220.

Zhu, Z., Cheng, F. & Chen, J. 2013. Investigation of effects of carbon coating on the electrochemical performance of Li 4 Ti 5 O 12/C nanocomposites. *Journal of Materials Chemistry A,* 1(33): 9484-9490.

Zulueta, A., Esteve, M.J. & Frígola, A. 2009. ORAC and TEAC assays comparison to measure the antioxidant capacity of food products. *Food Chemistry,* 114(1): 310-316.

Appendix

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.
- FWA 00002567, Approved dd 22 May 2002 and Expires 03/20/2022.
- IRB 0000 2235 IORG0001762 Approved dd 22/04/2014 and Expires 03/14/2020.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
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Faculty of Health Sciences Research Ethics Committee

1/03/2018

Approval Certificate
New Application

Ethics Reference No: 67/2018

Title: The effects of the trapping of methylglyoxal by flavonoids on antioxidant and antibacterial activity.

Dear Refilwe Joy Ndalane

The **New Application** as supported by documents specified in your cover letter dated 30/01/2018 for your research received on the 30/01/2018, was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 28/02/2018.

Please note the following about your ethics approval:
- Ethics Approval is valid for 3 years
- Please remember to use your protocol number (**67/2018**) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, or monitor the conduct of your research.

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

We wish you the best with your research.

Yours sincerely

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

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health).

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