THE TRAPPING OF METHYLGLYOXAL BY PHENOLIC ACIDS: EFFECT ON ANTIOXIDANT AND ANTIBACTERIAL ACTIVITY

By

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Abstract

Methylglyoxal (MGO) is a reactive carbonyl species found in Manuka honey reported to cause advanced glycation end products (AGE) formation. AGE's increase the risk for hyperglycaemia resulting in neuropathy, arteriosclerosis, retinopathy and Alzheimer's disease. Phenolic acids such as pyrogallol (PY) are known to trap MGO, lessening the harmful effects of MGO as an AGE precursor. However, MGO is also a very effective antibacterial agent therefore; its trapping could have negative side effects. Manuka honey contains both phenolic acids such as gallic acid (GA), caffeic acid (CA) as well as MGO and it is unknown whether trapping of MGO by phenolic acids reduces the antioxidant activity of phenolic acids or the antibacterial activity of MGO.

Phenolic acids PY, GA and CA were combined with MGO in a 1:1 and 1:2 ratio. The trapping of MGO with polyphenolic acids was determined with Liquid chromatography-mass spectrometry (LCMS). Total polyphenolic acids (TPC) was determined with the TPC assay. Antioxidant activity was determined with 2,2-diphenyl-2-picrylhydrazyl (DPPH), Trolox equivalent antioxidant capacity (TEAC) and Oxygen Radical Absorbance Capacity (ORAC) assays. The effect on cell number and viability was determined with crystal violet and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays on Caco-2 and SC-1 cells. Cellular antioxidant activity was determined with Dichlorodihydrofluorescein diacetate assay. Lastly, antibacterial activity was determined with the turbidity assay on Gram positive *B. subtilis* and Gram negative *E. coli* and the ultrastructural morphology of *B. subtilis* was further investigated with scanning electron microscopy.

PY was the only phenolic acid used with trapping ability, forming mono- and di- adducts with MGO reported with the LCMS results, resulting in a decrease in TPC and antioxidant activity measured with the DPPH assay. GA did not show any alteration when combined with MGO at 1:1 and 1:2 ratio in all antioxidant content and activity assays. The antioxidant content of CA in combination with MGO was decreased, although its antioxidant activity (DPPH) was increased at 1:2 ratio.

The antioxidant activity measured with the ORAC assay was increased with PY and CA combined with MGO. TEAC assay did not show any changes when phenolic acids were combined with MGO a 1:1 and 1:2 ratio. The cytotoxicity of phenolic acids combined with MGO did not cause a change in cell number or viability of SC-1 and Caco-2 cells. MGO and phenolic acids alone and in combination did no cause oxidative damage (without 2,2'-Azobis(2-

amidinopropane) dihydrochloride (AAPH). All phenolic acids in combination with MGO retained the ability to reduce AAPH induced oxidative damage.

The polyphenolic acids showed minor inhibition of the growth of *B. subtilis* and *E. coli.* PY only reduced the antibacterial activity of MGO at a 1:1 combination of *B. subtilis.* GA and CA did not alter the antibacterial activity of MGO when combined at 1:1 or 1:2 ratio.

This study showed that phenolic acids with the ability to trap MGO can be altered by the mono- and di-MGO adduct formation, altering its antioxidant activity and can further alter the antibacterial activity of MGO.

Keywords: Wound healing, Pyrogallol, Caffeic acid, Gallic acid, Methylglyoxal, Mono-adduct, di-adduct, Antioxidant, Antibacterial, scanning electron microscopy.

Declaration

I, Magalli Marcelline Magnoumba Legnanga declare that this research dissertation is my own work and has not been presented for any degree of another University.

Signed.....

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%	Percentage
°C	Degrees centigrade
3-DG	3-Deoxygucosulose
µg	Micrograms
µg/ml	Microgram per milliL
µl	MicroLs
µM	Micromolar

A

A10	Rat thoracic aorta cell line
A. baumannii	*Acinetobacter baumannii*
AAPH	2,2'-Azobis(2-amidinopropane) dihydrochloride
ABTS	2,2'-Azo-bis (3-ethylbenzothiazoline-6-sulfuric acid) diamonium salt
ADF	Glioblastoma cell line
Ag	Silver
AGEs	Advanced glycation end products
AGE-Alb	Advanced glycation end product - albumin
$AlCl_3$	Aluminium chloride
ANOVA	Analysis of variance
Arg	Arginine
ATCC	American Type Culture collection
AUC	Area under the curve

B

B. subtilis	*Bacillus subtilis*
BSA	Bovine serum albumin
BSO	Buthionine sulfoximine

C

Caco-2	Human colon adenocarcinoma cell line
CA	Caffeic acid
CH_3OH	Methanol

CO_2	Carbone dioxide
CONS	Coagulase-negative *S. aureus*
COOH	Carboxylic acid
CRAB	Carbapenem resistant *Acinetobacter baumannii*
CRE	Carbapenem resistant *Enterobacteriaceae*
CV	Crystal violet
Cys	Cysteine

D

DCFH-DA	Dichlorofluorescein diacetate
DCF	Dichlorofuorescein
DDC	Diethyldithiocarbamate
ddH_2O	Double distilled water
DIMC	Dimethoxycurcumin
DMEM	Dulbecco's modified essential medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DPI	Diphenylene iodonium
DPPH	2, 2-Diphenyl-2-picrylhydrazyl

E

E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetra acetic acid ($C_{10}H_{16}N_2O_8$)
EGCG	Epigallocatechin gallate
EPM	Extracellular polysaccharide matrix
EPS	Extracellular polysaccharide matrix
ET	Electron transfer
EtOH	Ethanol

F

FCS	Foetal calf serum
F-C reagent	Folin-Ciocalteu's reagent
FDA	Fluorescein diacetate

FRAP	Ferric reducing antioxidant power

G

G	Gram
G100	100% Growth
g/kg	Gram per kilogram
g/L	Gram per L
g/mol	Gram per mole
GA	Gallic acid
GAE	Gallic acid equivalents
GAE/kg	Gallic acid equivalents per kilogram
GO	Glyoxal
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
GSSG	Glutathione oxidized

H

h	Hour/hours
H_2O	Water
H_2O_2	Hydrogen peroxide
H. pylori	*Helicobacter pylori*
H. streptococci	*Hemolytic streptococci*
HAT	Hydrogen Atom Transfer
HDMS	Hexamethyldisilazane
HMF	5 Hydroxymethylfurfural
HPLC	High performance liquid chromatography
HO^-	Hydroxyl ion
HT	Hydroxytyrosol

I

isoPBS	Isotonic saline buffered PBS

K

$K_2S_2O_8$	Potassium peroxidisulfate
K. pneumonia	*Klebsiella pneumonia*
KH_2PO_4	Potassium dihydrogen phosphate
KCl	Potassium chloride

L

L	Litre
L. monocytogenes	*Listeria monocytogenes*
L-NAME	Nitro-L-arginine methyl ester
LASEC	Laboratory Scientific Equipment Company
LB	Luria Bertani
Lys	Lysine

M

M	Molar
mg	Milligrams
mg/kg	Milligram per kilogram
mg/L	Milligram per L
mg/ml	Milligram per milliL
ml	MilliL
ml/min	MilliL per minute
mm	Millimetre
mM	Millimolar
MW	Molecular weight
Max	Maximum
MCP-1	Monocyte chemotactic protein-1
MDRO	Multidrug resistant organisms
MDRP	Multidrug-resistant *P. aeruginosa*
MGO	Methylglyoxal
MIC	Minimum inhibitory concentration
Min	Minute
mRNA	Messenger ribonucleic acid

MRP	Maillard reaction products
MRSA	Methicillin resistant *Staphylococcus aureus*
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

N

nm	Nanometre
NAC	N-acetyl-cysteine
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
$NaHCO_3$	Sodium hydrogen carbonate/sodium bicarbonate
NaH_2PO_4	Sodium hydrogen phosphate
$Na_2HPO_4.2H_2O$	Sodium phosphate dibasic dihydrate
NaOH	Sodium hydroxide
NaP buffer	Nucleic acid preservation buffer
NO	Nitric oxide

O

O^-	Singlet oxygen
O_2	Oxygen
$O_2^{\cdot -}$	Superoxide ion
$O_2^{\cdot 2-}$	Peroxide anion
OD	Optical density
OH^-	Hydroxyl radical
OH	Hydroxyl group
ORAC	Oxygen radical absorbance capacity

P

P. aeruginosa	*Pseudomonas aeruginosa*
PBS	Phosphate buffered saline
pH	Logarithmic scale for the measurement of the acidity or alkalinity of an aqueous solution
P-value	Probability value
PY	Pyrogallol

R

r	Correlation
r^2	Coefficient of determination
RAGE	Receptor of advanced glycation end products
Rt	Retention time
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species

S

S. aureus	*Staphylococcus aureus*
S. pyogenes	*Streptococcus pyogenes*
S. typhimurium	*Salmonella typhimurium*
SC-1	Mouse embryonic fibroblast cells
SEM	Scanning electron microscopy
SH	Thiol group
SHR	Spontaneously hypersensitive rats
SH-SY-5Y	Neuroblastoma cell line
SOD	Superoxide dismutase

T

TE	Trolox equivalent
TEAC	Trolox equivalent antioxidant capacity
TIME	Time management Infection control Moisture imbalance and Epithelial advancement
TFC	Total flavonoid content
TPC	Total polyphenol content

U

U937	Macrophage derived cell line
UMF	Unique Manuka factor

V

v/v	Volume to volume
VEGF	Vascular endothelial growth factor
Vit A	Vitamin A/Retinol
Vit B3	Vitamin B3/Niacin
Vit C	Vitamin C/Ascorbic acid
Vit E	Tocopherol
VRE	Vancomycin resistant *Enterococci*
VRSA	Vancomycin resistant *S. aureus*
VSMC	Rat mesenteric artery smooth muscle cells

W

w/v	Weight to volume
w/w	Weight to weight
WKY	Wistar Kyoto rats

X

xg	Centrifugal force

1 CHAPTER 1: INTRODUCTION

Most wounds heal by themselves without requiring any intervention. In acute wounds (traumatic loss of tissue or a surgical procedure), the healing pathway is normal and results in the restoration of both function and structure of tissues. In chronic wounds (leg or foot ulcers, diabetic wounds and pressure sores, arterial and venous insufficiency and vasculitis), wound healing does not occur normally and the healing process is adversely affected by various factors which include necrosis, tissue hypoxia, high levels of inflammatory cytokines and infections. In such situations, the inflammation phase is longer, provoking a cascade of tissue responses that maintain a non-healing state (Lazarus *et al*., 1994).

Wound healing therapy involves cleaning and disinfection of the wound, reduction of bleeding and surgical debridement if required and then lastly, the wound is covered with a topical dressing. Ideal wound dressings must ensure bacterial eradication, prevent biofilm formation and reduce inflammation while promoting cellular regrowth (Soneja *et al*., 2005).

Besides prevention or eradication of infection, the key to successful wound healing is the reduction of inflammation and promotion of cellular regrowth. In the process of wound healing inflammatory cells like neutrophils, macrophage (phagocytes), endothelial cells and fibroblasts produce large amounts of pro-oxidants (e.g. superoxide ion) by the phagocytic isoform of NADPH oxidases. During wound healing, formed reactive oxygen species (ROS) play an important role as cell activation molecules (Soneja *et al*., 2005). However, a strict balance between oxidants and antioxidants must be maintained, as excessive amounts of ROS can cause cellular damage.

In vitro and *in vivo* research has shown that honey has antibacterial activity (Alzahrani *et al*., 2012; Armos, 1980; Bogdanov *et al*., 2008; Cooper & Molan, 1999). These effects are due to the high osmolarity (sugar content) of honey that limits bacteria growth (Molan, 1992). Secondly the presence of hydrogen peroxide (H_2O_2) in some honeys contributes to the antibacterial activity of these honeys, however, at high concentrations can cause tissue damage due to ROS formation (Roth *et al*., 1986). Manuka honey has been reported to have antibacterial activity against a wide range of bacteria including bacteria resistant to other treatments (Pieper, 2009; Stewart *et al.,* 2014). In Manuka honey, antibacterial activity is directly linked to the presence of methylglyoxal (MGO). MGO effectively kills *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*) (Talukdar *et al*., 2009) as well as methicillin and oxacillin resistant *S. aureus* (Stewart *et al*., 2014). Kilty *et al*., (2011) reported that MGO was also effective against biofilms of *Pseudomonas aeruginosa* (*P. aeruginosa*), *S. aureus* as

well as methicillin-resistant *S. aureus* although effective concentrations were several folds greater than required for plantonic bacteria (Kilty *et al*., 2011). This has resulted in the development of Manuka honey as a wound healing product that effectively clears up infections, including abscesses, surgical wounds, traumatic wounds, burns and ulcers. Manuka honey is classified according to its unique Manuka factor (UMF) (Alvarez-Suarez *et al*., 2014).

However, MGO is a highly reactive dicarbonyl that can modify rapidly via a non-enzymatic reaction with the free amino groups of lysine (Lys) and arginine (Arg) residues of proteins and peptides. This leads to the formation of advanced glycation end products (AGEs) (Alvarez-Suarez *et al*., 2014) that has an adverse effect on protein, nucleic acid and membrane function thereby adversely affecting normal cellular and tissue function.

Manuka honey in addition to MGO also contains polyphenols such as phenolic acids and these molecules contribute to the antibacterial and antioxidant activity of honey. Recent research has shown that polyphenols including phenolic acids can bind MGO, reduce MGO levels, and limit AGE formation. Polyphenols, such as pyrogallol, 1,2,4-benzenetriol, 1,3,5-trihydroxybenzen and 2.4.6-trihydroxybenzoic acid bind or trap MGO (Wang & Ho, 2012; Lo *et al.,* 2011).

Although there are several phenolic acids that are able to trap MGO, little is known about the consequence of this trapping on the antioxidant activity of phenolic acids as well as the antibacterial activity of MGO. Therefore, the aim of this study is to determine if trapping of MGO by phenolic acids reduces the antioxidant activity of phenolic acids while reducing the cellular toxicity and/or antibacterial activity of MGO.

2 CHAPTER 2: LITERATURE REVIEW

2.1 Wounds and wound healing

A wound is defined as an injury occurring in living tissue caused by an impact resulting in a cut or broken skin. Four phases of wound healing have been identified and these are haemostasis, inflammation, proliferation and remodelling.

Haemostasis occurs immediately, before proper healing responses start. Initially the platelets seal the damaged blood vessels by forming a platelet plug, and the blood vessels constrict. The platelets secrete factors and interact with the intrinsic clotting cascade and through this process fibrinogen is converted to fibrin. Fibrin aids and strengthens platelet aggregation. In the final stage of homeostasis, the platelets secrete growth factors and recruit neutrophils as well as monocytes, which initiate the next phase of wound healing, inflammation (Kerstein, 1997).

Inflammation is associated with erythema, warmth and swelling. In this phase neutrophils phagocytise microorganisms and debris and provides the first line of defence against infection. The fibrin is degraded and epithelial and fibroblast cell migrate into the wound site. Monocytes undergo extracellular matrix mediated differentiation into macrophages as these cells migrate from the blood vessels into tissue. Macrophages provide the second line of defence by phagocytising bacteria. These cells also secrete cytokines and growth factors required for the next phase of wound healing. In normal acute wounds, inflammation lasts four days post injury (Wahl & Wahl, 1992).

Proliferation is the third phase of wound healing and occurs from the fourth day until about the twenty-first day, and generally involves the replacement of damaged tissue. Fibroblasts secrete collagen, for regeneration of dermal tissue and pericytes re-establish the vascular network mediated by cytokines. The basal layer of keratinocyte divide, differentiate and re-establish the epithelial layer. The last phase and longest phase, can take up to two years, and involves fibroblast associated collagen remodelling and rearrangement to provide greater tensile strength (Heather *et al*., 2013).

Wounds are classified according to the time taken to heal. Acute wounds heal properly within 30 days and all phases of wound healing are as described above. Chronic wounds do not follow the normal phases of wound healing and characteristic of chronic wounds is that certain phases of wound healing are prolonged. Factors causing this delay in wound healing are necrosis, tissue hypoxia, high levels of inflammatory cytokines and infection. The outcome of

chronic wound healing is poor restoration of function and tissue structure and often these wounds are subject to relapse (Velnar *et al*., 2009).

2.2 Role of ROS in wound healing

For cellular proliferation and remodelling to occur there needs to be an infection free wound, with a cellular environment with reduced ROS levels and sufficient oxygenation and growth factors (Soneja *et al.,* 2005).

ROS is defined as oxygen species with higher reactivity than molecular oxygen, and includes $O_2^{\cdot-}$, hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-) and singlet oxygen (O^-). In a biological milieu, molecular oxygen can be reduced into superoxide anion ($O_2^{\cdot-}$), hydroxyl ion (HO^-), and peroxide anion ($O_2^{\cdot 2-}$). These oxidants can cause cellular oxidative damage. The term “redox homeostasis” is used when the cells prevent the accumulation of these oxidants, maintaining cellular homeostasis.

ROS plays an important role in the healing process. During coagulation, ROS is involved in platelet recruitment and activation, which leads to the formation of clots and also the release of cytokines and various growth factors required for the initiation of wound healing. In addition, ROS also promotes the process of re-epithelization with the activation of collagenase expression that leads to the degradation of the extracellular matrix leading to the migration of wound associated cells (Soneja *et al.,* 2005). Oxidants aslo provide signalling and defence against microorganisms, although levels must be tightly controlled to prevent oxidative stress and subsequent cellular damage (Sen, 2003). In the process of wound healing inflammatory cells like neutrophils, macrophage (phagocytes), endothelial cells and fibroblasts produce large amounts of pro-oxidants (e.g. $O_2^{\cdot-}$), through the phagocytic isoform of nicotinamide adenine dinucleotide phosphate (NADPH) oxidases. In acute wounds, the redox homeostasis is maintained by antioxidant enzymes, molecules such as GSH and endogenous dietary sources of antioxidants (Droge, 2002). In contrast, in chronic wounds, due to an extended inflammatory phase there is an uncontrolled and persistent production of ROS. In diabetic wound complications (which are considered chronic wounds due to the extensive time the wounds take to heal), oxidative stress is believed to be an important pathogenic factor (Soneja *et al.,* 2005).

2.3 Infection and wound healing

The main objective in the management of wound healing is to avoid any infections. Acute wounds heal easily and usually by themselves via the normal healing process. In contrast, chronic wounds take a long time to heal (Flanagan, 2003). Once the skin is injured the microorganisms normally on the skin can access the underlying tissues. The wound can be classified according to the state of infection and replication status of the microorganisms. Classification of infection includes contamination, colonization, local infection/critical colonization, and spreading invasive infection (Guo & DiPietro, 2010). Contamination refers to the presence of non-replicating organisms and colonization is defined as the presence of replicating microorganisms' adherent to the wound without causing tissue damage. Critical colonization is the transition phase between microorganism replication and local tissue responses. Finally, characteristic of an invasive infection is the presence of replicating organisms within a wound that cause tissue and host injury. The colonizing organisms progress to invade the tissues and the observed effect is dependent on the number of microbial host interactions, the amount of bacteria per gram of tissue, the virulence and pathogenicity of the organism and the ability of the host to respond with an immune response (Edwards & Hardings, 2004). It has been determined that 105 microorganisms per gram tissue results in wound infection and poor wound healing. Poly-microbial interactions also play an important role (i.e. less invasive microorganisms can be synergistic with more virulent forms, causing greater infection).

There are different types of bacteria that adversely affect wound healing and bacteria commonly found in wounds include *P. aeruginosa*, *S. aureus*, and *β-haemolytic streptococci* (Edwards & Harding, 2004). Depending on the extent of infection, these bacteria can cause significant wound deterioration and can occur as biofilms, which are defined as complex communities of aggregated bacteria embedded in a self-secreted extracellular polysaccharide matrix (EPM). Mature biofilms have a more protected environment within the EPM with channels for nutrients and waste products transfer and the EPM physically shields the bacteria from phagocytic activity. The EPM also increases their resistance against conventional antibiotic treatment (Edwards & Harding, 2004; Guo & DiPietro, 2010).

The chronicity in wounds begins with a persistent level of bacteria in tissue. These bacteria stimulate a prolonged inflammation phase, which results in extracellular matrix degradation and inhibition of re-epithelisation (Flanagan, 2003). Although bacteria can impair wound healing their presence in normal wounds is important for the inflammatory response. Clinically non-infective levels of bacteria accelerate the healing process with formation of granulation

tissue associated with increased neutrophil, monocyte, macrophage levels and increased collagen formation (Edwards & Harding, 2004). However, wounds do provide a favorable environment for microorganism colonization and therefore it is important to create an environment unfavourable for microorganism growth and biofilm formation, to aid in wound healing.

2.4 Wound dressings

There are different types of wound dressings for better healing of chronic wounds, including, silver (Ag). These Ag-containing creams are favoured topical ointments for large burns, marketed as Flamazine™ and Silvazine™ are used to treat and prevent infection in skin wounds by the slow release of silver that kill microbes by having multiple sites of antimicrobial action on target sites. Wound dressing containing slow release of Ag compounds include Silverton™, Actisorb™ and Acticoat™ (Silver *et al*., 2006). Hydrocolloids wound dressings, such as Comfeel Plus™, are suitable for necrotic, granulating and epithelising wounds and are effective treatment against pseudomonas. Hydrogel based wound dressings (Purilon™) are used for debridement of necrotic and sloughy wounds. Alginate wound dressings, contain mannuronic and guluronic acid residues forming a gel that provides a moist wound healing environment. This type of wound dressing is an effective haemostatic agent, used on bleeding wounds. Polyurethane foam wound dressings (Biatain™), are absorbent dressings with good fluid control. These are used in the granulation or epitheliasation of wounds with light to heavy exudate (Purser, 2007). Honey has been recently incorporated into wound dressings, an example of honey based dressing is Medihoney (leptospermum species). There are aslo various types of honey based wound dressings, such as Medihoney, Actilite (Manuka honey and Manuka oil) and Therahoney sheet dressings (Manuka honey).

As the focus of this study is the interaction between phenolic acids and MGO both found in honey the antioxidant and antibacterial effects of honey and the bioactivity of these constituent molecules will be discussed in greater detail.

2.5 Honey

Honey is a natural sweetener and has been used for both nutritional and medical purposes throughout the years. Honey has been used in the healing treatment of wounds, burns and infections (Bogdanov *et al*., 2008). It is produced by bees from plant nectar, extractions of plant sucking insects and plant secretions (Alvarez-Suarez *et al*., 2014). Honey is mainly constituted of carbohydrates (95%), however, also contains compounds such as organic

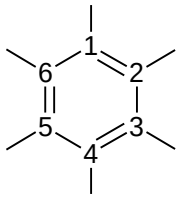
acids, amino acids, proteins, vitamins, minerals, aroma and polyphenols. Some of these polyphenols and their structures are summarised in Table 2.1. There are different types of honey, and their compositions are dependent on the botanical origin of the honey (Bogdanov *et al*., 2008). Honey is classified into four categories: blossom honey, which is obtained from the nectar of the flower; honeydew honey which is produced by bees after the honeydew collection, monofloral honey, where the bee forages mostly on one type of plant, and multifloral or polyfloral honey which has many botanical sources, none of which is predominant. Examples of the latter include forest and meadow blossom honey (Alvarez-Suarez *et al*., 2014). Honey contains several compounds, and its complex composition has been shown to be responsible for its various therapeutic activities that include antioxidant and antibacterial activity. The antioxidant activity is predominantly due to the presence of polyphenols while MGO is the main component that contributes to the antibacterial

2.5.1 Polyphenols as antioxidants

Major dietary sources of antioxidants are the polyphenols which consist of phenolic acids, flavanoids, stilbenes, and lignans. Phenolic acids can be divided into two different classes. The first is hydrobenzoic acids, which include gallic acid (GA), found mostly in tea and red fruits. The second class is hydroxycinnamic acids that are more common and include mainly p-coumaric and caffeic acid (CA) which are mostly found in coffee and fruits, such as kiwis and cherries (Tomas-Barberan, 2000). Of the flavanoids, flavonols are the most common and includes quercetin and kaempforol, mostly found in onions, broccoli and blueberries. Flavones are less common than the flavonols e.g. glycosides (luteolin) and are mostly found in parsley and celery. Flavanones include mainly aglycones (naringenin) and are found in grapefruit and tomatoes. Isoflavones, which include mostly genistein, are found mainly in soya and its processed products (Coward *et al.,* 1998). Flavanols can be divided into two groups, the catechins, found in red wine, chocolate and in green tea and the proanthocyannins found in cider, beer and peaches (Arts & Van De Putte, 2000). Lastly, the anthocyannins including cyanidin, are mostly found in cabbage, beans, and aubergines. Lignans are the third type of polyphenols and are mostly found in linseed, but are also found in small amounts in garlic and asparagus. Stilbenes are very rare, found in low quantities in red wine (Manach *et al.,* 2004). Polyphenols differ in structure. The phenolic acids have the simplest structure composed of one aromatic ring with a hydroxyl and carboxyl group attached (positions C_6 and C_1), Table 2.1.

The antioxidant activity of phenolic acids depends on their molecular structure, Table 2.1 & 2.2, that is, on the availability of phenolic hydrogens and on the possibility for stabilization of the resulting phenoxyl radicals formed by hydrogen donation (Rice-Evans *et al.,* 1996). In the cinnamic group, research has shown increased efficacy due to the presence of the catechol group. They exert their antioxidant activity by increasing the number of hydroxyl groups binding to the aromatic group. PY and GA with three hydroxyl groups have excellent antioxidant activity (Table 2.2) compared to 3-hydroxybenzoic acid with one hydroxyl group. The position of the hydroxyl groups bound to the aromatic ring also determines the antioxidant activity of phenolic acids. Highest antioxidant activity is found for ortho (C_1 and C_2 as well as C_1 and C_6) and para (C_1 and C_4) arrangements of the hydroxyl groups on the aromatic ring (Sroka, 2005). The hydroxyl group arrangement for PY is ortho and that for GA and CA is para (Sroka & Cisowski, 2003).

Table 2.1: Structure of some phenolic acids (Sroka, 2005)



Phenolic acids	Position				
	1	2	3	4	5
Caffeic acid	-CH=CH-COOH		-OH	-OH	
Pyrogallol	-OH	-OH	-OH		
Gallic acid	-COOH		-OH	-OH	-OH
3,5-Dihydroxybenzoic acid	-COOH		-OH		-OH
***p*-Couramic acid**	-CH=CH-COOH			-OH	
Ferrulic acid	-CH=CH-COOH		$-CH_3O$	-OH	
Salicylic acid	-COOH	-OH			
3,4-Dihydroxyphenylacetic acid	$-CH_2$-COOH		-OH	-OH	
3-Hydrobenzoic acid	-COOH		-OH		
Gentistic acid	-COOH	-OH			-OH
Syringic acid	-COOH		$-CH_3$	-OH	$-CH_3$

Table 2.2: Strong and weak antioxidative activity of phenolic acids (Sroka &Cisowski, 2003).

Strong antioxidant activity		Weak antioxidant activity	
Compound	**S_r (%)[a]**	**Compound**	**S_r (%)[a]**
Gallic acid	75 ± 2	**p-Couramic acid**	0.25 ± 0.01
Pyrogallol	79.5 ± 0.6	**4-Hydroxyphenylacetic acid**	0.11 ± 0.07
Caffeic acid	44 ± 0.5	**3-Hydroxybenzoic acid**	0.07 ± 0.15

[a] Antiradical activity of phenolic acids (S_r) measured with DPPH. Concentration of compounds in the sample was 0.0028 mg/ml

Flavanoids have a more complex structure, and consist of 2 aromatic rings and 1 heterocyclic ring with hydroxyl and carboxyl groups (C_6-C_3-C_6). This difference in structure is well known to be linked with their ability to either have more or less antioxidant activity. All flavanoids with the exception of isoflavones, have the same basic structure, composed of a flavanone nucleus with two aromatic rings formed with fifteen carbon atoms (A and B) interconnected with an heterocyclic C composed of three carbon atoms and one oxygen atom, Figure 2.1. Flavanoids can be modified via glycosylation, hydroxylation and alkylation, and are further classified into different groups. The position of hydroxylation and the state of their polymerisation can lead to further differentiation (Halliwell, 1995).

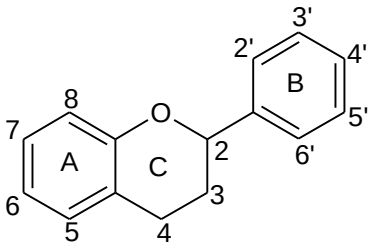
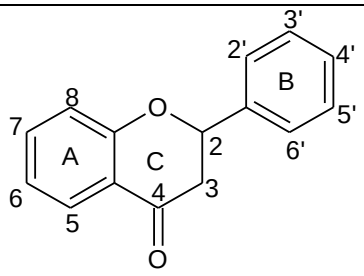


Figure 2.1: The flavonone nucleus (Halliwell, 1995).

The antiradical activity of flavanoids depends on many factors, the extent of glycosylation where blocking of the hydroxyl group at position 3 of the C-ring decreases antioxidant activity. The more hydroxyl groups bound to the aromatic B-ring the greater the antioxidant activity. The presence of a 2,3-double bond in the C-ring increase antioxidant activity while no group bonding or the presence of a methoxy group at the B-ring makes the flavonoid ineffective against free radicals, Table 2.3 & 2.4 (Burda & Oleszek, 2011). As shown in Table 2.4, substitution on the C_3 position reduces the antioxidant activity of several flavanoids.

Table 2.3: Structures of some flavanoids (Shadidi & Wanasundara, 1992).



Flavanoid	Position						
	3	5	7	2’	3’	4’	5’

Quercetin	-OH	-OH	-OH		-OH	-OH	
Flavanoid	**Position**						
	3	**5**	**7**	**2'**	**3'**	**4'**	**5'**
Kaempferol	-OH	-OH	-OH			-OH	
Kaempferide	-OH	-OH	-OH			$-OCH_3$	
Myricetin	-OH	-OH	-OH		-OH	-OH	-OH
Apigenin		-OH	-OH				
Laricytin-3,7,3'-*O*-triglucoside	-O-Glc	-OH	-O-Glc		$-OCH_3$	-OH	-O-Glc
Chrysin		-OH	-OH				
Taxifolin	-OH	-OH	-OH		-OH	-OH	
Narirutin		-OH	-O-Glc-Rha			-OH	

Table 2.4: Strong and weak antioxidative activity of some flavanoids (Burda & Oleszek, 2011).

Strong antioxidative activity		**Weak antioxidative activity**	
Compound	A_n(%)[a]	**Compound**	A_n (%)[a]
Kaempferol	65.3	**Laricytrin 3,7,3'-O-triglucoside**	-6.2
Galangin	64.9	**Quercetin3-O-glucoside-7-O-rhamnoside**	-6.2
Quercetin	63.6	**Rutin**	-10.2
Murin	63.5	**Kaempferol 3,7-O-dirhamnoside**	-17.5
Robinctin	61.7	**Apigenin**	-78.8

[a] Antioxidant nutrient percentage

Flavanoids exert their protective effect through three different mechanisms, free radical trapping, enzyme inhibition and metallic ion chelation. These effects are dependent on the degree of substitution and saturation. In free radical trapping, the flavanoids are oxidised by the radicals (R^*), stabilising the radical species as it donates/removes hydrogen, forming a flavonoxy radical (flavonoid (O^*)) which is stabilised by resonance, Figure 2.2.

$$\textbf{Flavanoid (OH) + } R^* \rightarrow \textbf{Flavonoid } (O^*) \textbf{ + RH}$$

Figure 2.2: Flavanoid stabilising free radicals (Sanhueza *et al.,* 1992)

Xanthine dehydrogenase (XD) is an enzyme involved in the production of free radicals and during cellular re-oxygenation, XD coverts molecular oxygen into the superoxide radical, $O_2^{\cdot-}$ Flavanoids inhibit xanthine oxidase (oxygenation form of XD) and traps $O_2^{\cdot-}$ (Sanhueza *et al.,* 1992). Metal ions such iron (Fe^{2+}) and copper (Cu^+) are essential for some of the physiological

function of cells, however these metal ions also catalyse the reduction of H_2O_2 to the hydroxyl radical as shown in Figure 2.3 (Van Acker *et al.,* 1995). This is known as the Fenton reaction.

$$\mathbf{H_2O_2 + Fe^{2+}(Cu^{+}) \rightarrow OH^{-} + OH^{-} + Fe^{3+}\ (Cu^{+})}$$

Figure 2.3: Reduction of (OH) into hydroxyl radical (Van Acker *et al.,* 1995).

Flavanoids are able to form a stable complex with transition metals such as Fe^{3+}, Cu^{2+}, Zn^{2+} and Al^{3+}. The stability of the complex depends on the flavonoid structure especially the presence of the catechol and pH, Figure 2.4 (LeNest *et al.,* 2004).

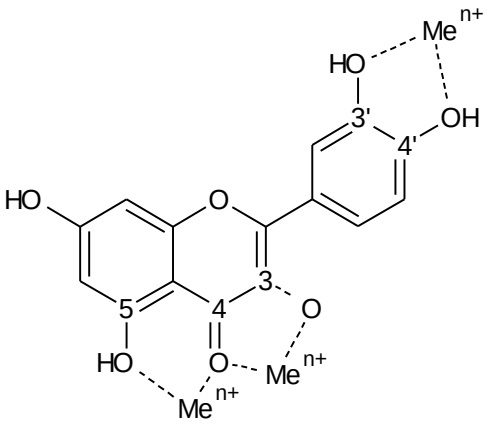


Figure 2.4: Chelation of flavanoids (LeNest *et al.,* 2004).

2.5.2 Honey as a source of polyphenols with antioxidant activity

Honey is a rich source of polyphenols and the type and content is dependent on the geographical region and floral source. Many honey types including honeys from southern Australia that are dark in colour is due to their high polyphenol content and have excellent antioxidant properties (Alvarez-Suarez *et al*., 2014). Honey was tested for its influence on the antioxidative capacity of plasma, in two studies. In the first study by Schramm *et al*., (2003) subjects were given maize syrup or buckwheat honeys in a dose of 1.5 g/kg body weight, this was compared to the sugar control (maize syrup). Honey showed an increase in both serum antioxidant capacity and antioxidant content. In the second study by Al-Waili (2003), it was shown that a daily honey serving of 1.2 g/kg supplemented in the diet increased plasma and cellular levels of antioxidants. Levels of vitamin C, β-carotene, glutathione reductase and uric acid were also increased by 47%, 3%, 7% and 12%, respectively.

The reported antioxidant and anti-cancer effects of Manuka honey are due to the presence of phenolic acids (CA, p-couramic acid, GA) and flavanoids (chrysin, apigenin and genistein) (Alvarez-Suarez *et al*., 2014). Alzahrani *et al*., (2012) found that Manuka honey compared to Acacia, Wild carrot and Portobello honeys contains the highest phenolic and antioxidant capacity. Manuka honey was also evaluated in its role against oxidative damage in an *in vivo* model, using young and middle-aged groups of rats. It was observed that Manuka reduced DNA damage and glutathione peroxide activity in the liver. This was due to the modulation of antioxidant enzyme activity by polyphenols (Jubri *et al*., 2013).

2.5.3 Honey and antibacterial activity

Ancient empires such as Serbia, China and Greece used moulds, soil and plants to treat bacterial infection while the Egyptians used wheatened bread applied directly to the infected site. Scientific endeavour has led to the isolation and characterisation of new antibiotics and these included the discovery of an antiseptic protein lysozyme and the isolation of penicillin from mould such as *Penicillium spp* by Sir Alexander Fleming. Penicillin specifically targets the synthesis of the cell wall of several Gram positive pathogens. In addition, several plant species were identified as important sources of novel antibiotic agents and these molecules served as lead compounds for the development of new antibiotics. A typical example is the coumarins, which have antioxidant activity and are being developed as potential antibacterial drugs. These compounds have shown good antibacterial activity against *E. coli, P. aeruginosa* and *Bacillus subtilis* (*B. subtilis*) (Wang *et al.,* 2014).

Antibacterial agents can be classified either as non-antibiotics or antibiotic (Tripathi, 1994). The non-antibiotic agents that are generally used to prevent infection and are used to create a bacteria free environment so that normal wound healing can occur.

Antibiotic agents are classified according to certain activities where broad based antibiotics are active against both Gram positive and Gram negative bacteria. Examples are the tetracyclines and fluoroquinolones. A narrow spectrum antibacterial refers to an antibacterial agent with limited effect against particular types or species of bacteria (Sanchez *et al*., 2006). Antibiotics have either a bactericidal or bacteriostatic effect. Bactericidal compounds such as aminoglycosides kill bacteria while bacteriostatic compounds such as the tetracyclines inhibit bacterial growth and replication. In addition, some antibiotics act as both bactericidal and bacteriostatic, depending on the state of the bacteria and also duration of exposure for example fluoroquinolones. Generally, antibiotics can be classified according to their chemical structure or mode of action.

The classification based on the mode of antibacterial action is according to specific cellular or biochemical targets. Antibiotics such as penicillin are inhibitors of cell wall synthesis while polymixin B disrupts the structure of the cell membrane (Sanchez *et al*., 2006). Antibiotics such as tetracycline inhibit protein synthesis by either binding to the 30S subunit or the 50S subunit of the ribosome, leading to the inhibition of the cell multiplication or even cellular death. DNA and RNA are also specific antibiotic targets and as a consequence DNA and RNA synthesis is inhibited. Examples of such antibiotics are the quinolones (Sanchez *et al*., 2006). Some antibacterial agents act on selected cellular processes necessary for the survival of bacteria and examples of these types of antibiotics are sulfonamides and trimethoprim that disrupt the folic acid pathway, which is essential for bacteria to produce precursors important for DNA synthesis (Sanchez *et al*., 2006).

Despite the availability of antibiotics drug resistance is increasing and specifically multidrug resistance is of major concern. Bacteria such as *S. aureus, E. coli, A. baumannii*, are causative agents of major infections, including urinary tract infection, pneumonia, septicaemia, skin and soft tissue infections, and are becoming increasingly resistant. Multidrug-resistant organisms (MDRO) have been shown *in vitro* to be resistant to more than one antimicrobial agent. One of the most important antibiotic resistant bacteria is methicillin-resistant *S. aureus* (MRSA), where 7% of all infections result in death. Other common multidrug resistant bacteria are Vancomycin-resistant *S. aureus* (VRSA), vancomycin-resistant *Enterococci* (VRE), carbapenem-resistant *A. baumannii* (CRAB) and carbapenem-resistant *Enterobacteriacecae* (CRE) (Ho, 2011).

Honey has been used for its capacity to heal wounds and avoid infection by inhibiting the growth of microorganisms and fungi. The antimicrobial effect of honey depends on its botanical origin and the various components found in honey. Its low water activity can inhibit bacterial growth, because of its high osmolarity (sugar content); also the glucose oxidase of honey produces an antibacterial agent, H_2O_2. H_2O_2 levels however, are dependent on honey catalase activity. H_2O_2 is not the only compound in honey responsible for its antibacterial activity, there are also aromatic acids, polyphenols (phenolic acids and flavanoids), and the low pH. Some honeys such as honeydew honey, can lose their antibacterial activity due to heat, storage and light, therefore it is advisable to keep honey in a stored cool dark place (Bogdanov *et al.,* 2008). In Manuka honey, antibacterial activity is directly linked to the presence of a 1,2 dicarbonyl compound MGO. MGO has been attributed to manuka's efficacy as a wound healing product that effectively clears up infections, including abscesses, surgical wounds, traumatic wounds, burns and ulcers. A study by Ahmed & Othman (2013), showed the different

microorganisms sensitive to Manuka honey, such as MRSA from colonised wounds, but also *in vitro* by cell division interruption, as listed in Table 2.5.

Table 2.5: List of microorganisms found to be sensitive to Manuka honeys (Ahmed& Othman, 2013).

Gram positive strains	Gram negative strains
Streptococcus pyogenes (S. pyogenes)	*A. baumanii*
MRSA	*P. aeruginosa*
S. aureus	*E. coli*
Hemolytic streptococci (H. streptococci)	*Salmonella typhimurium (S. typhimurium)*
Enterococcus	*Klebsiella pneumonia (K. pneumonia)*
Coagulase-negative *S. aureus* (CONS)	*Helicobacter pylori (H. pylori)*

Furthermore, Muller *et al.,* (2013), has shown that Manuka honey can also be used synergistically with rifampicin to eradicate *S. aureus* and MRSA strains. The authors showed that not only does Manuka have the ability to kill some bacteria, but can be used together with antibiotics in a synergistic manner to treat multidrug-resistant (MDR) bacteria. Manuka honey is classified according to its UMF (Alvarez-Suarez *et al.,* 2014) and this is classification is based on the MGO content of honey (Table 2.6) (Mavric *et al*., 2008). Manuka honey with a UMF >10 are available commercially for wound treatment and some of the commercial products that contain Manuka honey are Medihoney™, Actilite™ and Therahoney™.

Table 2.6: 1,2-Dicarbonyl compounds and 5-hydroxymethylfurfural (HMF) content of UMF Manuka honey compared to commercial honey (adapted from Mavric *et al.*, 2008)

Sample	3-DG	GO	MGO	HMF
Commercial honey	342	1.7	3.1	3.9
Samples (n=50)	(119 – 1451)	(n.d. – 4.6)	(n.d.– 5.7)	(1.0 – 7.5)
Manuka 1 "active 5"	1060 ± 54	0.7 ± 0.2	38.4 ± 5.0	3.0 ± 0.2
Manuka 2 "active"	668 ± 30	3.0 ± 1.0	347 ± 20	22.6 ± 0.5
Manuka 3 "active"	563 ± 26	3.9 ± 1.0	411 ± 24	17.6 ± 0.6
Manuka 4 "UMF 10"	747 ± 40	1.2 ± 0.5	416 ± 35	21.3 ± 1.1
Manuka 5 "UMF 20"	807 ± 39	4.2 ± 1.1	743 ± 40	43.9 ± 2.0
Manuka 6 "UMF 25"	697 ± 44	7.0 ± 1.0	761 ± 25	n.a.

Data is given in mg/kg as median, minimum and maximum value; for samples of Manuka honey. Data are mean ±SD resulting from triplicate analysis; Abbreviations are as follows: 3-DG, 3-deoxyglucosulose; GO, glyoxal; MGO, methylglyoxal; HMF, 5 hydroxymethylfurfural; n.d., not detectable, below 0.2 mg/kg; n.a., not analyzed.

2.6 Methylglyoxal

MGO is a reactive dicarbonyl species (RCS) that appears as a yellow hygroscopic liquid. It is present in three forms, mainly monohydrade 71%; dehydrate 28% and anhydrated form 1%.

MGO can be generated *in vivo*, during glycolysis in cells, metabolism of ketone body degradation of theonine, and by the fragmentation of trisophosphates. It is generated *in vivo* through the Maillard reaction (chemical interaction involving carbohydrates and amino compounds), also from Schiff's base and Amadori compounds (Wang & Ho, 2012).

2.6.1 Methylglyoxal as a reactive dicarbonyl agent

RCS such as MGO have shown to play an important role in mediating carbonyl stress in human cells and are associated with proliferating signalling pathways. RCS in the cells can modify amino acids such as lysine and arginine residues of proteins and peptides leading to the formation of AGEs. AGE causes carbonyl stress, followed by oxidative stress and tissue damage. Furthermore, an accumulation of AGEs is associated with hyperglycaemia in both diabetes types resulting in neuropathy, arteriosclerosis and retinopathy. The exogenous formation of MGO can also be due to sugar autoxidation, by retro-aldol condensation with the help of oxygenation, this process occurs mostly in foods with high carbohydrate content, such as honey (Lo *et al*., 2011).

2.6.2 Antibacterial activity of methylglyoxal

The dicarbonyl compounds present in Manuka honey are 3-deoxygluculose (3-DG), glyoxal (GO) and MGO (Table 2.6). Mavric *et al.,* (2008), has shown the correlation between the antibacterial activity of Manuka honey and its MGO content. MGO effectively kills *E. coli* and *S. aureus* (Talukdar *et al.,* 2009) as well as methicillin and oxacillin resistant *S. aureus* (Stewart *et al.,* 2014). Kilty *et al.,* (2011), reported that MGO was also effective against biofilms of *P. aeruginosa, S. aureus* as well as methicillin-resistant *S. aureus* although effective concentrations were several folds greater than that required for plantonic bacteria (Kilty *et al.,* 2011). These studies showed that pure MGO at similar concentrations found in Manuka honey had the same killing effect on the different strains of bacteria, and these studies confirmed that MGO is the major antibacterial component of Manuka honey. Roberts *et al.,* (2014), found that Manuka honey reduced the swarming and swimming motility of *P. aeruginosa* due to de-flagellation. The expression of the major structural protein flagellin was reduced as well as flagellin-associated genes, fliA, fliC, flhF, fleN, fleQ and fleR. De-flagellation of bacteria by Manuka honey would limit bacteria mobility, reduce bacterial adhesion and prevent biofilm formation. Recently, Rabie *et al.,* (2016) reported that this effect was directly due to MGO.

2.6.3 Interaction between polyphenols and methylglyoxal

Phenolic acids directly scavenge free radicals while flavanoids, scavenge free radicals, chelate metal ions and activate antioxidant pathways as described in Section 2.5.1. Lo *et al.,* (2011) described the ability of certain phenolic acids to bind MGO, thereby reducing MGO levels and consequently RCS formation. Wang and Ho (2012), show that flavanoids with catechin-like structures have strong MGO trapping ability. Theaflavins could reduce MGO levels by 66% due to the trapping of two MGO molecules. Similarly the trapping of MGO with genistein, shows the formation of mono and di-MGO adducts, Figure 2.5.

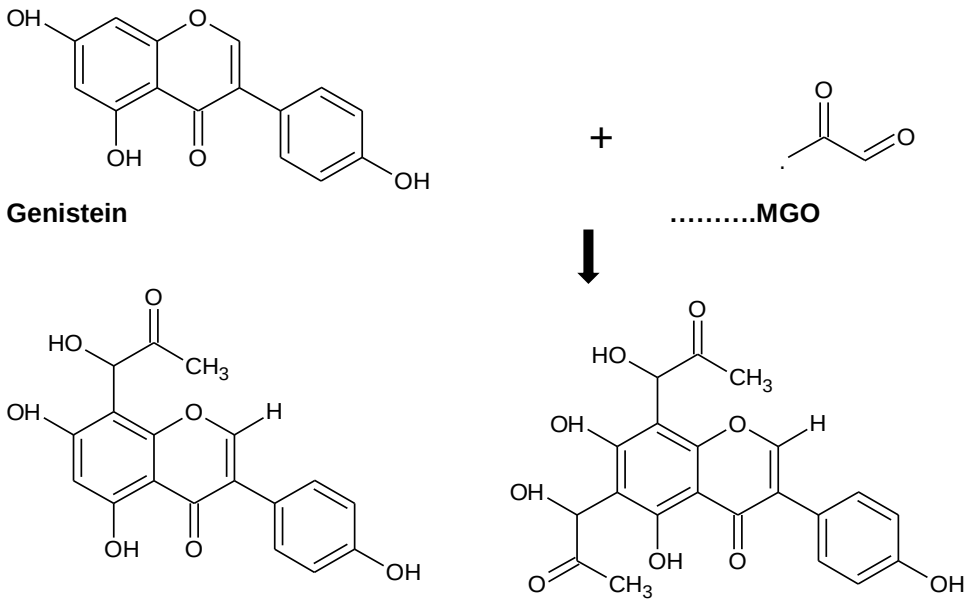


Figure 2.5: The mono and di-MGO adduct formation from genistein trapping (Wang & Ho, 2012)

Lo *et al.,* (2011), investigated the ability of MGO to bind phenols, Figure 2.6. MGO was found not to react with phenols with a benzene structure and a mono-hydroxyl substitute, such as 3-hydroxybenzoic acid, Figure 2.6A. Likewise benzenetriols with 1-COOH and 3-OH group such as GA, Figure 2.6b, also did not bind MGO. However 2,4,6,-trihydroxybenzoic acid, Figure 2.6C, and benzenetriols isomers such as PY showed high reactivity, Figure 2.6D. Mass spectrometry identified the formation of mono- and di- MGO adducts as shown in Figures 2.7A and B for PY. Navarro and Morales (2015) reported that after 168 hrs, GA trapped 99.3 ±0.71% MGO. In the same study, hydroxytyrosol (HT), hydroxytyrosol acetate, 3,4-dihydroxyphenylacetic acid, pyrocatechol and CA were also found to trap MGO. For HT the adduct that forms is 3,4-dihydroxyphenylacetic acid –MGO adduct (Figure 2.8).

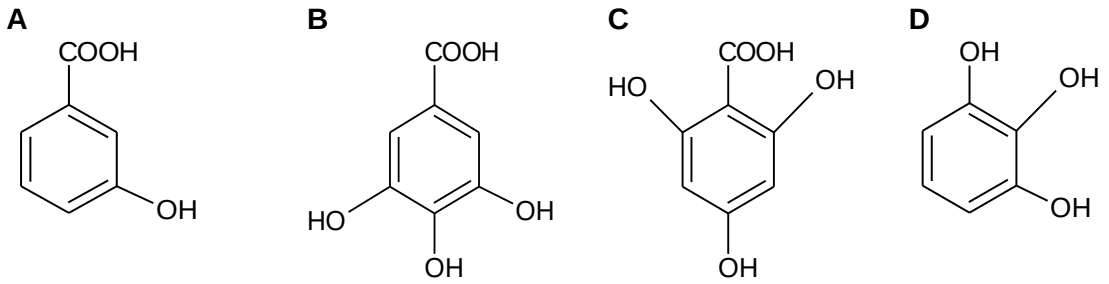


Figure 2.6: Structure of A) 3-hydroxybenzoic acid, B) GA, C) 2,4,6,-trihydroxybenzoic acid and D) PY (Lo *et al.,* 2011).

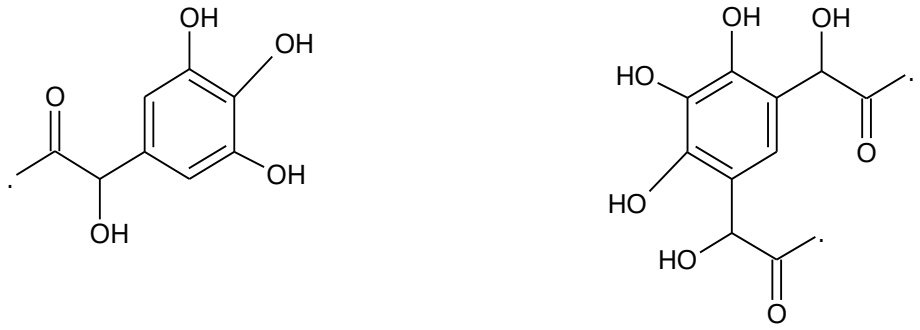


Figure 2.7: PY and MGO trapping forming A) mono- and B) di-MGO adducts (Lo *et al.,* 2011).

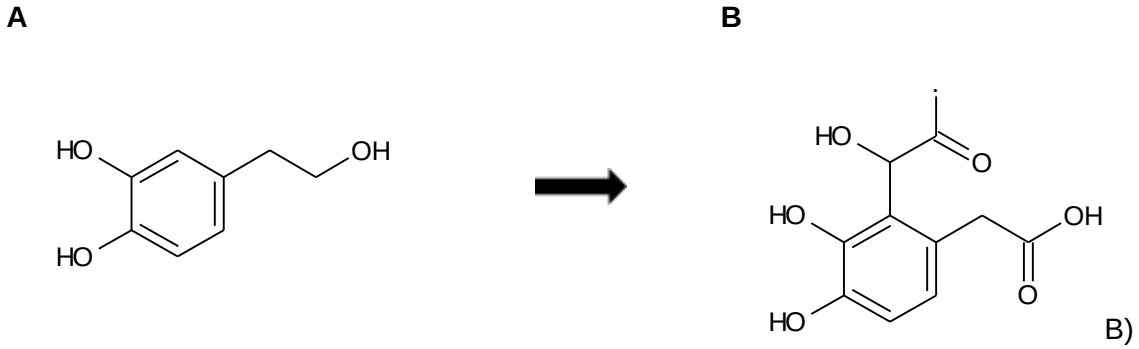


Figure 2.8: A) Hydroxytyrosol and B) 3,4-dihydroxyphenylacetic acid –MGO adduct (Navarro and Morales, 2015)

CA (Table 2.1) is a hydroxycinnamic acid, and is a precursor to ferulic acid and subsequent biosynthesis of curcumin. In a study by Hu *et al.,* (2012), curcumin was found to effectively trap MGO, and its trapping was due to the presence of the diketone group and not its phenol group (Figure 2.9). In this study ferulic acid was found not to trap MGO. Whether CA a common phenolic acid found in Manuka honey (Section 2.5.2) will bind and trap MGO is unknown. In a recent study, Navarro and Morales (2015) reported that after 168 h, CA trapped 90.6 ± 1.48% MGO. These longer incubation times also resulted in 99.3 ±0.71% MGO trapping by GA.

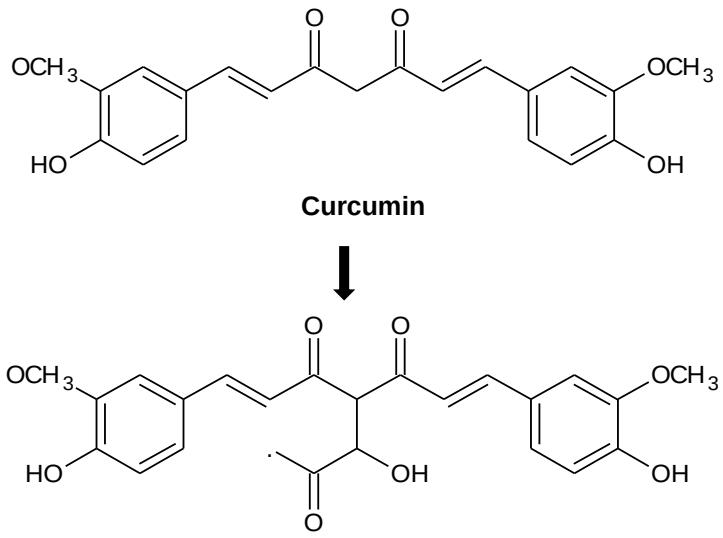


Figure 2.9: Formation of curcumin-MGO adduct (Hu *et al.,* 2012).

High MGO content of Manuka honey and the presence of phenolic acids such as caffeic acid, isoferrulic acid, p-coumaric acid, gallic acid, 4-Hydrobenzoic acid and Syrinigic acid has been reported (Alvarez-Suarez *et al*., 2014). It is therefore unknown whether these compounds occur/remain in honey as separate entities or if they form combinations such as polyphenol-MGO adducts and if so, what impact do they have on the antioxidant and antibacterial activity of Manuka honey.

Although mono- and di-MGO adduct formation has been described, it is unknown whether the antioxidant activity of phenolic acids is altered i.e. do the observed structural changes decrease or enhance antioxidant activity as has been shown for other types of substitution (Table 2.4). Likewise, with MGO trapping by phenolic acids it is unknown whether the antibacterial effects of MGO become lost, unaltered or increased.

2.7 Aim

The aim of this study is to determine if trapping of MGO by phenolic acids reduces the antioxidant activity of phenolic acids while reducing the cellular toxicity and/or antibacterial activity of MGO.

The aim was achieved through the following objectives:

1. Determine the effect of MGO on the antioxidant properties of phenolic acids; caffeic acid, gallic acid and pyrogallol (MGO+caffeic acid, MGO+gallic acid and MGO+pyrogallol) by determining:

a. total polyphenol content with the Folin-Ciocalteu method (TPC).
b. antioxidant activity with 2,2-diphenyl-2-picrylhydrazyl (DPPH), Trolox equivalent antioxidant capacity (TEAC) and Oxygen Radical Absorbance Capacity (ORAC) assays.
c. cellular antioxidant activity with the dichlorodihydrofluorescein diacetate (DCFH-DA) assay.

2. Determine whether cell viability (MTT assay) and number (Crystal Violet) was reduced following exposure of SC-1 and Caco-2 cells to MGO and phenolic acids (caffeic acid, gallic acid and pyrogallal) alone and in combination (MGO+caffeic acid, MGO+gallic acid and MGO+pyrogallol).
3. Determine if the interaction between MGO and phenolic acids (caffeic acid, gallic acid and pyrogallol):
    a. alters the antibacterial activity of MGO in *E. coli* (Gram negative) and *B. subtilis* (Gram positive) bacteria measured with the turbidity assay.
    b. reduces MGO induced morphological changes to *B. subtilis* evaluated with scanning electron microscopy.

3 CHAPTER 3: THE ANTIOXIDANT PROPERTIES OF PHENOLIC ACIDS AND METHYLGLYOXAL ALONE AND IN COMBINATION

3.1 Introduction

Medicinal UMF honey such as Manuka have a high MGO content which varies from 38 – 761 mg/ kg (Table 2.6) which is equivalent to 0.69 mM – 14.07 mM MGO (Mavric *et al*., 2008). Phenolic acids found in Manuka honey are CA, isoferrulic acid, p-coumaric acid, GA, 4-Hydrobenzoic acid and Syrinigin acid (Alvarez-Suarez *et al*., 2014).

Phenolic acids trap MGO as described in Section 2.4 and depending on the concentration and structure, these phenolic acids can trap MGO possibly reducing the cellular toxicity of MGO. Furthermore, this can result in the formation of novel phenolic acid derivatives with unknown bioactivity.

The antioxidant capacity of phenolic acids is due to their reactivity of the phenol moiety. The antioxidant activity of phenolic acids is via several mechanisms, such as radical scavenging by atom donation, electron donation and singlet oxygen quenching to stabilise free radicals (Robbins, 2003). Different assays to evaluate different aspects of antioxidant activity and the most commonly used are the DPPH and TEAC assays that measures radical scavenging by electron transfer (ET) as indicated in Figure 3.1.

$$M(n) + e \text{ (from AH)} \rightarrow AH^{\bullet +} + M(n-1)$$

Figure 3.1: Reaction involving assays with electron-transfer reaction (Huang *et al*., 2005)
M(n): probe/oxidant; e: electron; $AH^{\bullet +}$: oxidised antioxidant; M (n – 1): reduced probe

HAT based assays monitor competitive reaction kinetics and a typical such assay is the ORAC assay which involves hydrogen atom transfer reactions (Ghiselli *et al.,* 2000). In the ORAC assay, 2,2'- azobis(2-amidinopropane) dihydrochloride (AAPH) generates peroxyl radicals, and the ability of the antioxidants to protect an indicator molecule, fluorescein is measured (Figure 3.2). The ORAC assay is considered to be physiologically relevant as the radical scavenged is widely found in biological systems.

$$ROO^{\bullet} + PH \rightarrow ROOH + P^{\bullet}$$
$$P^{\bullet} + ROO^{\bullet} \rightarrow ROOP$$
$$ROO^{\bullet} + AH \rightarrow ROOH + A$$
$$A + ROO^{\bullet} \rightarrow ROOA$$

Figure 3.2: Schematic diagram of the principle of the ORAC assay, adapted from Huang *et al.,* (2005). PH=probe, ROO• =peroxyl radicals generated from AAPH, AH= antioxidant

According to Lo *et al.* (2011), phenols such as PY, 1,2,4-trihydroxybenzene, 1,3,5-trihydroxybenzene effectively trap MGO while GA does not trap MGO. In contrast, Navarro and Morales (2015) reported that phenolic acids with a C3-OH will trap MGO and these include HT, hydroxytyrosol acetate, 3,4-dihydroxyphenylacetic acid, pyrocatechol and CA. Interestingly in contrast to the study of Lo *et al*., (2011) these authors found that GA trapped MGO. Lo *et al*., (2011) identified that a para- > meta- > and ortho-benzenediol structure was required for MGO trapping. Except for GA all phenolic acids that trapped MGO had a typical meta diol arrangement. Differences between studies may be related to incubation times, buffers used, pH, molar ratios and methods of quantification.

Using PY, in this chapter the effect of MGO trapping on antioxidant properties of PY will be determined. Due to contradictory reports, the ability of MGO to trap GA will be determined. Furthermore, the ability of CA will also be determined.

3.2 Materials

3.2.1 Reagents, equipment and disposable plastic ware

Sodium phosphate dibasic dehydrate ($Na_2HPO_4 \bullet 2H_2O$), sodium phosphate (NaH_2PO_4), sodium chloride (NaCl), sodium carbonate ($NaHCO_3$) and methanol (CH_3OH) of analytical quality and were obtained from Merck Chemicals, Modderfontein South Africa (SA). Folin-Ciocalteu's (F-C) reagent, Trolox, 2, 2-diphenyl-2-picrylhydrazyl (DPPH) and 2,2'-azo-bis (3-ethylbenzothiazoline-6-sulfuric acid) diamonium salt (ABTS), Fluorescein, 2,2'-azo-bis(2-amidinopropane) dihydrochloride (AAPH), potassium peroxodisulfate ($K_2S_2O_8$), was obtained from the Sigma-Aldrich Company, Atlasville, SA. Phenolic acids samples: pyrogallol (PY) was obtained from Merck chemicals; Modderfontein SA and caffeic acid (CA), gallic acid (GA) and MGO were obtained from Sigma-Aldrich Company, Atlasville, SA.

Equipment used were: Lambda LS5OB spectrophotometer, from Perkin Elmer, Boston, MA, USA, Germany supplied by Separations Scientific, Honeydew, SA. An E-max plus microplate reader from Biochrom Ltd Cambridge, England. A plate shaker and Eppendorf pipettes from Eppendorf AG Hamburg, Germany supplied by the Laboratory Scientific Equipment Company (LASEC), Cape Town, SA.

Disposable plastic ware includes: 96 well plates, 50 ml, 15 ml tubes and pipette tips (10, 25, 100, 200, and 1000 µl) and were obtained from Greiner Bio-one also supplied by LASEC, Cape Town, SA.

3.3 Methods

3.3.1 Samples preparation

A stock solution of 40% MGO (6.50 M) was diluted according to the requirements for each assay. Equimolar solutions of phenolic acids : MGO (1:1) were prepared containing final concentrations of 1 mM MGO and 1 mM phenolic acid. These solutions were 1 mM MGO:1 mM PY, 1 mM MGO:1 mM GA and 1 mM MGO:1 mM CA.

Secondly 1:2 molar ratio solutions of phenolic acid : MGO were also prepared containing final concentrations of 2 mM MGO and, 1 mM phenolic acid. These solutions were 2 mM MGO:1 mM PY, 2 mM MGO:1 mM GA and 2 mM MGO:1 mM CA. All samples were diluted with double distilled water (ddH_2O). Depending on the sensitivity of each assay, optimized serial dilutions were used.

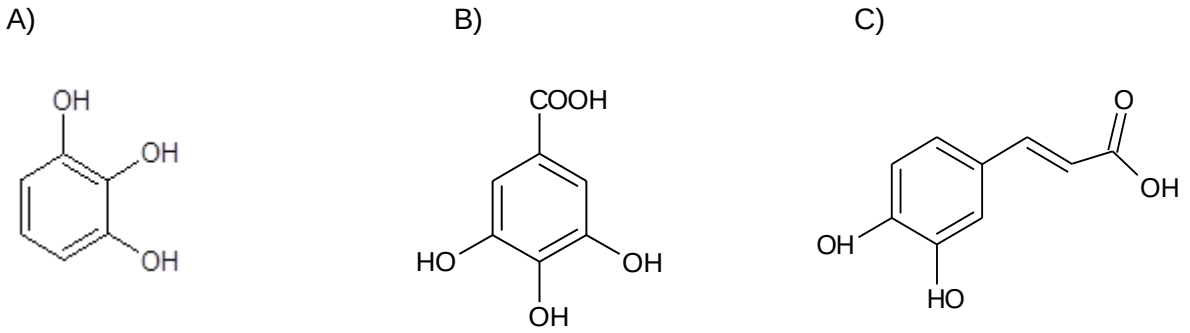


Figure 3.1: Structure of the phenolic acids used in this study, A) PY, B) GA and C) CA (Lo *et al.,* 2011).

3.3.2 Buffers

3.3.2.1 Phosphate buffered saline (PBS)

PBS was used for the preparation of the phenolic acid : MGO solutions. A 0.2 M concentration of PBS was made 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.

For the ORAC assay a 0.1 M phosphate buffer ph 7.4 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. The pH was adjusted to 7.00 with NaOH when required.

3.3.3 Liquid chromatography-mass spectrometry (LCMS) quantification

Analyses were performed using a High-Resolution Mass Spectrometer (Waters Synapt G2) with prior separation of the analytes on a Waters T3 HSS column. Peak areas for the samples were integrated automatically by the TargetLynx software (Waters) and the analyte compounds quantified by extrapolation of calibration curves produced using fresh calibration standards prepared from concentrations of PY, GA, CA, at final concentrations of 166 µM. Subsequent dilutions were then performed to make a range of calibration standards used to calibrate the instrument response to the compounds of interest. Phenolic acids were measured in ESI+ve mode using extracted ions at m/z (M+H+) for each of the compounds. The area under curve was determined for each unreacted phenolic acid peak and the data was reported as percentage phenolic acid remaining compared to phenolic acid samples not containing MGO.

3.3.4 Determination of antioxidant content

3.3.4.1 Total polyphenolic content

In this assay the F-C reagent binds to the phenolic compounds causing a colour change, therefore the antioxidant content of samples is measured by a gain or loss in colour intensity (Huang *et al.,* 2005).

Serial dilutions (final concentrations 0 – 0.25 mM) of each phenolic acid and MGO alone and in combination (as described in 3.3.1) were prepared. A volume of 50 µl F-C reagent, followed by 50 µl of $NaHCO_3$ was added to a 30 µl volume of each sample. The samples were mixed well and the absorption was then read at 630 nm with an E-max plus microplate reader. All data was expressed as absorbance against phenolic acid concentration and the gradient of each serial dilution graph was calculated and compared.

3.3.5 Determination of antioxidant activity

3.3.5.1 2, 2-diphenyl-2-picrylhydrazyl (DPPH)

In the DPPH assay, the odd electron in the DPPH free radical gives a strong purple absorbance maximum at 570 nm. The colour turns from purple to yellow when the odd electron of DPPH radical becomes paired with hydrogen from a free radical scavenging antioxidant (XOH) to form the reduced DPPH-H as indicated below (Huang *et al.,* 2005).

DPPH solution was prepared as a stock solution (0.006 g dissolved in 25 ml of methanol and sonicated for 20 min) and a working solution (was prepared by diluting the stock solution 5x). Both solutions were kept in the dark until used (Huang *et al.,* 2005). A serial dilution (final concentrations 0 – 0.01 mM) of each phenolic acid and MGO alone and in combination was prepared. To 15 µl of each sample (Section 3.3.1), 285 µl of DPPH was added. The samples were mixed well and after 15 min the absorbance was measured at 570 nm with an E-max plus microplate reader. A volume of 15 µl of ddH_2O added to the DPPH was used as the vehicle control. The concentration gradients of phenolic acids vs phenolic acid : MGO combinations were compared.

3.3.5.2 Trolox equivalent antioxidant capacity (TEAC)

The TEAC method is a colorimetric assay based on the capacity of a sample to scavenge the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ATBS) radical. An ABTS solution was prepared by dissolving 0.0021 g ABTS in 5 ml of PBS and 0.0041 g of potassium persulfate, $K_2S_2O_8$ in 5 ml of PBS. Both solutions were mixed together and left for 12 – 16 h in the dark. A working solution was then prepared by diluting the stock solution 30x. Both solutions were kept in the dark until used (Huang *et al.,* 2005).

Serial dilutions (final concentrations 0 – 0.007 mM) of each phenolic acid and MGO alone and in combination (prepared as described in section 3.3.1) were prepared. To a 10 µl volume of each sample, 290 µl ABTS working solution was added. The samples were mixed well and then after 30 min the absorbance was measured at 630 nm with an E-max plus microplate reader. A vehicle control was included whereby ABTS solution was added to 10 µl of ddH_2O. All data was expressed as absorbance against phenolic acid concentration and the gradient of each serial dilution graph was calculated and compared.

3.3.5.3 Oxygen Radical Absorbance Capacity (ORAC)

To determine the antioxidant activity of each phenolic acid and MGO, alone and in combination, the ORAC assay based on a modified method of Ou *et al*., (2002) was used. AAPH was used as a peroxyl radical generator and fluorescein is a fluorescent probe.

A stock solution was prepared by dissolving 3.32 g fluorescein in 10 ml PBS (Huang *et al*., 2005). A volume of 10 µl of each phenolic acid and MGO, alone and in combination (final

concentration for phenolic acids 0.005 mM and 0.005 mM/0.01 mM for MGO) was added to 165 µl of the fluorescein working solution followed by 25 µl AAPH (65 mg/ml). The samples were mixed well and the microplate placed into the plate reader and was incubated and read at 37˚C. The fluorescence was measured every minute for 2 h at an excitation wavelength of 485 nm and at an emission wavelength of 520 nm. A 1 mM trolox solution was used to generate a standard curve, yielding final concentrations of 0 – 0.05 mM. The final ORAC values of the samples were calculated as µM TE.

3.3.6 Data management and statistical analysis

All experiments were performed at least in triplicate and data was reported as mean ± (standard error of mean) SEM. Significant differences between samples were determined either using the students T-TEST or one way ANOVA with Tukey's post-hoc analysis.

3.4 Results and discussion

The effect of MGO trapping by phenolic acids on the antioxidant content and activity of PY, GA and CA was determined. Two different solutions were used and these were a 1:1 and a 2:1 ratios of MGO : phenolic acids. For all reactions, there is a reaction equilibrium and by adding an excess of MGO the reaction is then driven towards the formation of increased amounts of product that in the case of the present study is increased trapping of MGO by phenolic acids. In a study by Lo *et al*., (2011) it was shown that PY traps MGO by direct binding and forming a mono MGO adduct while GA was found not to bind MGO. Based on these finding PY and GA was used in this study. The MGO binding capacity of CA is unknown. To confirm that this reaction was occurring samples containing MGO : phenolic acid in a 1:1 ratio were sent to the Central Analytical Facility, Stellenbosch University, SA for analysis. The levels of phenolic acids was determined, first in Figure 3.2 and changes in retention times (Rt) and the presence of the predicted MGO adducts was determined in Figure 3.3. A peak representing PY, with a Rt = 5.27 ml and a molecular mass of 125.0 g/mol was identified. Two addition peaks with Rt of 8.3 ml, with molecular weight of 197.0 g/mol and Rt=9.35 with a molecular weight of 269.1 g/mol which represents the mono- and di-MGO adduct/s respectively were identified. (Figure 3.3). For GA a single peak with a Rt of 6.05ml and a molecular weight of 169.0 was identified. This confirms previous findings of Lo *et al*., (2011), that MGO and GA do not form adducts. Analysis showed that CA did not form mono- or di-MGO adducts. However, it is unknown whether the presence of MGO may alter the antioxidant activity of GA and CA.

The amount of unreacted phenolic acid in each sample was determined. The recovery as percentage was 99.12% and 105.00% for GA and CA respectively. For PY, the percentage recovery was 79.5%, which indicates that although adduct formation did occur, only a small portion of 20.5% was involved in adduct formation. Although these samples are a mixture of phenolic acids and MGO and for PY, adducts this probably represents the equilibrium ratio in honey.

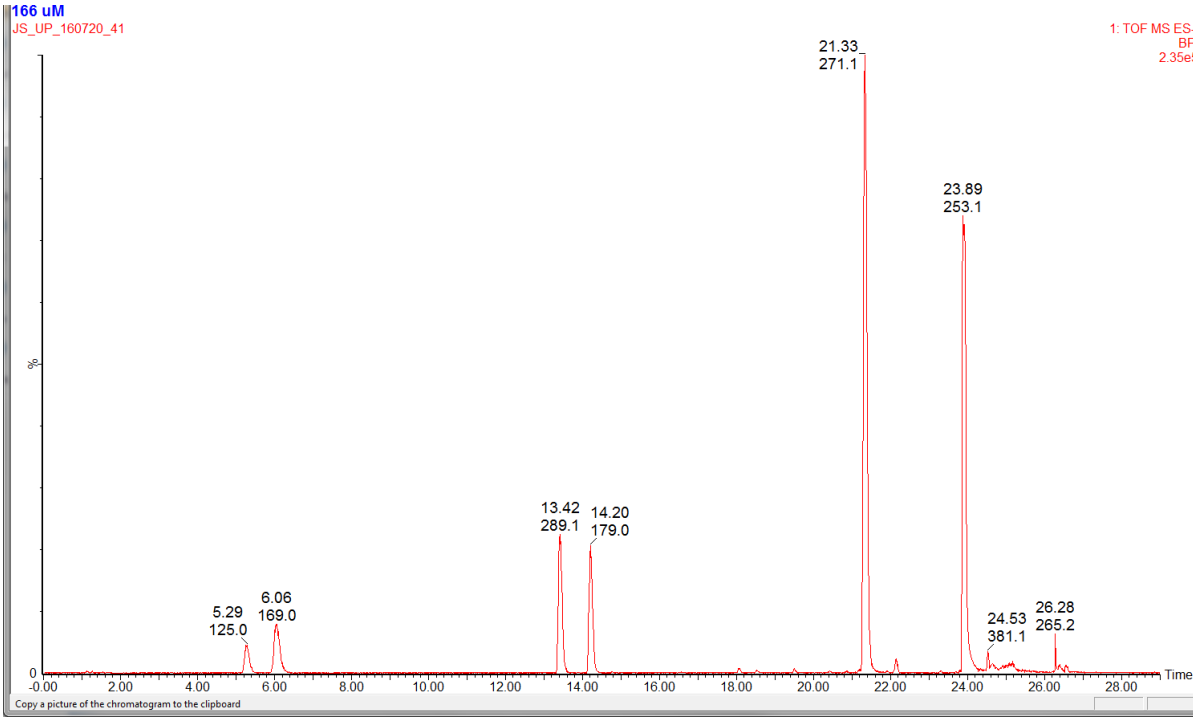
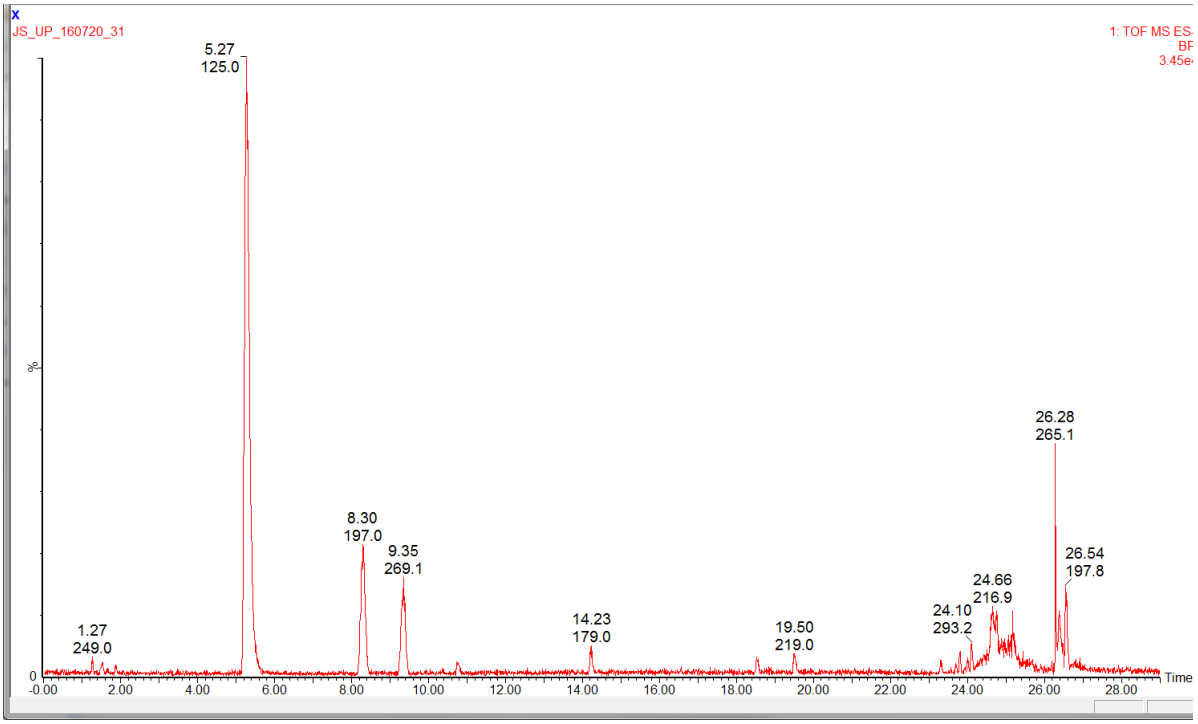


Figure 3.2: Liquid chromatography mass spectrometry chromatograms of polyphenols. Peaks with increasing retention time (Rt) are i) PY (Rt=5.29), ii) GA (Rt= 6.06), iii) Catechin (Rt= 13.42), iv) CA (Rt= 14.2), v) naringenin (Rt= 21.33), vi) chrysin (Rt=23.89). Conditions were as described in section 3.3.3.



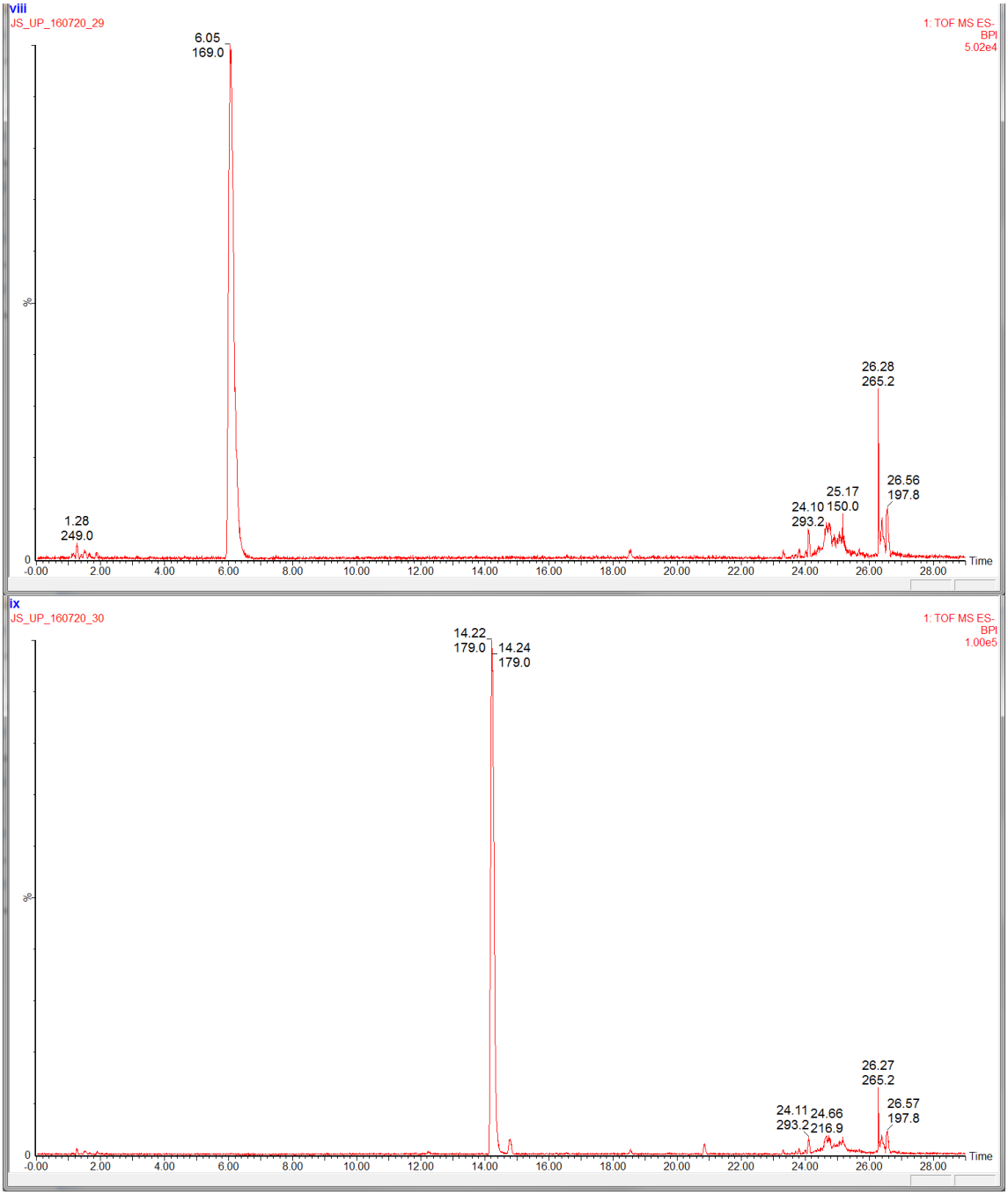


Figure 3.3: Liquid chromatography mass spectrometry chromatograms of MGO and x) PY, viii) GA and ix) CA in 1:1 ratio. Conditions were as described in section 3.3.3.
KEY: CA= Caffeic acid; GA= Gallic acid; MGO= Methylglyoxal; PY= Pyrogallol.

Table 3.1: Concentration, %change in sample phenolic acid content due to mono- and di MGO adduct formation

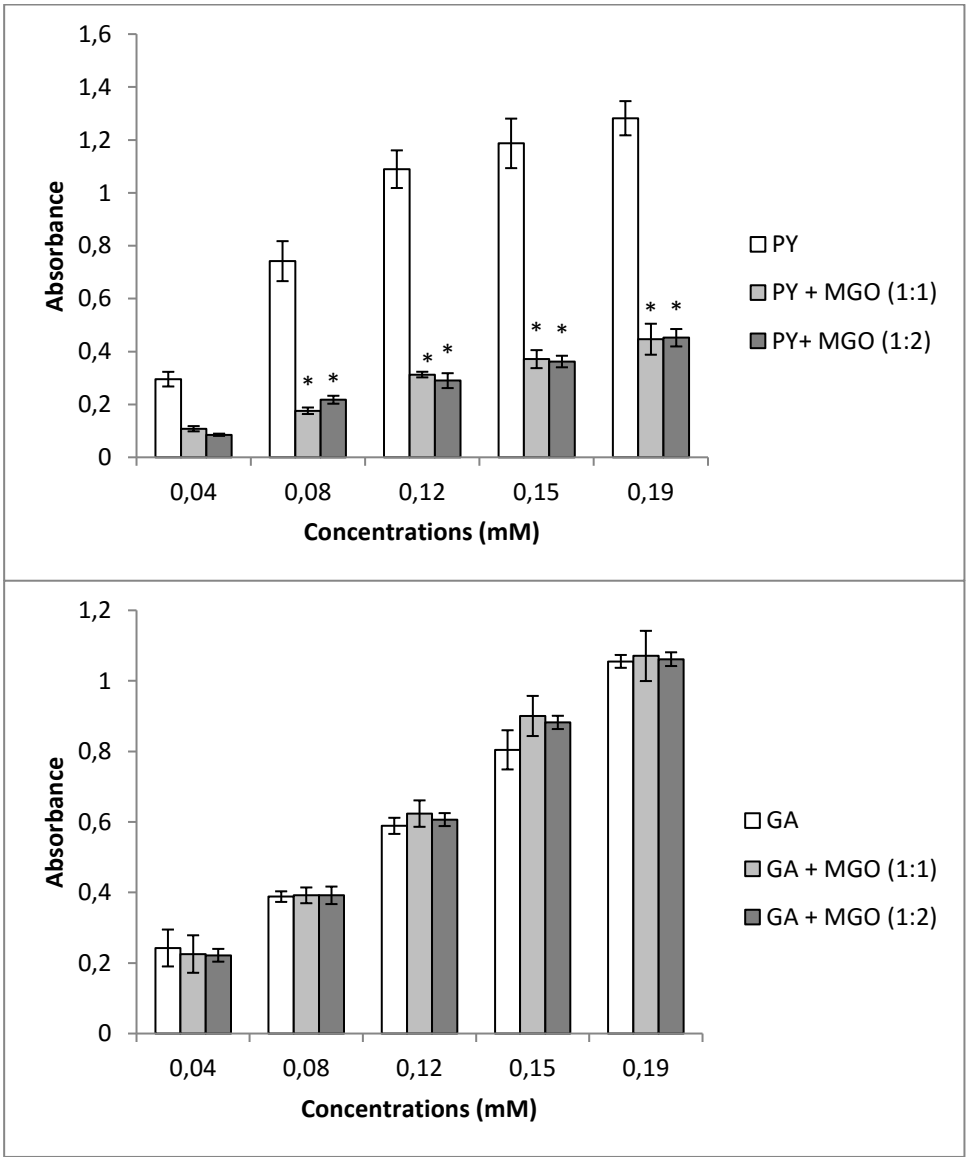
	µM	% Change	Formation
PY	263.93	79.5	Mono and di-MGO
GA	329.05	99.12	Unchanged
CA	349.00	105.12	Unchanged
Initial Concentration	332.00	100.00	–

3.4.1 Antioxidant content and activity

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

3.4.1.1 Antioxidant content

TPC being an ET assay which is also used to measure total polyphenol content (TPC). The effect of MGO on the TPC of PY, GA and CA was determined as shown in Figure 3.4.



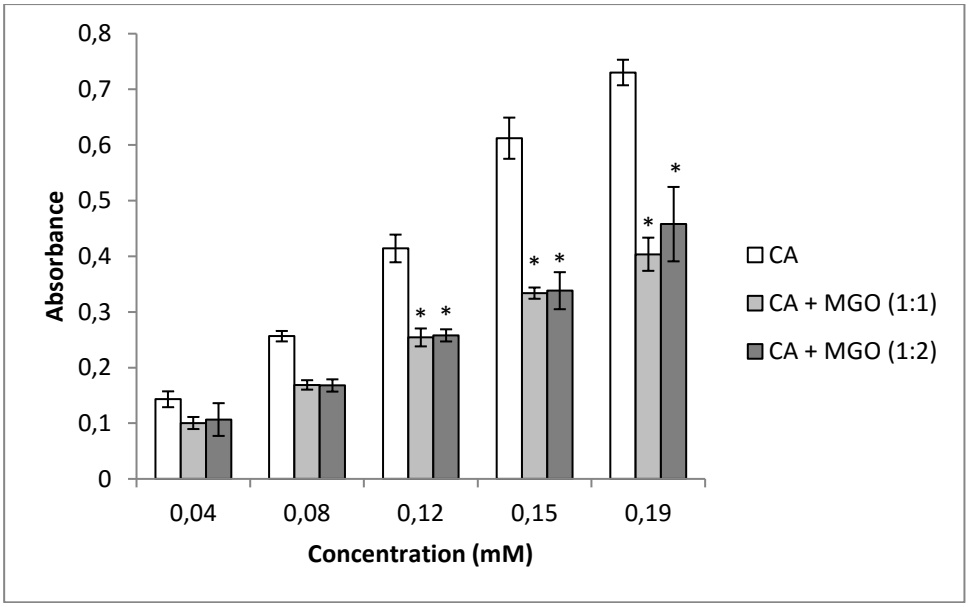


Figure 3.4: Effect of MGO on the antioxidant content of phenolic acids, PY, GA and CA. Data is an average of three experiments ± SEM. * indicates significant differences of combined compounds compared to each polyphenol alone p≤0.05.
KEY: CA= Caffeic acid; GA= Gallic acid; MGO= Methylglyoxal; PY= Pyrogallol; SEM= Standard error of mean, TPC= Total polyphenols content.

With increasing concentrations of PY there is an increase in TPC (line equation and gradient, for PY from 0.04 to 0.19mM, y=7.0066 x - 0.0922, R^2= 0.94). In combination with MGO there is a decrease in measured TPC. For the ratio of MGO:PY 1:1 y= 2.3462x + 0.0102 (R^2= 0.99) and for MGO:PY 2:1 y= 2.35x + 0.0085 (R^2 = 0.99) (Figure 3.4). Addition of PY results in a 3 fold in decrease in TPC for the 1:1 ratio and a 2.98 fold decrease in TPC for the 2:1 ratio. This implies that the trapping of MGO by PY trapping at equimolar concentrations is complete and at higher MGO concentrations, no further trapping of MGO occurs.

For GA no MGO trapping occurs and for GA alone, MGO:GA 1:1 and MGO:GA 2:1 there is a linear increase in TPC with gradients of, y=5.2637 x - 0.0053 (R^2= 0.99), y= 5.4585x + 0.0043 (R^2= 0.99) and y= 5.1511x + 0.0185 (R^2 = 0.99), respectively in Figure 3.4. This implies that GA does not trap MGO at any of the concentrations evaluated.

For CA with increasing concentrations there is a linear increase in TPC y=3.874 x - 0.0132, R^2= 0.99). In combination with MGO there is a decrease in measured TPC. For the ratio of MGO:CA 1:1 y= 2.0827x + 0.0099 (R^2= 0.98) and for MGO:CA 2:1 y= 2.2832x + 0.0019 (R^2 = 0.99). Addition of MGO resulted in decreases at both the 1:1 and 1:2 ratios in TPC with 1.86 fold and 1.7 fold respectively. Differences between CA combined with 1 mM MGO or 2 mM MGO were not significant which similar to PY, optimal trapping of MGO by CA occurs at equimolar concentrations.

Both PY and CA trapped MGO although PY was more effective in trapping MGO than CA. GA did not bind MGO. With trapping for the 1:1 ratios there was a 3, 1.86 and 0 fold decrease in TPC for PY, CA and GA, respectively.

The results obtained for PY and GA confirm the findings of Lo *et al.,* (2011) that reported that some phenolic acids such as PY trap MGO while others such as GA does not bind MGO. In this study Lo *et al.,* (2011) by measuring the changes in MGO levels determined the extent of phenol trapping of MGO. A series of benzoic acids with one, two and three hydroxyl groups were evaluated. In this study a mixture of MGO and polyphenols were prepared at a ratio of 2:1 MGO : phenolic acid and after 1hr the percentage unreacted MGO was determined with HPLC. Benzenes with one and two –OH did not bind MGO whereas compounds with 3 -OH could bind MGO, with a decrease in MGO levels from 100% to 35% -50%. The most effective triolbenzene was 1,3,5-trihydroxyl-benzoic acid. Both PY and GA are benzenetriols could have the possibility to trap MGO. 2,4,6 –trihydoxybenzoic acid was found to have the highest MGO binding ability while GA which is a 3,4,5 trihydroxybenzoic acid does not bind MGO which indicates that the position of the –COOH is important for the ability of benzoic acids to trap MGO and this was reported to be due to steric hindrance and the effect of carbon electron charges.

Curcumin was found to effectively trap MGO and its trapping was due to the presence of the diketone group and not its phenol group (Hu *et al.,* 2012). Curcumin has two methoxy groups while dimethoxycurcumin (DIMC) has four methoxy groups as seen in Figure 3.5. These methoxy groups inhibit the hydroxyl groups that participate in the reaction that forms adducts associated with the benzene ring. DIMC was still able to trap MGO due to the presence of the diketone group. Curcumin is synthesised from ferrulic acid. Hu *et al.,* (2012) found that ferrulic acid was unable to trap MGO. Ferrulic acid contains a methoxy group whereas CA does not have methoxy groups and has two -OH groups, and MGO may bind.

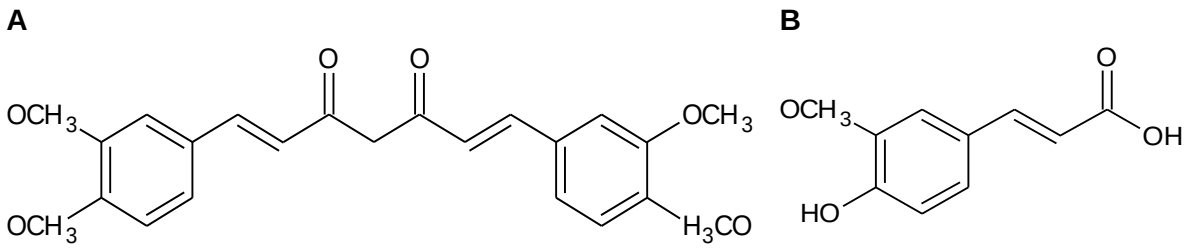


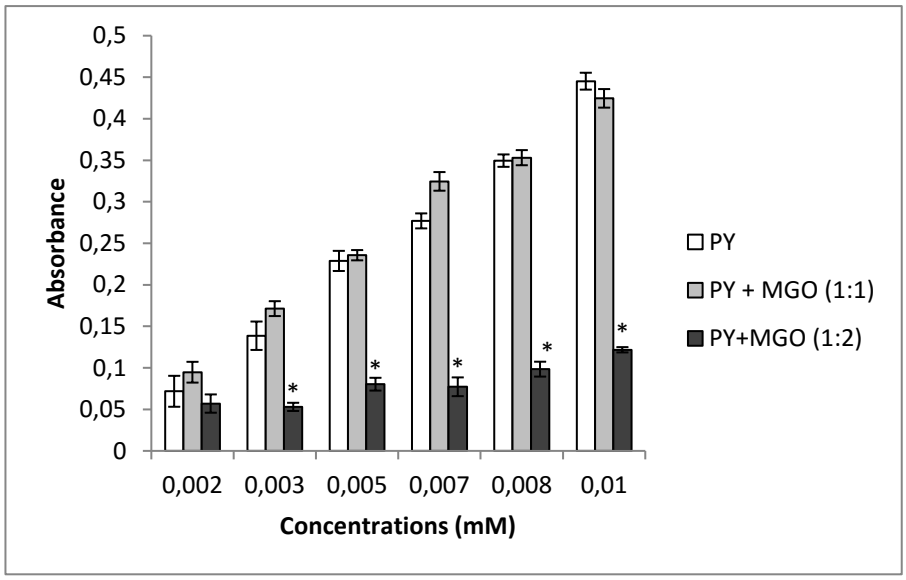
Figure 3.5: Structure of A) DIMC and B) ferrulic acid (Hu *et al*., 2012)

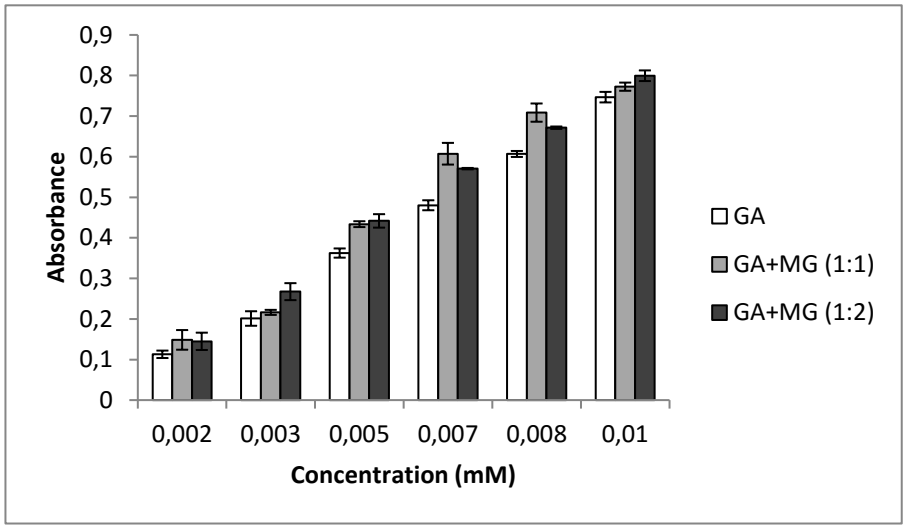
In the presence of MGO, CA like PY causes a decrease in measured TPC (Figure 3.4) which implies that MGO does bind CA. The mechanism of binding is unknown although it can be speculated that there is no steric hindrance due to a methoxy group thus MGO can bind.

For phenolic acids : MGO in a ratio of 1:1, Navarro and Morales (2015) reported a time dependent decrease form 0-168 h in the TPC of HT and at 8 h and 168 h (7 days) there was a 20% and a 69% decrease in TPC indicating trapping of MGO by phenolic acids is a slow time dependant reaction.

3.4.1.2 Antioxidant activity

In DPPH, PY showed a linear increase with gradient y= 43.49x – 0.0016, R^2=0.99, in combination with MGO a 1:1 ratio a gradient of y= 41.648x + 0.0209, R^2=0.99, showed PY retained its activity, and at 2:1 ratio a gradient of y= 10.124x + 0.0191, R^2=0.89 showed a decrease in antioxidant activity of 4.3 fold. GA maintained its antioxidant activity when incubated with MGO. For CA a linear increase with gradient y= 8.3143x + 0.004, R^2=0.96, in combination with MGO at 1:1 ratio a gradient of y= 12.962x + 0.0014, R^2=0.96, showed an increase of antioxidant activity of 1.6 fold, and at 2:1 ratio a gradient of y= 15.417x + 0.0031, R^2=0.99 showed an increase of 1.9 fold, as seen in Figure 3.6. At a ratio of 2:1, the antioxidant activity of PY was reduced by 4.3-fold, unchanged for GA and increased by 1.9-fold for CA. No studies could be found that evaluated the antioxidant activity of phenolic acid and MGO in combination.


0,5
0,45
0,4
0,35
0,3
0,25
0,2
0,15
0,1
0,05
0
Absorbance
0,002
0,003
0,005
0,007
0,008
0,01
Concentrations (mM)
PY
PY + MGO (1:1)
PY+MGO (1:2)
*


0,9
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0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
Absorbance
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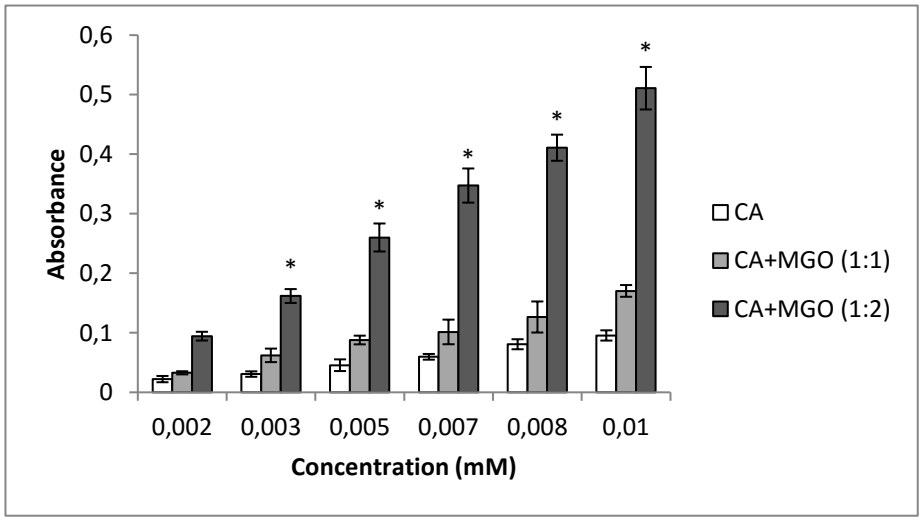
0,003
0,005
0,007
0,008
0,01
Concentration (mM)
GA
GA+MG (1:1)
GA+MG (1:2)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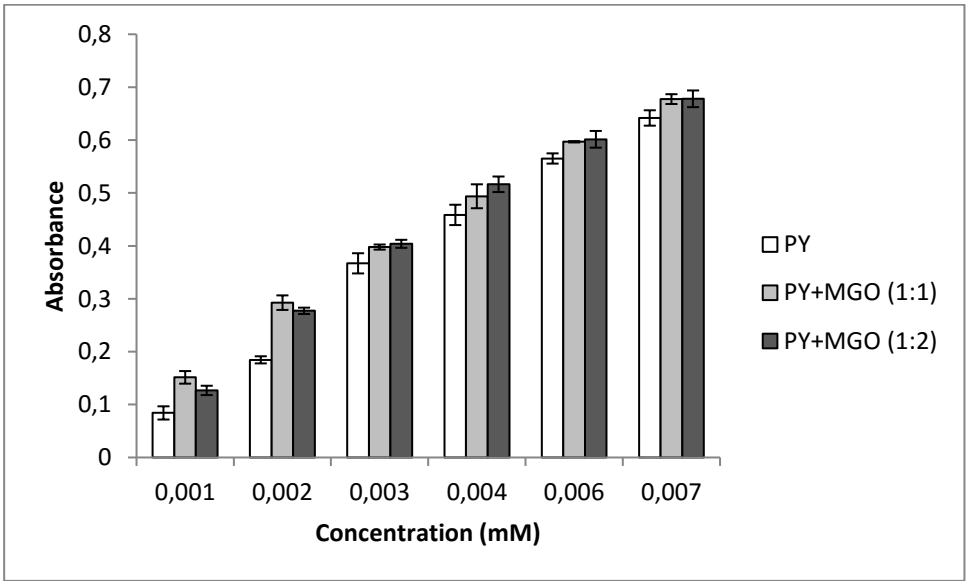
0,5
0,4
0,3
0,2
0,1
0
Absorbance
0,002
0,003
0,005
0,007
0,008
0,01
Concentration (mM)
CA
CA+MGO (1:1)
CA+MGO (1:2)
*

Figure 3.6: **Effect of MGO in ratio of 1:1 and 1:2 with phenolic acids on the antioxidant activity measured with the DPPH assay of PY, GA and CA.** Data is an average of three experiments ± SEM. * indicates significant differences in antioxidant activity of phenolic acid when compared with phenolic acid and MGO at each concentration, p≤0.05.
KEY: CA= Caffeic acid;; GA=Gallic acid; MGO=Methylglyoxal; PY= Pyrogallol, SEM= Standard error of mean, DPPH= 2, 2-Diphenyl-2-picrylhydrazyl.

The TEAC assay follows the same mechanism as DPPH being an ET assay. For PY, GA and CA in a 1:1 and 1:2 combination with MGO there was no change in antioxidant activity measured with the TEAC assay (Figure 3.7). For phenolic acids : MGO in a ratio of 1:1, Navarro and Morales (2015) reported a time dependent decrease form 0-168 hours in the antioxidant activity of HT measured with the ATBS TEAC and FRAP TEAC assays.

At 8 h and 168 h (7 days) there was a 3.0% and a 53% decrease in antioxidant activity measured with the TEAC assay indicating trapping of MGO by phenolic acids is a slow time dependant reaction. For the FRAP and TEAC assay, there was a 4.8% and a 79.9% decrease in antioxidant activity after 8hrs and 168 h, respectively. In the present study antioxidant activity was measured after 2 h and this may account for the lack of change in antioxidant activity measured with TEAC assay. This also implies that the DPPH assay is a more sensitive assay for the measurement of the effect of MGO trapping on the antioxidant activity of phenolic acids.



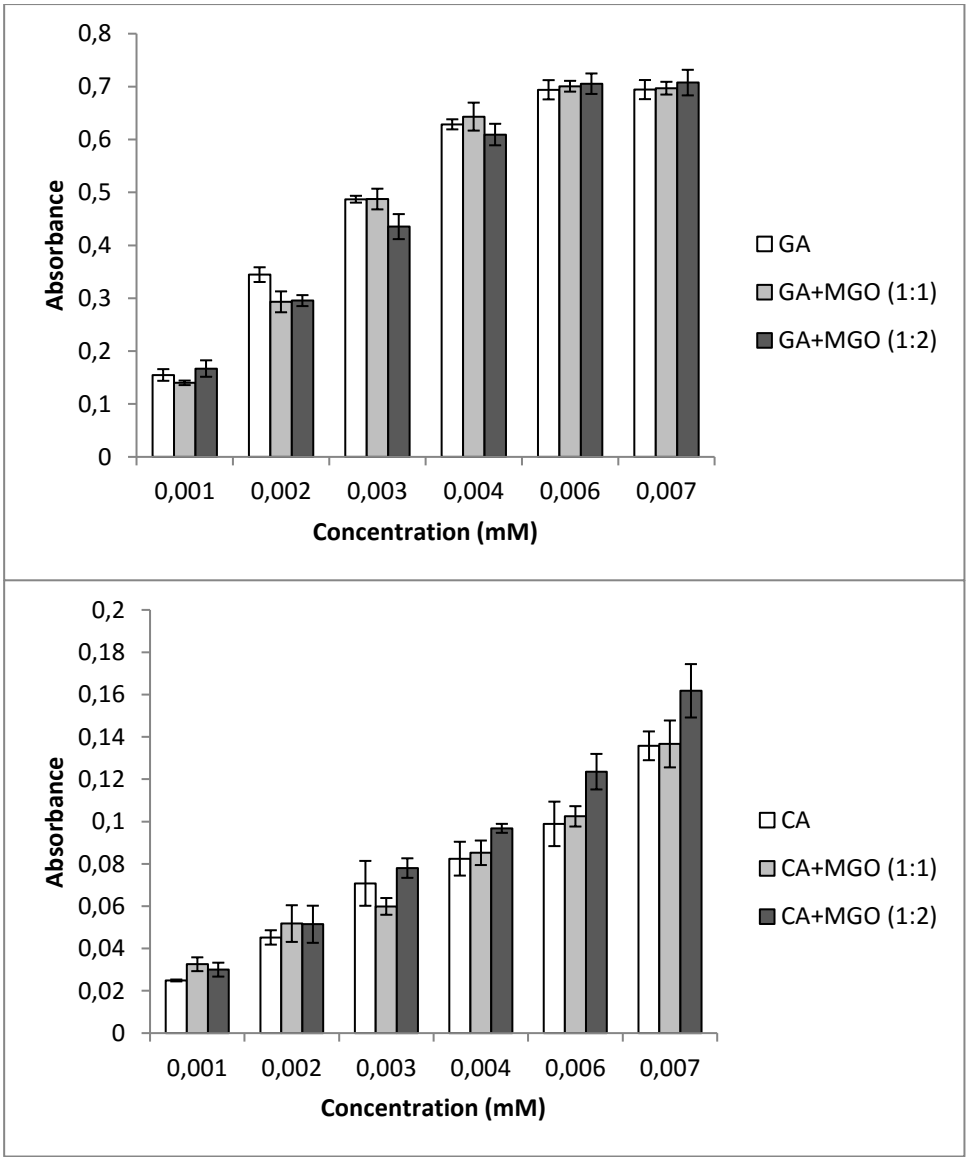
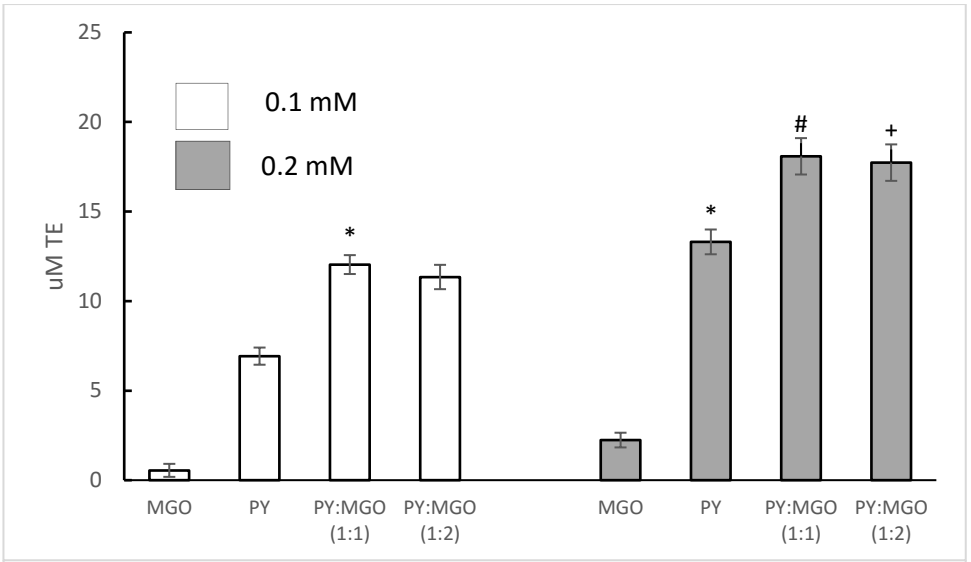


Figure 3.7: Effect on the antioxidant activity (TEAC) of PY, GA and CA when combined with MGO. Data is an average of three experiments ± SEM. * indicates significant differences in antioxidant activity of each phenolic acid when compared with phenolic acid combined with MGO, p≤0.05**.**
KEY: CA= Caffeic acid; GA= Gallic acid; PY= Pyrogallol, MGO= Methylglyoxal; SEM= Standard error of mean, TEAC= Trolox equivalent antioxidant capacity.

The ORAC assay measures the ability of phenolic acids such as PY, GA and CA to protect fluorescein against peroxyl radical damage. Compared to PY alone, antioxidant activity was significantly increased for 0.1 mM PY : MGO (1:1), 0.2 mM PY : MGO (1:1) and 0.2 mM PY : MGO (1:2) compared to PY alone, Figure 3.8. The antioxidant activity GA was unchanged for all combinations and concentrations. For CA a significant increase in antioxidant activity was observed for 0.1 mM CA : MGO (1:2), 0.2 mM CA : MGO (1:1) and 0.2 mM CA : MGO (1:2) compared to CA alone. No studies could be found that evaluated the effect of MGO trapping on HAT based assays.


25
20
15
10
5
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uM TE
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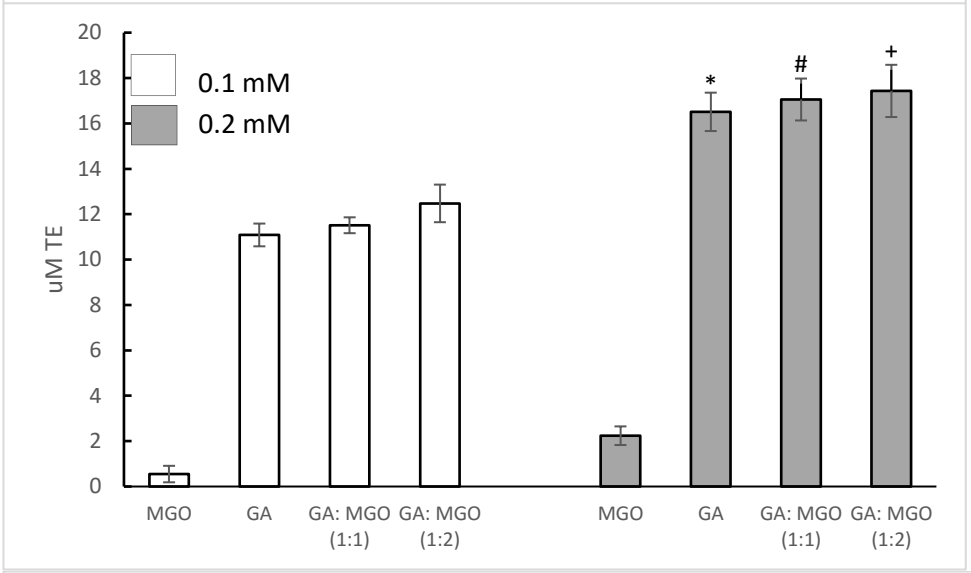
PY:MGO (1:2)


20
18
16
14
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10
8
6
4
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uM TE
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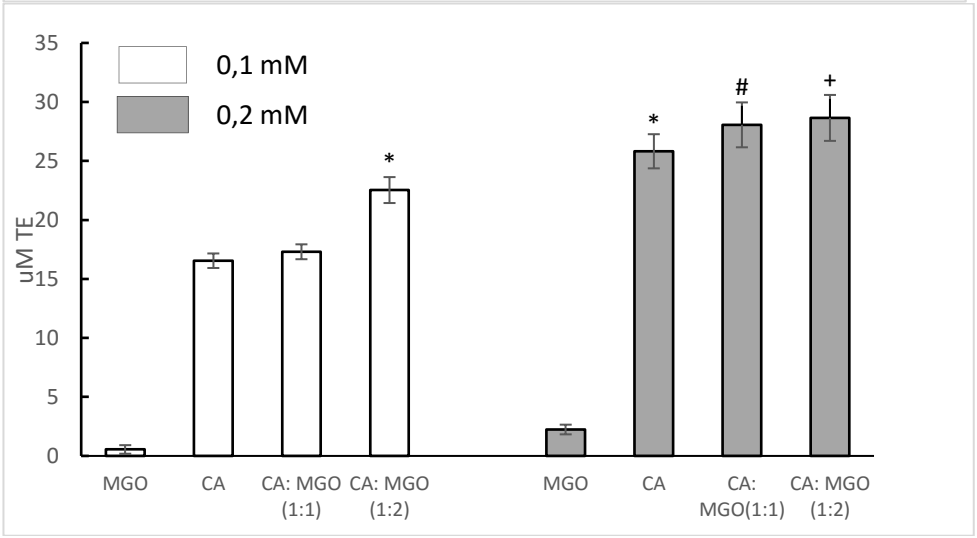
GA
GA: MGO (1:1)
GA: MGO (1:2)
MGO
GA
GA: MGO (1:1)
GA: MGO (1:2)


35
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25
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15
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5
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uM TE
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MGO
CA
CA: MGO (1:1)
CA: MGO (1:2)
MGO
CA
CA: MGO(1:1)
CA: MGO (1:2)

Figure 3.8: **Effect on the antioxidant activity (ORAC) of PY, GA and CA alone and in combination with MGO.** Data is an average of three experiments ± SEM. * indicates significant differences in antioxidant activity compared to polyphenol alone # indicates significant differences compared to polyphenol:MGO at 1:1 and 2:2 ratio, and + indicates significant differences compared to polyphenol:MGO at 1:2 and 2:4 ratio, p≤0.05**.**
KEY: CA= Caffeic acid; GA= Gallic acid; PY= Pyrogallol; MGO= Methylglyoxal; SEM= standard error of mean, ORAC= Oxygen radical absorbance capacity.

Trapping of MGO by phenolic acids results in new molecules, which have unique properties and bioactivities. Although the degree of trapping of MGO by PY was only 30% and absent for GA and increased for CA (DPPH assay) and increased for PY, unchanged for GA and slightly increased for CA (ORAC assay), findings are that these phenolic acids derivatives have unique properties. Future studies should focus on the synthesis of these adducts and the evaluation of bioactivity such as antioxidant and anti-inflammatory properties.

The MGO content of UMF 10 Manuka honey is 747 ± 40 mg/kg that is calculated to be equivalent to 13.5 mM. The TPC of UMF 10 honey is 119.23 mg GAE/kg (Magoshi, 2017) and this is equivalent to 7.17 mM GA. The ratio of MGO : polyphenols in Manuka UMF 10 honey is 1: 1.88. Phenolic acids present in Manuka honey are CA, isoferulic acid, p-coumaric acid, GA, 4-hydroxybenzoic acid and syringing acid. Based on the structural requirements defined by Navarro & Morales (2015) only CA and GA in Manuka honey can trap MGO.

The pH of honey is acidic and for UMF 10 Manuka honey was found to be 3.92 (Magoshi, 2017). Navarro & Morales (2015) determined the effect of pH on the ability of phenolic acids to trap MGO. The effect of different PBS solutions (saline, 10, 50 and 100 mM pH 7.4 as well as 100 mM at pH 5.5), sodium acetate buffers (pH 4.5 and 7.5) as well as water and alkaline water, pH 8.5 on MGO trapping was determined. For the trapping of MGO by HT, trapping was > 95% for all buffers at pH 7.4. At pH 4.5, 5.5 and 8.5 as well as in water trapping was reduced to <10%. However, in the present study where phenolic acids and MGO were prepared in water a significant degree of adduct formation occurred such as mono- and di adduct formation for PY (Figure 3.3). This also implies that at a low pH as found in 100% UMF10 Manuka honey, levels of MGO adducts will be low, however with dilution of this honey in for example in buffer pH 7.4 or cell culture media adduct formation may occur especially if samples are stored in these buffers for extended times. This may also be a confounding factor when quantifying MGO levels in honey.

Although indications are that MGO phenolic adducts may not form in honey due to its acidity, it may be important to determine whether unidentified phenolic molecules are not MGO polyphenol adducts as this effect of pH is based on one study by Navarro & Morales (2015).

3.5 Conclusion

PY traps MGO with the formation of the mono- and di-MGO adducts and this results in a decrease in TPC and antioxidant activity measured with the DPPH (ET assay - hydrophobic) assay. However, antioxidant activity measured with the ORAC (HAT assay) was increased and this could be due to different mechanisms of the ET vs. HAT assays. GA does not bind MGO and the presence of MGO does not alter the TPC or the antioxidant activity of GA measured with the DPPH and ORAC assays. CA and MGO did not result in adduct formation although the TPC was reduced, but antioxidant activity measured with the DPPH and ORAC assays was increased. For all combinations activity measured with the TEAC assay (ET assay – hydrophilic) was unaltered.

4 CHAPTER 4: THE CELLULAR ACTIVITY OF METHYLGLYOXAL AND PHENOLIC ACIDS ALONE AND IN COMBINATION

4.1 Introduction

MGO can be generated from sugar autoxidation, by the Maillard reaction, which is a process that mainly occurs in food containing many carbohydrates (Wang & Ho, 2012) such as honey.

Physiological concentrations of MGO in human plasma is between 100 – 600 nM (McLellan *et al*.,1992, Nemet *et al*., 2004) while in diabetic patients levels are 1.6 – 2.7 mM (Tan *et al*., 2008). Treatment of diabetic wounds with honey with high MGO levels is of concern, as MGO is cytotoxic. Concentrations of 0.2 – 10 mM causes damage to a wide range of cell types specifically targeting the antioxidant enzymes glutathione reductase (GR), SOD and catalase (Table 4.1). *In vivo* studies also identify the antioxidant pathways as specific targets (Table 4.1).

Table 4.1: Effect of MGO on antioxidant enzymes activity adapted from Kalapos, 2008.

***In vitro* studies**					
Enzyme	**Species**	**Tissue/cell type**	**Inhibition (%)**	**Concentration**	**Incubation time (h)**
GR	Bovine	Not mentioned	87.5	1 mM	24
GR	Rat (WKY)	Aortic VSMC	50.0	0.6 mM	24
GR	Rat (SHR)	Aortic VSMC	70.0	0.6 mM	24
GPx	Rat (WKY)	Aortic VSMC	40.0	0.6 mM	24
GPx	Rat (SHR)	Aortic VSMC	76.0	0.6 mM	24
CAT	Human	ADF (glioblastoma)	±40% ↑	0.2 mM	24
CAT	Human	SH-SY 5Y (neuroblastoma)	± 100% ↑	0.2 mM	24
CAT	Mouse	Liver	25.0	10 mM	2
SOD	Mouse	Liver	16.0	10 mM	0.5
SOD	Human	ADF (glioblastoma)	± 20% ↑	0.2 mM	24
SOD	Human	SH-SY 5Y (neuroblastoma)	± 100% ↑	0.2 mM	24
DT-diaphorase	Mouse	Liver	15.0	10 mM	1
***In vivo* studies**					
Enzyme	**Species**	**Tissue**	**Inhibition (%)**	**Concentration**	**Incubation time (h)**
SOD	Mouse	Liver	14	400 mg/bw kg	6
CAT	Mouse	Liver	33	400 mg/bw kg	6
CAT	Mouse	Spleen	64	400 mg/bw kg	6
DT-diaphorase	Mouse	Liver	10	400 mg/bw kg	6

GR glutathione reductase, GPx glutathione peroxidase, CAT catalase and SOD = superoxide dismutase. Wistar Kyoto rats (WKY); spontaneously hypersensitive rats (SHR); neuroblastoma cell lines (SH-SY-5Y); glioblastoma cell line (ADF)

This is due to the interaction of MGO with specific amino acids (Figure 4.1). MGO reduces cellular antioxidant defence systems by forming a hemithioacetal adduct with the –SH groups of the cysteine residue. Other amino acids to which MGO binds are the guanidine group of Arg, the Ɛ-amine residue of Lys and to sulfhydryl group of Cys (Kalapos, 2008). Inhibition of enzyme activity, protein glycation and GSH depletion can lead to cellular malfunction and oxidative stress (Figure 4.1). Binding and modification of nucleic acids can also lead to genotoxicity and mutagenicity (Ahmed *et al*., 2003).

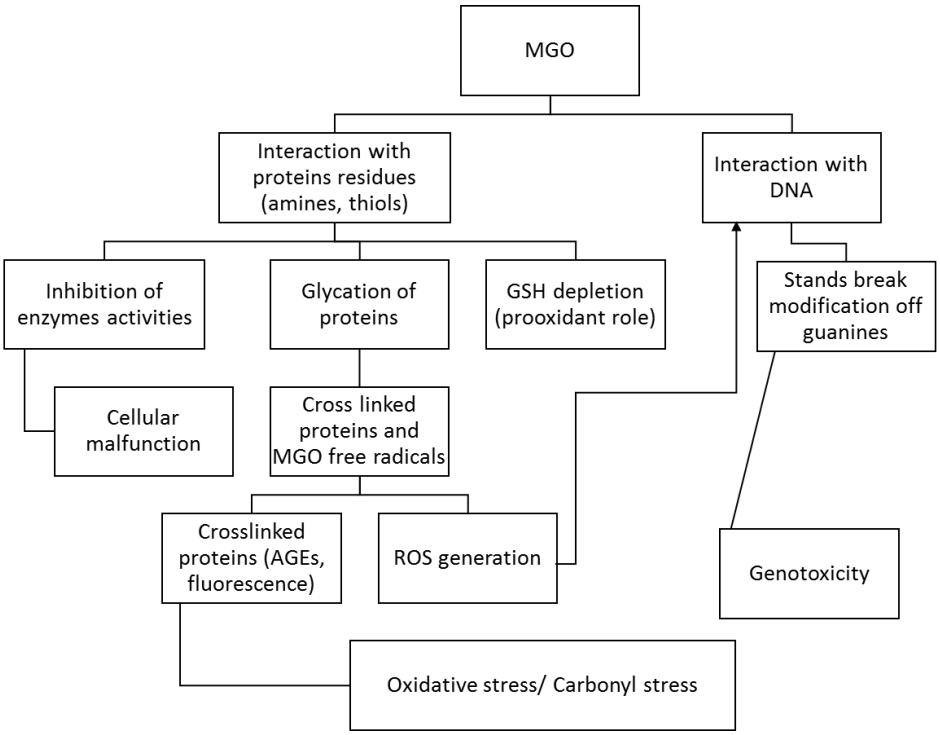

Figure 4.1: Effect of MGO on protein and DNA structure, leading to enzyme inhibition, cellular malfunction leading to cellular and tissue damage (adapted from Kalapos, 2008).

Anti-glycation agents function as trapping agents for reactive carbonyl compounds such as MGO thereby preventing oxidative stress and further formation of AGEs. However it is unknown if anti-glycation molecules such a polyphenols (Kalapos, 2008) directly trap MGO with the formation of adducts or protect cells and tissue against ROS (Figure 4.1).

The aim of the research in this chapter is to determine if trapping of MGO by phenolic acids, alters the cytotoxicity of MGO in the SC-1 and Caco-2 cell line. To determine if MGO exposure leads to ROS formation and/or makes these cells more sensitive to the effects of oxidative agents.

4.2 Materials

Potassium dihydrogen phosphate (KH_2PO_4), ethylenediaminetetraacetic acid (EDTA), and potassium chloride (KCl), were of analytical quality and were obtained from Merck Chemicals, Modderfontein, SA. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Dimethyl sulfoxide (DMSO), Dulbecco's modified essential medium (DMEM), trypsin powder, antibiotic solution (each ml containing 10 mg streptomycin, 10000 units penicillin and 25 µg amphotericin B), paraformaldehyde, gluteraldehyde, Crystal Violet, acetic acid (CH_3COOH) were obtained from the Sigma-Aldrich Company, Atlasville, SA. Foetal calf serum (FCS) was obtained from BIOCOM diagnostics, SA. Phenolic acids samples: were used as described in Section 3.2.1

Lambda LS5OB spectrophotometer from Perkin Elmer, Boston, MA, USA supplied by Separations Scientific, Honeydew, SA. The microplate reader and the well plates were the same as used in section 3.2.1.

4.3 Methods

4.3.1 Samples, buffers and media

A stock solution of 40% MGO (6.50 M) was diluted as described in Section 3.3.1.Likewise PBS was prepared as described in Section 3.3.2.. For the cell culture of Caco-2 and SC-1 cells DMEM was made 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 filtering the solution, using 0.2 µm sterile filters (GVS Life sciences, USA). The medium was supplemented with 1% of antibiotics solution and 10% of Foetal Calf Serum (FCS). PBS- EDTA consisting of 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4 and 0.53 mM EDTA was used in addition to trypsin for the trypinisation of the Caco-2 cell lines. The SC-1 cell lines were trypsinised with trypsin only. The trypsin solution was a 5% solution (5 g trypsin in 100 ml PBS). The PBS-EDA and the trypsin solutions were sterilized as described above.Once experiments were complete, the cell lines were stored in freezing medium which was made by mixing 10% DMSO and 80% FCS made up in DMEM. All media and solutions were kept at 4˚C.

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

For the following experiments, the SC-1 (fibroblast) and Caco-2 (colon adenocarcinoma) cell lines, obtained from the American Type Culture Collection (ATCC) both adherent cells were

maintained in DMEM, each supplemented with 10% FCS (DMEM/FCS) and a 1% antibiotic solution. Vials containing the cells were thawed rapidly in warm water at 37˚C. The cells were suspended in 5 ml medium supplemented with FCS, and collected by centrifugation. The supernatant was removed and the cells were suspended in fresh medium and were counted then plated at $4x10^4$ cells per ml in 25 cm^2 and 75 cm^2 cell culture flasks and were maintained until confluency at 37˚C at 5% CO_2. Once confluent, the cells were passaged with a 5% trypsin solution prepared in PBS.

For the SC-1 cells (doubling time = 30 h), the cells were passaged by removing the medium from the confluent monolayer then adding 1 ml of a 5% trypsin solution and the flask were placed at 37˚C for 1 – 2 min. A 5 ml volume of DMEM was then added to the trypsin solution containing detached cells, and transferred to a 15 ml centrifuge tube and the cells were collected by centrifugation at 792 xg for 2 min. The medium was removed and the cells were re-suspended in 5 ml DMEM/FCS. The numbers of cells were determined by counting a 10 µl aliquot of cells using a haemocytometer.

For the Caco-2 cells (doubling time = 62 h), the cells were passaged by removing the medium from the confluent monolayer. The monolayer was rapidly rinsed with a 5 ml, 0.53 mM EDTA/PBS solution. The Caco-2 cells were then processed further as described for the SC-1 cell line.

These cell lines were either used for experiments described below or stored for later use at -70˚C. For storage, the cells were suspended in cell culture freezing medium at a concentration of $2x10^5$ cells/ml. A volume of 1.8 ml was transferred to the freezing vials and stored by slow freezing (first placed on ice, then in -20˚C and finally in a -70˚C freezer), cells were stored for a maximum of 6 months with minimal loss of viability. For longer storage the cells were stored in liquid nitrogen (-196˚C).

4.3.3 Cytotoxicity of phenolic acids and MGO, alone and in combination on SC-1 and Caco-2 cells

4.3.3.1 Cell exposure

Cells (SC-1 and Caco-2) were plated in a 96 well plate (90 µl) at a concentration of $2X10^4$ cells/ml and left overnight to attach. The cells were then exposed to 10 µl of MGO alone or in

combination with phenolic acids at a final concentration of 0.1 mM MGO : 0.1 mM PY, GA or CA or 0.2 mM MGO : 0.1 mM PY, GA or CA for 24 h at 37˚C and 5% CO_2.

4.3.3.2 Cell viability – MTT assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay depends on conversion of the yellow water soluble dye by mitochondrial succinate dehydrogenase to a water insoluble purple formazan product, which can be solubilised by various organic solvents (Vega-Avila & Pugsley, 2011). The amount of formazan produced is directly proportional to cell function, therefore cell viability (Vega-Avila & Pugsley, 2011). However, research has shown that MTT is also reduced in other cellular compartments in the cytoplasm and plasma membrane (Bernas & Dobrucki, 2002) and consequently other assays such as CV assay is used to confirm results.

Following exposure, 10 µl of 1 mg/ml of MTT dissolved in PBS was added to the media of either SC-1 or Caco-2 cells exposed to serial dilutions of phenolic acids and MGO alone or in combination. After 3 h incubation at 37˚C, 5% CO_2, the medium was removed and the plates dried. A 100 µl volume of a 25% DMSO in EtOH solution was then added to each well. This was followed by gentle shaking to achieve complete solubilize the MTT formazan crystals. The absorbance was read at 570 nm and the data was expressed as percentage cell viability compared to control, which were cells exposed to PBS.

4.3.3.3 Cell number – Crystal Violet (CV) assay

CV is a positively charged dye that stains the negatively charged components of the cell i.e. DNA and protein such as protein with a high aspartic and glutamic acid content. The extent of staining up is proportional to cell number (Mosmann, 1983; Vega-Avila & Pugsley, 2011). The exposed cells were fixed with 10 µl of 20% formaldehyde for 30 min. The medium was then removed and the plate dried. The attached cells were then stained with 100 µl of 0.1% (w/v) CV solution prepared in 200 mM (0.75%) formic acid for 30 min. The plates were rinsed with H_2O and air dried before dissolving the bound dye with 100 µl of 10% acetic acid. The absorbance was read at 620 nm and the data was expressed as percentage cell number compared to control, which were cells exposed to PBS.

4.3.3.4 Oxidative damage – DCFH-DA assay

DCFH-DA is a cell permeable compound hydrolysed by cellular esterases of live cells to DCFH (non-fluorescent). Once in contact with free radicals the hydroxyl group in DCFH is removed to stabilize radicals making DCFH fluorescent DCF. Cellular antioxidant activity is the measure of the ability of antioxidants to prevent or reduce the conversion of DCFH to DCF consequently reducing fluorescence (Wang & Joseph, 1999).

Both SC-1 and Caco-2 cells were plated at a concentration of $2x10^4$ cells/ml in a volume of 100 µl in 96 well plates. After 24 h incubation to allow the cells to attach and adapt to the cell culture environment, a volume of 50 µl DCFH-DA working solution (75 µM) was added to each well, to a final concentration of 25 µM. The plate was then further incubated for 1 h at 37˚C. The medium containing the DCFH-DA solution was then removed and cell culture plates washed once gently with PBS and blotted dry. A volume of 40 µl of each phenolic acid and MGO alone or in combination (as described in Section 3.1.1) was added to each well of the cell culture plates, with a final concentration of 0.5 mM, followed by 40 µl of 15 mM AAPH (final concentration 7.5 mM). Change in fluorescence was measured immediately over 0 – 60 min, every 2 min at an excitation wavelength of 485 nm and an emission wavelength of 520 nm. A vehicle control included a well with PBS only whereas a positive control included a well with AAPH only. The gradient of the change in fluorescence was calculated, and the data expressed as % damage where AAPH alone causes 100% damage using the following equation:

% Damage = [(Sample-PBS)/(AAPH-PBS)]*100

4.3.4 Data management and statistical analysis

All experiments were performed at least in triplicate and data was reported as mean ± (standard error of mean) SEM. Significant differences between samples were determined either using the students T-TEST or one way ANOVA with Tukey's post-hoc analysis.

4.4 Results and discussion

4.4.1 Cytotoxicity of MGO and phenolic acids alone and in combination

In this study SC-1 and CaCo-2 cells were used in order to observe toxicity of MGO and phenolic acids alone and in combination. The SC-1 cell line are fibroblast cells while the Caco-

2 cells represent epithelial cells. Two different cells types were used as the response of these cells to MGO may vary.

The current study showed that SC-1 cell line (Table 4.2) exposure to MGO caused an increase in cell number and viability and this was not significantly different to the control no MGO added. PY, GA and CA exposure did cause a change in SC-1 cell number. PY and CA did not affect cell viability while in contrast GA a significant increase in cell viability was observed. At a 1:1 MGO : PY, GA or CA ratio SC-1 cell number and viability was unchanged. At a ratio of 2:1 only MGO in combination with GA caused a statistically significant decrease in cell number but not cell viability.

In this current study Caco-2 cell line (Table 4.3) exposure to MGO for 24 h resulted no changes in cell number or viability. Likewise exposure to PY, GA or CA alone did not alter cell number or viability. For MGO: PY, GA and CA combinations there was no significant change in cell number or viability.

Table 4.2: Effect of MGO alone and in combination with PY, GA and CA on SC-1 cell number and viability.

CV (Cell number)			
	0 mM MGO	**0.1 mM MGO[1]**	**0.2 mM MGO[2]**
	100 ± 15.78%	126.50 ± 15.86%	110.76 ± 9.70%
0.1 mM PY	106.50 ± 5.38%	110.04 ± 9.83%	119.78 ± 10.39%
0.1 mM GA	109.67 ± 15.56%	99.32 ± 15.93%	90.04 ± 4.47%
0.1 mM CA	105.60 ± 5.38%	105.31 ± 13.32%	118.63 ± 14.53%
MTT (Cell viability)			
	0 mM MGO	**0.1 mM MGO**	**0.2 mM MGO**
	100 ± 5.15%	112.05 ± 6.26%	101.99 ± 4.42%
0.1 mM PY	100.71 ± 3.66%	103.50 ± 1.99%	104.91 ± 2.61%
0.1 mM GA	112.41 ± 0.55%*****	100.08 ± 1.29%	90.60 ± 4.31%[#]
0.1 mM CA	106.68 ± 8.72%	103.92 ± 4.23%	113.59 ± 3.74%

[1] Ratio 1:1, [2] Ratio 1:2. Data is an average of three experiments ± SEM. * indicates significant differences of samples compared to control (100% cell viability). [#] indicates significant differences between individual phenolic acids and phenolic acids combined with MGO.
KEY: CA= Caffeic acid; GA= Gallic acid; PY= Pyrogallol, MGO= Methylglyoxal; SEM= Standard error of mean, CV= Crystal Violet MTT= 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

Table 4.3: Effect of MGO alone and in combination with PY, GA and CA on Caco-2 cell number and viability.

CV (Cell number)			
	0 mM MGO	**0.1 mM MGO[1]**	**0.2 mM MGO[2]**
	100 ± 21.88%	85.08 ± 1.22%	90.04 ± 8.29%

	0 mM MGO	0.1 mM MGO[1]	0.2 mM MGO[2]
0.1 mM PY	103.25 ± 3.31%	87.85 ± 8.52%	85.30 ± 5.72%
0.1 mM GA	81.52 ± 7.12%	81.36 ± 10.40%	89.21 ± 10.12%
0.1 mM CA	103.79 ± 6.59%	98.82 ± 9.82%	88.18 ± 10.57%
MTT (Cell viability)			
	0 mM MGO	**0.1 mM MGO**	**0.2 mM MGO**
	100 ± 23.07%	90.72 ± 2.25%	86.79 ± 3.41%
0.1 mM PY	87.26 ± 6.78%	84.61 ± 3.62%	85.67 ± 3.60%
0.1 mM GA	84.67 ± 7.20%	88.99 ± 9.09%	92.47 ± 18.52%
0.1 mM CA	92.78 ± 4.36%	85.62 ± 8.30%	87.78 ± 9.82%

[1] Ratio 1:1, [2] Ratio 1:2. Data is an average of three experiments ± SEM. No significant differences for both assays compared to control (100% cell number or viability) was observed.

KEY: CA= Caffeic acid; GA= Gallic acid; PY= Pyrogallol, MGO= Methylglyoxal; SEM= Standard error of mean, CV= Crystal Violet MTT= 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

In a study by Kalapos *et al*., (1991) the authors identified that at MGO concentrations < 1 mM, an increase of glucose formation is observed, between 1 and 5 mM MGO, glucose formation is static, while at concentrations > 5 mM MGO, a plateau is observed. At 0.1 mM MGO in the SC-1 cells an increase in cell number and viability is observed although not statistically significant and this may be due to increase gluconeogenesis identified by Kalapos *et al*., (1991) at low MGO concentrations.

Kalapos (2008), proposed three targets of MGO toxicity. Firstly the direct inhibition by MGO of enzymes results in defective cellular function, secondly GSH binding that leads to GSH depletion and thirdly a direct interaction of MGO with DNA causing the emergence of genotoxicity that can lead to carcinogenicity.

Carbonyl stress is the result of antioxidant enzyme inhibition (Table 4.1 and Figure 4.1) as well as the inhibition of enzymes involved in intramitochondrial transport (Leoncini *et al*., 1980). These effects together with the depletion of GSH results in the accumulation of ROS as cellular control mechanisms and pathways are dysfunctional. ROS accumulation leads to oxidative membrane, protein and DNA damage. If this damage to the cellular macromolecules is not repaired, cellular death via apoptosis or necrosis occurs. The last phase of toxicity is the result of the carcinogenicity of dicarbonyls and surviving cells either regenerate or are transformed to genetically altered cells with tumorigenic potential.

As shown in Table 4.1, 0.6 - 10 mM MGO has an inhibitory effect on enzymes associated with the elimination of free radicals such as GR, GPx, catalase and SOD. Seo *et al*., (2014) investigated the potential of MGO to cause toxicity/ apoptosis and impaired mitochondrial

function in HepG2 (hepatocarcinoma cells). The HepG2 cells were exposed to 3 and 10 mM MGO for 36 h and observed a significant decrease in the cell viability measured with the MTT assay. MGO was found to cause the activation of caspase-3 a marker of mitochondrial mediated apoptosis. In contrast, in the present study no changes in cell number or viability was observed and this may be related to the concentrations and exposure times used. As shown in Table 4.1 changes in the levels of antioxidant enzymes after 24 h exposure was only observed at MGO concentrations ≥ 0.2 mM. In the HepG2 cell line Seo *et al*., (2014) only observed changes in cell viability after 36 h exposure to 3 and 10 mM MGO.

MGO cytotoxicity mainly occurs through the increase of oxidative stress and apoptosis induction. Some cells are more sensitive to apoptosis following exposure to MGO. For example, Jurkat cells are more sensitive to H_2O_2 generated ROS compared to HeLa cells, which are less H_2O_2 sensitive (Nakagawa *et al*., 2004). Differences in sensitivity may be related to differences in enzymatic activity associated with different cellular detoxifying pathways such as glyoxalase, aldehyde dehydrogenase and carbonyl reductase pathways (Kalapos, 1999). The important detoxifying enzyme is glyoxalase that plays an important role against glycation and oxidative stress.

Busch *et al*., (2010) proposed several cell surface proteins as receptors binding to AGEs, namely receptor of advanced glycation end products (RAGE) detected in various tissues such as endothelial cells, vascular smooth muscle cells, peripheral blood mononuclear cells, neural tissue, lung tissue and skeletal muscle cells. The expression of these receptors is dependent on disease state e.g. RAGE expression in endothelial cells is increased in diabetic patients. These receptors that bind AGE activate pro-inflammatory transcriptional factor NF-$_{\kappa}\beta$ and also induce the generation of ROS through activated NADPH oxidase (Yang, 2011). Furthermore, RAGE expression in diabetic mice has been found to lead to the development of diabetic nephropathy. In human kidney podocytes and endothelial cells RAGE activation was found to cause hypertrophy with cell apoptosis and pro-inflammatory cytokine generation (Busch *et al*., 2010). The authors have shown that MGO is liable to cause toxicity from diabetic complications, aging, and neurodegenerative disorders, depending on the targeted cells.

Phenolic acids known for their antioxidant activity are able to defend against glycation either by directly trapping MGO (Kalapos, 1999) or at the later stages of toxicity scavenging generated radicals, thereby reducing oxidative stress (Allaman *et al*., 2015).

MGO induces AGE formation and the AGE-albumin (AGE-Alb) formation assay is widely used to evaluate how polyphenols can reduce AGE formation. AGE-Alb is generated by incubating

of human serum albumin (50 mg/ml) with D-glucose (0.5 mol/L) in phosphate buffer. After several weeks, AGE-Alb will form (Hong *et al*., 2015). This formed AGE-Alb inhibited the proliferation of human peritoneal mesothelial cells in a dose- and time-dependent manner. The AGE-Alb increased the mRNA and protein expression of cytokines including vascular endothelial growth factor (VEGF) and monocyte chemotactic protein-1 (MCP-1), by activation of p38MAPK signalling, leading to the development of diseases such as diabetes mellitus, inflammation as well as vascular disease. Increased levels were attenuated following the addition of antioxidant N-acetyl-cysteine (NAC). Cell culture medium contains albumin and therefore AGE-Alb can form, however due to the short exposure times used in this study, levels AGE-Alb would be minimal, and consequently the major effect of MGO is its direct GSH binding ability and direct inhibition of antioxidant enzyme activity.

These effects would not be observed if change in cell number and viability is measured and therefore future studies should evaluate the levels of GSH and activity of antioxidant enzymes.

4.4.2 Cellular antioxidant activity of MGO and phenolic acids alone and in combination

The degree of ROS formation can be determined can be determined in cell culture using the DCFH-DA assay. This assay is fluorescent and based on the ability of the compound to quench free radicals and therefore reduce the fluorescence of DCF (Wang & Joseph, 1999).

AAPH is a water-soluble azo compound, used as a free radical generator, and during degradation generates O_2 and two carbon radicals. The carbon radicals may combine to produce stable compound or react with O_2 to generate peroxyl radicals. MGO, PY, GA and CA alone at all concentrations evaluated did not cause an increase in radical formation (Figure 4.2). Wu & Juurlink (2002), showed that MGO is able to generate ROS and Wang & Ho (2012), showed that accumulation of MGO in cells may cause carbonyl stress which is the first step that induces the formation of H_2O_2 which would increase oxidative stress and result in tissue damage.

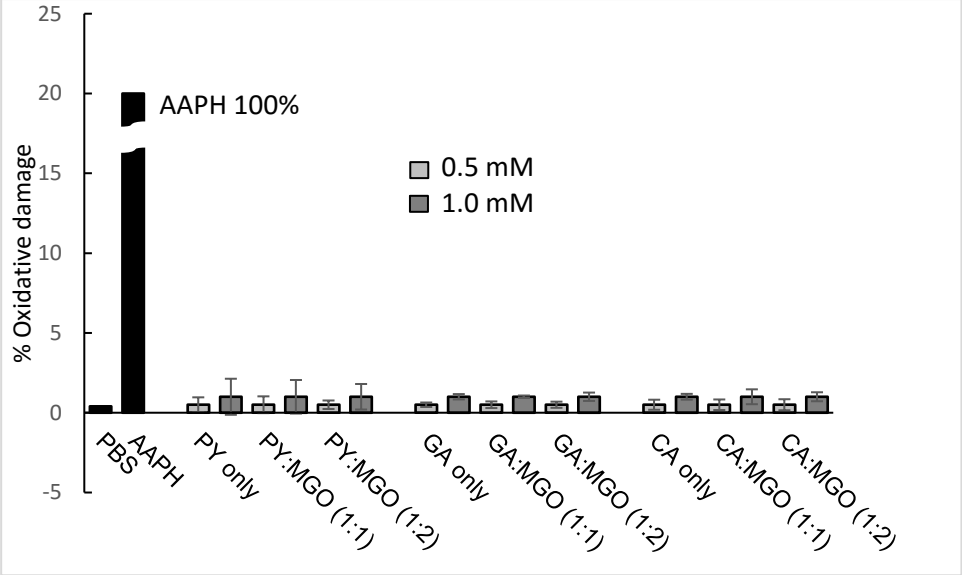

Figure 4.2: <u>Effect of MGO alone and in combination with PY, GA and CA without AAPH oxidative damage in SC-1 cells.</u> Data is an average of three experiments and represented as mean ± SEM.
KEY: CA= Caffeic acid; GA= Gallic acid; PY= Pyrogallol, MGO= Methylglyoxal; SEM= Standard error of mean, AAPH= 2,2'-Azobis(2-amidinopropane) dihydrochloride.

In the present study, 0.5 and 1 mM MGO did not cause an increase in DCF after 60 min exposure (Figure 4.2). Only in combination with AAPH, 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 (Table 4.4). Wu & Juurlink (2002) showed that MGO increased oxidative stress by inactivating antioxidant enzymes such as GR and GPx due to MGO glycation (Table 4.1). However, these enzymes are not the only MGO targets and AGE formation can cause membrane destabilisation, changes in permeability as well as structural and functional changes to protein. This will make cells more susceptible to AAPH induced oxidative damage.

Exposure to MGO for 24 h did not cause a statistically significant change in the cell number and viability of SC-1 and Caco-2 cells, however the DCFH-DA assay reveals that these cells may be more susceptible to AAPH induced oxidative damage. In the SC-1 cell line, PY, GA and CA did not effectively scavenge AAPH radicals. In contrast, in the Caco-2 cell line, PY, GA and CA effectively reduced the oxidative effects of AAPH from 100% to 56.07 ± 12.44%, 81.67 ± 33.90% and 65.72 ± 4.04%, respectively (Table 4.5).

Compared to SC-1 cells exposed to MGO and AAPH, PY, GA and CA effectively reduced measured cellular levels of oxidative damage. Of the three phenolic acids evaluated CA was the most effective with levels being reduced from 358.86 ± 38.51% to 60.64 ± 3.93% for 0.5 mM MGO and from 298.83 ± 49.04% to 90.45 ± 17.64% for 1 mM MGO. In the Caco-2 cell

line, CA effectively reduced the oxidative effects of 0.5 mM MGO from 306.42 ± 64.83% to 57.10 ± 25.79% and 1 mM MGO from 185.18 ± 7.60% to 53.98 ± 12.08%. Although at 1 mM MGO, GA was the phenolic acid which most effectively reduced measured levels of oxidative damage.

Table 4.4: Effect of MGO alone and in combination with PY, GA and CA on AAPH-induced oxidative damage in SC-1 cells.

DCHF-DA assay (Cellular antioxidant activity)			
	0 mM MGO	**0.5 mM MGO[1]**	**1 mM MGO[2]**
	100 ± 17.93%	358.86 ± 38.51%*	298.83 ± 49.04%*
0.5 mM PY	132.77 ± 19.26%	143.32 ± 20.34%	122.23 ± 26.88%+
0.5 mM GA	106.04 ± 24.62%	115.45 ± 51.44%	93.93 ± 16.17% #+
0.5 mM CA	109.09 ± 23.17%	60.64 ± 3.93% #+	90.45 ± 17.64% #+

[1] Ratio 1:1, [2] Ratio 2:1. Data is an average of three experiments and represented as mean ± SEM. * indicates significant differences compared to control (AAPH), # indicates significant differences compared to 0.5 mM MGO and + indicates significant differences compared to 1 mM MGO.
KEY: CA= Caffeic acid; GA= Gallic acid; PY= Pyrogallol; MGO= Methylglyoxal; SEM= Standard error of mean; DCFH-DA= Dichlorofluorescein diacetate; AAPH= 2,2'-Azobis(2-amidinopropane) dihydrochloride.

Table 4.5: Effect of MGO alone and in combination with PY, GA and CA on AAPH-induced oxidative damage in Caco-2 cells.

DCHF-DA assay (Cellular antioxidant activity)			
	0 mM MGO	**0.5 mM MGO[1]**	**1 mM MGO[2]**
	100 ± 9.31%	306.42 ± 64.83%*	185.18 ± 7.60%
0.5 mM PY	56.07 ± 12.44%*	73.85 ± 7.07%#	62.01 ± 23.26%#
0.5 mM GA	81.67 ± 33.90%	95.97 ± 15.82%#	48.14 ± 16.64%#
0.5 mM CA	65.72 ± 4.04%	57.10 ± 25.79%#	53.98 ± 12.08%#+

[1] Ratio 1:1, [2] Ratio 2:1. Data is an average of three experiments and represented as mean ± SEM. * indicates significant differences compared to control (AAPH), # indicates significant differences compared to 0.5 mM MGO and + indicates significant differences compared to 1 mM MGO.
KEY: CA= Caffeic acid; GA= Gallic acid; PY= Pyrogallol; MGO= Methylglyoxal; SEM= Standard error of mean; DCFH-DA= Dichlorofluorescein diacetate; AAPH= 2,2'-Azobis(2-amidinopropane) dihydrochloride.

Many studies have shown that in different cell-types, the addition of MGO to the incubation medium led to a concentration dependent rise of oxidation-sensitive fluorescence of DCFH-DA. In a macrophage-derived cell line (U937) exposed to 0.3 mM MGO (Okado *et al*., 1996), in rat mesenteric artery smooth muscle cells (VSMC) exposed to 0.1 mM MGO (Chang *et al*., 2005) and in rat thoracic aorta cell line (A10) exposed to 0.5 mM MGO (Wu, 2005) the addition of MGO led to a concentration-dependent elevation of DCF fluorescence.

Kalapos (2008) further treated these cells with buthionine sulfoximine (BSO) that reduces GSH levels by inhibiting gamma-glutamylcysteine synthetase activity. This inhibition resulted in increased fluorescence. Whereas the addition of NAC and GSH suppressed DCF

fluorescence and measured oxidative damage. Brebowicz (2004), cultured cortical neurons prepared from rat fetuses and treated them with 50 µM MGO for 30 min. Mesothelial cells were also exposed to 0.15 mM MGO for 8 h. The author found that intracellular generation of free radicals increased DCF fluorescence and the associated increase in free radical formation caused a decrease in cell division. A study by Chang *et al*., (2005), showed that rat VSMCs treated with MGO increased DCF fluorescence that was inhibited following the addition of antioxidant enzyme, SOD. Addition of CAT did not provide any protection against MGO induced oxidative damage. The NOX family of NADPH oxidases are key producers of cellular ROS. Inhibition of NAD(P)H oxidase with the inhibitor, diphenylene iodonium (DPI) and nitric oxide (NO) synthase inhibitor with nitro-arginine-methylester (L-NAME) results in reduced oxidative damage. These researchers concluded that MGO induced peroxynitryl radical formation due to superoxide anion and NO formation.

MGO is known to increase the sensitivity of cells to oxidative stress. In the present study MGO at 0.5 – 1 mM MGO alone did not cause oxidative damage, however with the addition of AAPH and increase in DCF fluorescence was observed. PY is known to bind MGO and depending on the reaction, equilibrium in solution there would be MGO, PY and the MGO-PY adducts. This would result in two different effects PY would reduce MGO levels and the free unbound PY would directly scavenge AAPH generated radicals. The chemical antioxidant assays reveal that PY reduces measured TPC and antioxidant activity determined with the DPPH while antioxidant activity measured with the ORAC assay was increased. The source of radicals in the ORAC and DCHF-DA assay is AAPH. From the findings in Chapter 3, it can be concluded that PY binds MGO but also has heightened scavenging activity.

In contrast, GA does not bind MGO (section 3.4.4.1 and 3.4.4.2) and therefore can only scavenge AAPH generated radicals. This reduces the observed toxicity of MGO in combination with AAPH. For CA, the TPC is reduced (section 3.4.4.1) but antioxidant activity measured with the DPPH assay is increased, although the antioxidant radical scavenging activity of CA is unchanged in TEAC and ORAC (section 3.4.4.2). Therefore, the effect of CA is direct scavenging of AAPH generated radicals.

Du *et al*., (2001) used Jurkat cells and incubated them with 0.25 mM MGO with the presence of 50 µM diethyldithiocarbamate (DDC) 30 min. DCC inhibits SOD which leads to intracellular O_2^- accumulation. The authors found that a 2-fold increase of luminescence was detected in comparison to controls after 30 min. The findings of this study was similar to the present study where an increase in oxidative damage is only observed with MGO in combination either an additional source of radicals or the inhibition of the antioxidant pathway.

Exposure of SC-1 and Caco-2 cells to MGO for 24 h did not cause significant changes in cell number or viability. However, as for the DCFH DA assay, these cells may be more vulnerable to the effects of ROS.

Additional cellular late onset effects are the mutagenic, carcinogenic and teratogenic effects of MGO, as AGE formation is a slow process. MGO in combination with H_2O_2 forms an acetyl radical that readily reacts with DNA (Nukaya *et al*., 1993). Phenolic acid trapping of MGO and/or direct radical scavenging will also reduce this effect. Although evaluation will require long term cultures and evaluation of effects with carcinogenesis and tumorigenesis assays.

Manuka honey contains both MGO and polyphenols. In the DCHF DA assay, honey effectively reduces AAPH induced oxidative damage. In Manuka honey, usually diluted in PBS buffer, some MGO trapping by polyphenols such a phenolic acids and flavanoids may occur. MGO may be cytotoxic, however direct scavenging of the AAPH generated radicals, this effect cannot be observed. It would be of value to determine the effect of Manuka honey on intracellular levels of antioxidant enzymes and GSH.

4.5 Conclusion

In conclusion, PY, GA and CA alone and in combination did not cause a decrease in SC-1 and Caco-2 cell number and viability. In combination with AAPH, MGO increased cellular oxidative damage measured in the SC-1 and Caco-2 cells. All phenolic acids evaluated reduced this effect with CA being the most effective although the exact mechanism involved is unknown. Possible observed effects are due to MGO inhibiting, cellular antioxidant pathways thereby increasing the susceptibility of these cells to oxidative damage. Phenolic acids, irrespective of the mechanism involved reduced cellular oxidative damage.

5 CHAPTER 5: THE ANTIBACTERIAL ACTIVITY OF METHYLGLYOXAL AND PHENOLIC ACIDS ALONE AND IN COMBINATION

5.1 Introduction

Chronic wounds occur mostly in patients with increased risk of bacterial invasion/infection, such as trauma, arterial insufficiency, or systemic diseases like rheumatoid arthritis and diabetes. The healing process is prolonged due to an expanded inflammation phase, but also due to defective re-epithelialization and impaired matrix remodeling (Harding, 2002). The colonization of chronic wounds changes overtime, and with time Gram-positive organisms are the predominant bacteria type found in these wounds. In addition, bacterial populations in wounds are arranged in organized biofilms protecting them not only from antimicrobial therapy but also from the immune system of the host. Biofilms are complex communities of collective bacteria embedded in a self-secreted extracellular polysaccharide matrix (EPS) (Bjarnsholt *et al*., 2008, Davis *et al*., 2008). Their growth and persistence within wounds is a contributing factor to impaired healing. Multidrug resistant organisms (MDRO) further complicate and delay wound healing (Edwards & Hardings, 2004).

Gram positive and negative bacteria differ in the structure of their cell walls, where the cell wall of Gram positive bacteria consist of a thick peptidoglycan layer and a plasma membrane whereas, the cell wall of Gram negative contains an outer membrane, a thin peptidoglycan layer and an inner membrane. MGO targets both Gram positive and negative bacteria irrespective of the composition of the cell wall. It is effective against chronic wounds *Pseudomonas* spp. *S. aureus* and *P. aeruginosa* (Majtan *et al*., 2013). Cooper *et al*., (2010) studied the effect on honey on biofilms and found that some honeys, particularly Manuka honey can be used as a bactericidal as it is effective against *P. aeruginosa* and MRSA biofilms *in vitro*. MGO also inhibited *S. aureus* and *P. aeruginosa* biofilm formation (Kilty *et al*., 2011). Roberts *et al*., (2014) showed that Manuka honey inhibited the expression of the major structural protein flagellin as well as flagellin-associated genes, fliA, fliC, flhF, fleN, fleQ and fleR. Rabie *et al*., (2016) showed that this effect was specifically due to MGO. De-flagellation of bacteria by Manuka honey and specifically MGO limits bacteria mobility, reduces bacterial adhesion and prevents biofilm formation.

The aim of the research undertaken in this chapter is to determine if trapping of MGO by phenolic acids reduces the antibacterial activity MGO and the effects of MGO alone and in combination with phenolic acids on bacteria morphology.

5.2 Materials

5.2.1 Reagents, equipment and disposable plastic ware

Formaldehyde, gluteraldehyde and poly-L-lysine were obtained from the Sigma-Aldrich Company, Atlasville, SA. Ethanol (EtOH), Hexamethyldisilazane (HDMS), osmium tetraoxide, sodium potassium phosphate and ethanol, tryptone, yeast extract were of analytical quality were obtained from Merck Chemicals, Modderfontein SA. Phenolic acids samples: were used as described in Section 3.2.1

Equipment used included: scanning electron microscope-Zeiss Ultra plus FEG from Oberkochen, Germany. The microplate reader and the well plates were used as described in Section 3.2.1.

5.2.2 Bacteria

Two model bacteria strains were used in this study and these were Gram positive *B. subtilis* (Strain 13933) and negative *E. coli.* (Strain 700928). These bacteria were obtained from the Department of Biochemistry, Faculty of Natural and Agricultural Sciences, University of Pretoria, supplied by the American Type Culture collection (ATCC).

5.3 Methods

5.3.1 Samples, buffers and media preparation

A stock solution of 40% MGO (w/w 6.50 M) was diluted as described in Section 3.3.1, MGO : Phenolic acid solutions were prepared as described in Section 3.3.1. Molar ratio solution of 1:1, 0.5 mM phenolic acids : 0.5 mM MGO and 1:2, 0.5 mM phenolic acids : 1.0 mM MGO were prepared in PBS as described in Section 3.3.1

Luria Bertani (LB) broth was prepared by mixing 10 g of tryptone, 5 g of yeast extract and 10 g of NaCl in 1 litre of ddH_2O. The pH of the solution will be adjusted to 7.5, then autoclaved to achieve sterility. Broth was kept at 4˚C when not in use.

5.3.2 Turbidity assay

Previous studies found the minimum inhibitory concentrations (MIC) were 1 mM and 0.8 mM for *E. coli* and *B. subtilis*, respectively (Rabie *et al*., 2016). The effect of 1 mM MGO alone and in combination with PY, GA and CA was determined using the turbidity assay as described by

Prinsloo *et al*., (2013). Briefly, bacteria were cultured overnight, diluted 100 times in LB broth and left to proliferate until an optical density (OD_{600}) of 0.4 – 0.7 was reached. The bacteria were then diluted with LB broth to a starting OD_{600} of 0.02 before being plated in sterile 96 well microtiter plates. To a volume of 50 µl of 0.02 LB cultures, 50 µl of the samples (final concentration of samples 0.5 mM for phenolic acids, 0.5 mM and 1 mM for MGO and bacteria, OD_{600} of standardized culture, 0.01) was added. Sterile controls contained LB broth only, whereas growth controls contained bacteria and LB broth. Plates were then incubated in the dark at 37˚C with shaking at 150 rpm for 24 h. Data was analysed according to Sherlock *et al*., (2010). Optical density was determined before (T0) and after (T24) incubation at 600 nm. The OD at 24 h was subtracted from the OD at 0 h. The growth control (bacteria and broth) was assigned 100% growth (G100). Percentage inhibition of growth was then determined using the formula:

%Inhibition= 100-[(T24-T0)/G100]*100

5.3.3 Scanning electron microscopy (SEM)

5.3.3.1 Poly L-lysine coating of coverslips

To prepare poly L-lysine coated glass coverslips, clean glass cover slip were washed with an alkaline solution of 10% NaOH in 60% EtOH for 2 h. The coverslips 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 glass coverslip was then coated with 1 m 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 left to dry at room temperature for 3 days before use.

5.3.3.2 Scanning electron microscopy

In order to evaluate the effect of MGO and phenolic acids, PY, GA and CA alone and in combination on bacteria morphology, scanning electron microscopy (SEM) was undertaken. Following exposure under conditions described in Section 3.3.1, a 100 µl volume of the exposed bacteria in suspension was transferred to the wells of 24 well plates containing poly-L-lysine coated cover slips. After 90 min incubation at 30˚C to ensure attachment, the bacteria were fixed immediately for 1 h using a solution of 2.5% formaldehyde and 25% gluteraldehyde in a 0.075 M phosphate buffer (pH 7.4)(NaP buffer). After fixation the samples were rinsed 3 times for 10 minutes with NaP buffer. After rinsing the samples were placed in a secondary

fixative of 1% osmium tetraoxide for 30 minutes. Again the samples were washed 3x with NaP buffer as described above. The samples were then dehydrated using increasing concentrations of ethanol for 10 min each (30%, 50%, 70% and 90%). Samples were further dehydrated 3x in 100% EtOH for 10 min each. The samples were dried using HDMS for 1 h and were then mounted with carbon tape on aluminium stubs before being coated with carbon. The samples were then viewed with a Zeiss Ultra plus FEG SEM. Similar to the turbidity assay growth controls contained only bacteria and LB broth.

5.3.4 Data management and statistical analysis

All MIC experiments were performed at least in triplicate and data was reported as mean ± (standard error of mean) SEM. Significant differences between samples were determined either using the students T-TEST or one way ANOVA with Tukey's post-hoc analysis.

5.4 Results and discussion

5.4.1 Antibacterial activity of MGO and phenolic acids against *B. subtilis*

In this study the effect of MGO trapping by phenolic acids, PY, GA and CA on the antibacterial activity of MGO was determined. The microbial turbidity assay provides a relationship between the concentration of the antibacterial compound and the observed growth of bacteria where increased turbidity is associated with increased number of bacteria (Prinsloo *et al*., 2013). The MIC is defined as the minimum concentration of an antibacterial agent that inhibits bacterial growth. MGO effectively inhibits *B. subtilis* and *E. coli* antibacterial activity with a MIC of 0.8 mM and 1 mM respectively (Rabie *et al*., 2016).

In *B. subtilis*, 0.5 mM and 1 mM MGO inhibited *B. subtilis* by 86.76 ± 8.82 %, and 91.51 ± 8.47%, respectively in Table 5.1. Polyphenols, PY, GA and CA at a concentration of 0.5 mM, a concentration several fold lower that the MIC of each phenolic acid (Table 5.3) inhibited *B. subtilis* by 24.03%, 23.62% and 30.19%, respectively.

In combination with 1 mM MGO, only PY causes a significant decrease in the antibacterial activity of MGO with a decrease in activity from 86.76 ± 10.17% to 71.72 ± 11.16%. This difference may be due to the trapping of MGO by PY and this reduces the amount of free MGO that can inhibit *B. subtilis* bacteria. At a higher ratio of PY:MGO of 1:2, MGO is in an excess and therefore no difference is seen between MGO and PY:MGO (1:2). As GA does

not trap MGO, no change in antibacterial activity is observed as shown in Table 5.1. Likewise, CA in combination with MGO has a similar antibacterial effect to GA.

Table 5.1: Antibacterial activity of MGO and phenolic acids, PY, GA and CA alone and in combination against *B. subtilis*.

	B. subtilis		
	0 mM MGO	**0.5 mM MGO[1]**	**1 mM MGO[2]**
	0 ± 12.13%	86.76 ± 8.82%	91.51 ± 8.47%
0.5 mM (130 µg/ml) PY	24.03 ± 7.18*#	71.72 ± 14.99*	91.13 ± 16.63
0.5 mM (170 µg/ml) GA	23.62± 12.71%*#	85.14 ± 17.84%	99.55± 18.14%
0.5 mM (180 µg/ml) CA	30.19 ± 6.23%*#	87.15± 18.77%	99.17± 18.07%

[1] Ratio 1:1, [2] Ratio 1:2 Data is an average of three experiments and reported as means ± SEM. * indicates significant differences compared to 0.5 mM MGO, whereas # indicates significant differences compared to 1 mM MGO, p≤0.05.
KEY: CA= Caffeic acid; GA= Gallic acid; PY= Pyrogallol; MGO= Methylglyoxal.

5.4.2 Antibacterial activity of MGO and phenolic acids against *E. coli*

MGO at a concentration of 0.5 mM and 1 mM inhibited *E. coli* by 48.93 ± 8.35 %, and 88.83 ± 8.74 %, respectively in Table 5.2. Polyphenols, PY, GA and CA at a concentration of 0.5 mM inhibits *B. subtilis* by 28.47%, 25.12% and 35.13%, respectively. A concentration of 0.5 mM is below the MIC reported for each phenolic acid (Table 5.3). Compared to 0.5 mM MGO no phenolic acids combined with MGO (1:1 ratio) caused a significant decrease in the % inhibition. Likewise, at a 1:2 ratio, no significant reduction in the % inhibition due to phenolic acid trapping of MGO was observed. In combination with 0.5 mM and 1 mM MGO, PY, GA and CA did not reduce the antibacterial activity of MGO.

Table 5.2: Antibacterial activity of MGO and phenolic acids, PY, GA and CA alone and in combination against *E. coli.*

	E. coli		
	0 mM MGO	**0.5 mM MGO[1]**	**1 mM MGO[2]**
	0 ± 7.92%	48.93 ± 8.35%	88.83 ± 8.74%
0.5 mM (130 µg/ml) PY	28.47 ± 5.15*#%	47.74 ± 4.11%	96.48 ± 0.41%
0.5 mM (170 µg/ml) GA	25.12 ± 2.76%*#	57.59 ± 6.30%	99.14± 0.16%
0.5 mM (180 µg/ml) CA	35.13 ± 2.32%*#	51.93 ± 6.09%	99.47± 0.13%

[1] Ratio 1:1, [2] Ratio 1:2 Data is an average of three experiments and reported as means ± SEM. * indicates significant differences compared to 1 mM MGO, whereas # indicates significant differences compared to 2 mM MGO, p≤0.05.
KEY: CA= Caffeic acid; GA= Gallic acid; PY= Pyrogallol; MGO= Methylglyoxal.

B. subtilis was more sensitive to the antibacterial effect of MGO compared to *E. coli*. At 0.5 mM and 1 mM, *B. subtilis* was inhibited by 86.76 ± 10.17 %, and 91.51 ± 9.26%, respectively compared to 48.93 ± 8.35% and 88.83 ± 8.74% for *E. coli*.

Extensive research has been done on the bactericidal effect of MGO on both Gram positive and negative bacteria (Kamiya & Kamiya, 2001; Hayashi *et al*., 2014). The reported MIC by Rabie *et al*., (2016) for *B. subtilis* and *E. coli* was 0.8 and 1.0 mM respectively, confirming that *B. subtilis* is more sensitive to the effect of MGO. Mavric *et al*., (2008), also evaluated the antibacterial effect of MGO on *E. coli* and *S. aureus* and observed an MIC between 1.1 – 1.8 mM, which is similar to the results of the present study. In multidrug-resistant *P. aeruginosa* (MDRP) known to be resistant to antimicrobial drugs such as ciproflaxin and imipenem (Hayashi *et al*., 2014), the antibactericidal effect of MGO on *P. aeruginosa*, and MDRP was 1.7 and 7.1 mM respectively.

MGO in eukaryotic cells can modify amino acids such as lysine and arginine residues of proteins and peptides leading to the formation of AGEs. This formation causes tissue damage and furthermore an accumulation of AGEs is associated with structural cell damage with DNA changes (Alvarez-Suarez *et al*., 2014). DNA modification and inhibition of protein expression can lead to inhibition of the formation of bacteria structures such as flagella and pili that are required for motility and adhesion (Booth *et al*., 2003).

Chaki *et al*., (2010), compared the MIC of MGO alone and honey containing MGO (H-MGO), on various Gram positive and negative strains. Against Gram positive, *S. aureus* the MIC of the H-MGO was 0.2 mM and for MGO was 0.5 mM. Against Gram negative, the MIC for H-MGO was 1.5 mM and for MGO 2 mM. This indicates that other molecules besides MGO inhibits bacteria and this could be due to the presence of polyphenols. In the present study phenolic acids found in honey also inhibited although to a lesser degree bacteria growth than MGO. Furthermore, this confirms that polyphenols, including phenolic acids contribute to the antibacterial activity of honey (Hayashi *et al*., 2014).

In Tables 5.1 and 5.2, the phenolic acids on their own showed low antibacterial activity against *B. subtilis* at 24%, 24% and 30% inhibition for PY, GA and CA, respectively. Similarly, activity against *E. coli* was low at 29%, 25% and 35% inhibition for PY, GA and CA, respectively. Taguri *et al*., (2006) and Vaquero *et al.,* (2007) evaluated the antibacterial activity of polyphenols against several strains of Gram positive and negative bacteria, and the findings of these studies related to the effect of PY, GA and CA is summarized in Table 5.3. Compared to MGO, the antibacterial activity of PY, GA and CA is low, however synergism may enhance

activity while trapping of MGO by phenolic acids such as PY may reduce the antibacterial activity of MGO as observed in Table 5.1.

Table 5.3: MIC range of polyphenols on different species of *B. subtilis* and *E. coli* (Taguri *et al*., 2006; Vaquero *et al*., 2007)

Polypenols	*B. subtilis* (µg/ml) [mM]	*E. coli* (µg/ml) [mM]
PY	≥267 [2.12]	≥83 [0.66]
GA	1600 – 2000 [9.4 – 11.7]	600 – 3200 [3.525 – 18.8]
CA	≥1600 [8.88]	500 – 2667 [2.775 – 14.8]

KEY: CA= Caffeic acid; GA= Gallic acid; PY= Pyrogallol.

5.4.3 Effect on morphology of *B. subtilis* when exposed to MGO and phenolic acids

In this study, only PY in combination with MGO, reduced the % inhibition of MGO in *B. subtilis* and not *E. coli*. As expected GA did not alter the antibacterial activity of MGO in both strains and indications are that CA also does not affect the antibacterial activity of MGO. Therefore, further morphological studies were undertaken in *B. subtilis*.

Figures 5.1 (A – L) are representative SEM micrographs of *B. subtilis* exposed to MGO, phenolic acids and controls. In figures 5.1A and B, the controls, show normal rod shaped bacteria with flagella responsible for movement of bacteria (grey arrows) (Guttenplan & Kearns, 2013) and pili (thin white arrows) responsible for the stabilisation of mating bacteria during DNA transfer and attachment to surface and protection (Lillington *et al*., 2014). At a lower magnification (10000x), bacterial density is high and the bacteria are attached or in close association with each.

In Figures 5.1C and D, *B. subtilis* exposed to 1 mM MGO at a lower magnification, bacteria density is lower and at a higher magnification, the pili and the flagella are fewer. The presence of large holes (thick white arrow), implies cell wall damage and loss of cellular content. Figures 5.1E and F showed the exposure to 2 mM MGO, shows lower cell density (lower magnification), pili and flagella are absent, cell wall damage and the presence of holes is observed in almost all bacteria (thick white arrows). The typical rod shape of *B. subtilis* is absent and the bacteria have a more rounded structure.

In Figures 5.1G and H, exposure to 1 mM PY, results in bacteria morphologically similar to the control, although these bacteria are more rounded and a few have small holes (thick white arrows, higher magnification).

In Figures 5.1I and J exposure of *B. subtilis* to PY: MGO, 1:1 combination, at a low magnification, are more densely arranged than the bacteria exposed to 1 mM MGO. Similar to bacteria exposed to 1 mM MGO, these bacteria also have cell wall damage and the presence of holes and flagella (grey arrows) as well as pili (thin white arrows) are almost non-existent. This is due to the high 71.72 ± 14.99% growth inhibition (Table 5.1) by PY in combination with MGO. Figures 5.1K and L shows *B. subtilis* exposed to the combination of MGO with PY at a 1:2 ratio, bacteria density is low, and the holes in the bacteria wall are less prominent compared to 2 mM MGO exposure. In Figures 5.1E and F, the flagella and pili and flagella are absent and the bacteria are more rounded.

Although, PY does, trap MGO, at the concentrations used in this study, although PY reduces the antibacterial activity of MGO, the remaining unreacted MGO still can kill bacteria causing a loss of flagella and pili as well as cell wall damage with probable loss of cellular content.

Figures 5.2G and H show exposure to 1 mM GA. At a low magnification, the density of bacteria exposed to GA is reduced. Remaining bacteria are rod shape although small holes in the bacteria (thick white arrows) are present. Some flagella (grey arrows, lower magnification) and pili Figure 5.2G, and pili Figure 5.2H (thin white arrows, higher magnification). At a GA: MGO ratio of 1:1 ratio (Figures 5.2I and J), pili (thin white arrows) are present, flagella are absent and cell leakage has occurred. In Figures 5.2K and L the exposure of GA with MGO at a 1:2 ratio showed the bacterial cells with a more rounded shape, although holes are absent. The pili (thin white arrows) are present in both figures and flagella (grey arrows) can be seen at a higher magnification. GA does not trap MGO and both GA and MGO at the concentrations evaluated inhibit bacteria growth. Scanning electron microscopy, indicates rather than a reduction in antibacterial activity the effects of MGO is enhanced.

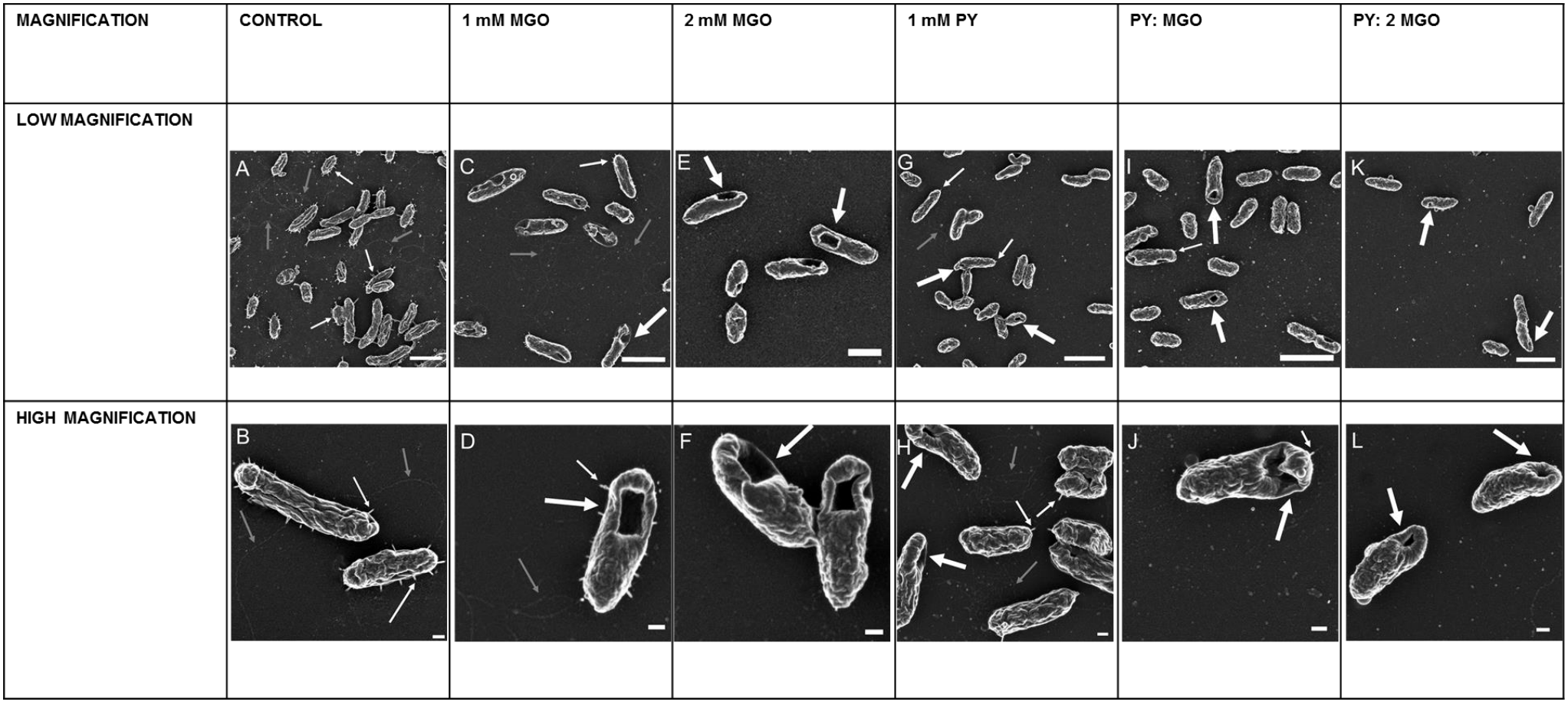

Figure 5.1: SEM micrographs of *B. subtilis* exposed to increasing concentrations of MGO alone and in combination with PY. (A) and (B) Control; (C) and (D) 1 mM MGO; (E) and (F) 2 mM MGO; (G) and (H) 1 mM PY; (I) and (J) PY:MGO at a 1:1 ratio; (K) and (L) PY:MGO at a 1:2 ratio. Thin white arrows indicate the pilus; grey arrows indicate the flagella; thick white arrow indicates a hole in the cell or leakage of cell content. Scale bar of A, C, E, G, I and K equals 2 µm, whereas scale bar of B, D, F, H, J and L equals 200 nm.
KEY: CA= Caffeic acid; GA= Gallic acid; PY= Pyrogallol; MGO= Methylglyoxal; SEM= scanning electron microscopy.

Figures 5.3G and H illustrate *B. subtilis* exposed to 1 mM CA, the bacteria cells show similar features compared to the control, presence of pili (grey arrows) and flagella (thin white arrows), same density and attachment, although some cells appear rounder. Figures 5.3I and J the combination of CA with MGO at 1:1 ratio shows most of the cells did not retain their rod shape become deformed an cell leakage (thick white arrows) is noticeable in most bacteria, although some bacteria do remain attached to each other. Pili and flagella are absent. Finally, the exposure to CA and MGO at a 1:2 ratio illustrated in Figures 5.3K and L show that the bacteria have more pronounced holes (thick white arrows), flagella are absent and pili (thin white arrows) are present and some bacteria are still attached to each other.

Structural observations are that even although PY, GA and CA cause 24.03 ± 7.18; 23.62 ± 12.71 and 30.19 ± 6.23 % inhibition respectively, the effect on bacteria is more than expected, highlighting the ability of electron microscopy to identify small structural changes to bacteria that leads to inhibition of growth as measured with the turbidity assay and eventually the death of bacteria. In addition, these morphological changes suggest that some degree of synergism related to antibacterial activity occurs. Trapping of MGO by PY results in only a 20% decrease in PY levels (Table 3.1) and although antibacterial activity against *B. subtilis* is reduced, because both MGO and PY, have antibacterial activity, under the conditions used there is no major loss of antibacterial activity (15% loss, Table 5.1).

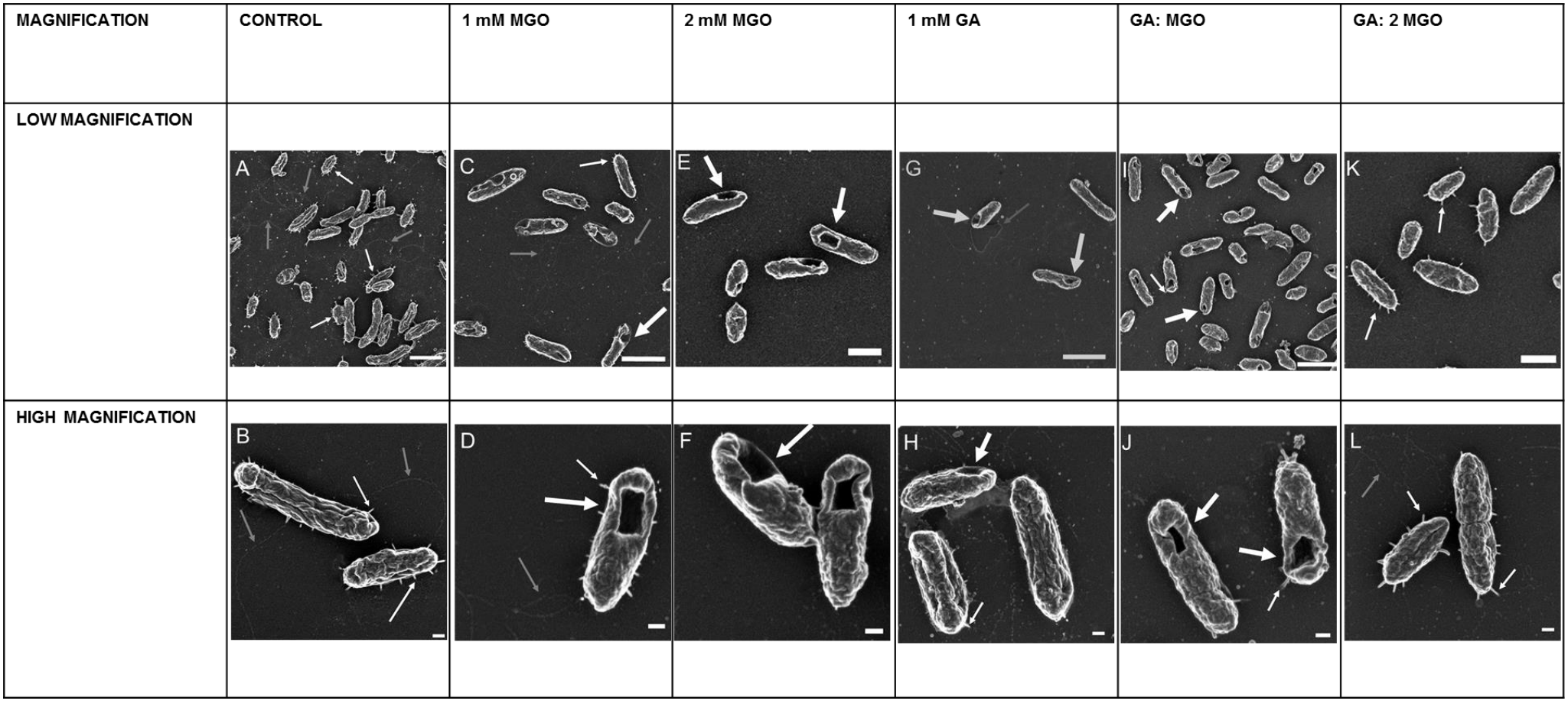

Figure 5.2: SEM micrographs of *B. subtilis* exposed to increasing concentrations of MGO alone and in combination with GA. (A) and (B) Controls; (C) and (D) 1 mM MGO; (E) and (F) 2 mM MGO; (G) and (H) 1 mM GA; (I) and (J) GA:MGO at a 1:1 ratio; (K) and (L) GA:MGO at a 1:2 ratio. Thin white arrows indicate the pilus; grey arrows indicate the flagella; thick white arrow indicates a hole in the cell or leakage of cell content. Scale bar of A, C, E, G and I equals 2 µm and K equals 1 µm, whereas scale bar of B, D, F, H, J and L equals 200 nm.
KEY: CA= Caffeic acid; GA= Gallic acid; PY= Pyrogallol; MGO= Methylglyoxal; SEM= scanning electron microscopy.

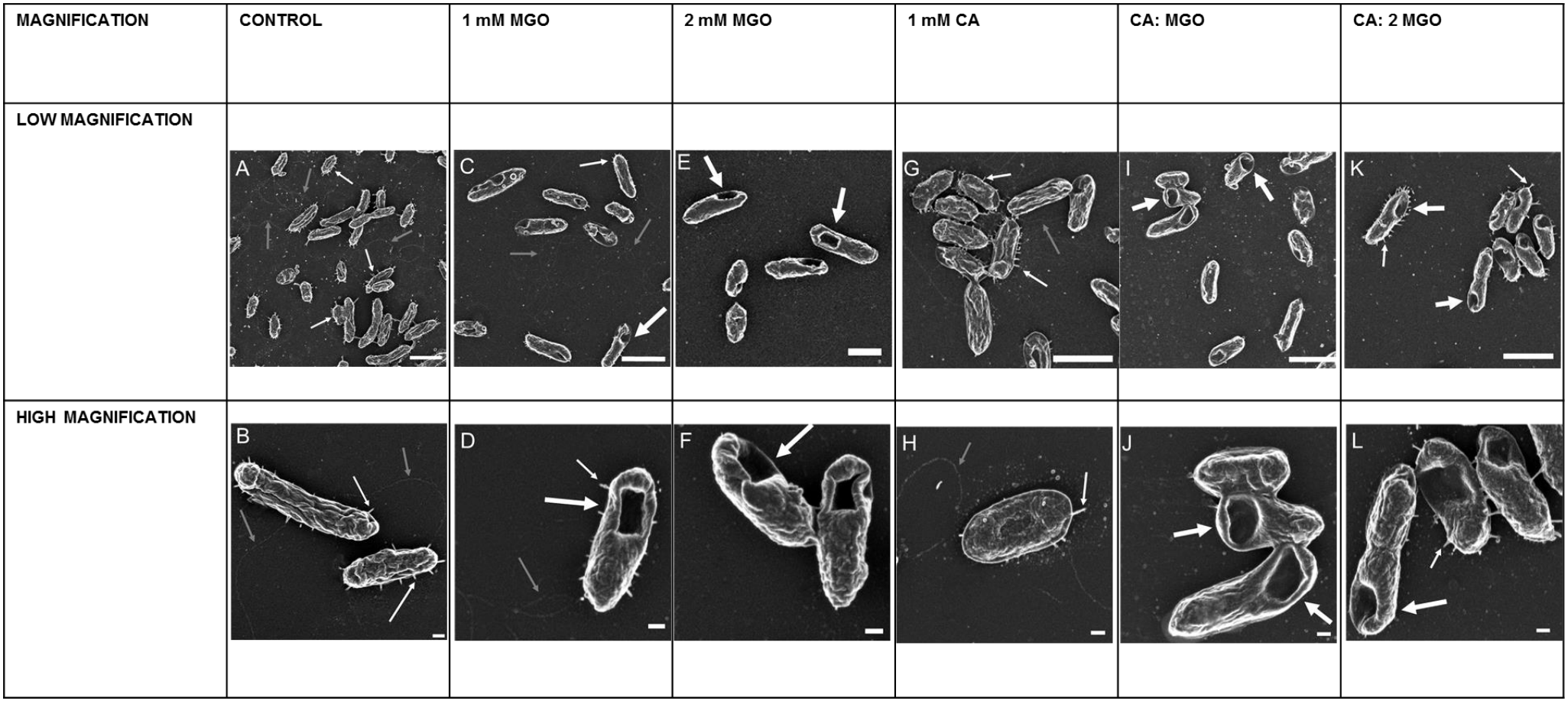

Figure 5.3: SEM micrographs of *B. subtilis* exposed to increasing concentrations of MGO alone and in combination with CA. (A) and (B) Controls; (C) and (D) 1 mM MGO; (E) and (F) 2 mM MGO; (G) and (H) 1 mM CA; (I) and (J) CA:MGO at a 1:1 ratio; (K) and (L) CA:MGO at a 1:2 ratio. Thin white arrows indicate the pilus; grey arrows indicate the flagella; thick white arrow indicates a hole in the cell or leakage of cell content. Scale bar of A, C, E, G, I and K equals 2 µm, whereas scale bar of B, D, F, H, J and L equals 200 nm.
KEY: CA= Caffeic acid; GA= Gallic acid; PY= Pyrogallol; MGO= Methylglyoxal; SEM= scanning electron microscopy.

Borges *et al*., (2013) evaluated the antimicrobial activity of GA and ferrulic acid (FA), against *E. coli*, *P. aeruginosa*, *S. aureus* and *L. monocytogenes*. The author found that GA with a MIC of 1500 µg/ml against *E. coli*, could affect the physiochemical properties of the bacteria, with GA causing the bacteria to become more hydrophilic by changing the bacterial surface charge. At a concentration of 100 µg/ml GA caused cytoplasmic membrane damage and at 1000 µg/ml bacterial intracellular K^+ leakage occurred. This implies that GA induces changes to membrane permeability due to cell wall damage and as was observed in the present study (Figure 5.2). Synergism between MGO and phenolic acids related to antibacterial activity is an important aspect for future research.

5.5 Conclusion

MGO inhibited the growth of the bacteria evaluated by > 90%, while some inhibition was observed for the phenolic acids. Only PY reduces the % inhibition of *B. subtilis* by MGO at a ratio of 1:1. PY, GA and CA in combination with MGO did not alter the % inhibition of MGO in *E. coli*. Ultrastructural analysis of PY, GA and MGO showed that all these constituent molecules of honey caused ultrastructural damage to bacteria. In combination, no changes in the major morphological features associated with the inhibition of bacterial growth were observed.

6 CHAPTER 6: CONCLUDING DISCUSSION

The beneficial effects of Manuka honey are attributed to the presence of phenolic acids and MGO (Mavric *et al*., 2008). Manuka is often included as an ingredient in wound healing products and this is due to the ability of MGO to eradicate bacteria and phenolic acids that can reduce levels of reactive oxygen species and thereby reducing inflammation (Ahmed & Othman, 2013). The safety of MGO in wound dressings has been questioned as MGO can inhibit GSH levels and antioxidant enzymes as well as cause the formation of AGE (Kalapos, 2008). This is especially a problem in diabetic patients where blood MGO levels are increased (Tan *et al*., 2008). Reduced MGO levels and antioxidant enzyme activity makes cells within the wound site highly susceptible to oxidative effects of ROS that can lead to cell death.

Recent studies (Lo *et al*., 2011) have reported that polyphenols can trap MGO, reducing the toxicity of MGO. Phenolic acids that have been reported to trap MGO are PY, 1,2,4-benzenetriol, 1,3,5-trihydroxybenzen, 2.4.6-trihydroxybenzoic acid and HT (Lo *et al*., 2011, Navarro and Morales, 2015). This results in the formation of mono- and di- MGO adducts. It is unknown if adduct formation reduces the antioxidant activity of phenolic acids. Two branched MGO – functional groups attached to benzene ring (Figure 2.7, 2.8 & 2.9) may create steric hindrances or prevent oxidation associated with antioxidant activity. In this study the effect of these interactions on the antioxidant activity of three phenolic acids, PY, GA and CA found in Manuka honey was evaluated (Alvarez-Suarez *et al*., 2014). Lo *et al*., (2011) reported that PY trapped MGO but GA did not trap MGO and therefore these phenolic acids also served as positive and negative controls.

This study was based on the reported ability of phenolic acids such as PY to have the ability to bind/trap MGO, therefore lessening the harmful effects of MGO as a potential advanced glycation end product (AGE) precursor. Both MGO and phenolic acids are found in honey at various concentrations depending on the type of honey. Whether trapping of phenolic acids by MGO reduces the antioxidant activity of these phenolic acids or alternatively the antibacterial activity of MGO altered is unknown. The presence of these adducts in honey may also account for some of the unidentified polyphenol-like molecules found in Manuka honey which have a high phenolic acid and MGO content.

Summary of results

MGO and each phenolic acid in a 1:1, 1 mM were evaluated using LCMS for trapping under the experimental conditions used in this study. Findings were that PY trapped MGO with the

formation of mono- and di-MGO adducts. Both GA and CA did not bind MGO. The degree of PY and MGO binding was only 20%. Differences between this study and that of Lo *et al., (*2011) and Navarro and Morales (2015) was that these authors evaluated changes in MGO levels following derivitisation. A problem with MGO quantification is that MGO is highly reactive and can bind free amino acids, protein and DNA. Therefore, it was much simpler to quantify changes in unreacted polyphenol levels and in addition it was possible to identify the adducts that formed.

This study also showed the formation of mono and di-MGO adducts with the trapping of MGO by PY, resulting in a decrease in TPC and antioxidant activity measured with the DPPH (ET assay - hydrophobic) assay. However, antioxidant activity measured with the ORAC (HAT assay) was increased. PY in combination with MGO did not cause a change in cell number or viability of SC-1 and Caco-2 cells. MGO and PY alone and in combination did not cause oxidative damage but these cells became more susceptible to AAPH-induced oxidative damage. PY in combination with MGO retained the ability to reduce AAPH-induced oxidative damage. PY showed minor inhibition of the growth of Gram positive *B. subtilis* and Gram negative *E. coli.* At the MIC of MGO, mono- and di-adduct formation by PY only reduced the antibacterial activity of MGO at a 1:1 combination of *B. subtilis.* Evaluation of the ultrastructure of bacteria exposed to PY and MGO alone and in combination showed that both PY and MGO caused cellular damage and any trapping of MGO by PY did not substantially reduce the effect of MGO on bacteria.

GA did not trap MGO and the measured total polyphenol content of GA was unaltered for GA: MGO at 1:1 and 1:2 ratio. Antioxidant activity was unchanged for both ET assays (DPPH and TEAC), and HAT assay (ORAC) based assays.

GA in combination with MGO did not cause a change in cell number or viability of SC-1 and Caco-2 cells. GA alone did not cause oxidative damage, although in combination with MGO, GA was able to decrease the oxidative damage caused by MGO, which made the cells (SC-1 and Caco-2) more susceptible to AAPH-induced oxidative damage. GA to a lesser degree than MGO inhibited bacterial growth. In combination, no change in bacterial inhibition or in the ultrastructural features of *B. subtilis* was observed.

CA is a phenolic acid present in Manuka honey, a hydrocinnamic acid which resembles the structure of ferrulic acid. In the study by Hu *et al*., (2012), the authors identified curcumin to have the ability to trap MGO although it was further noted that the ferrulic acid part of curcumin had no effect on the trapping of MGO. In this study it was not known whether CA would be

able to trap MGO as it resembled the ferrulic acid structure but has a –OH group on its C_3. Navarro and Morales (2015) reported that CA trapped MGO after 168 h using antioxidant activity assays. In the present study it was found that CA did not trap MGO (HPLC), although the combination of CA and MGO has the ability to alter the antioxidant content of CA, a decrease was observed at 1:1 and 1:2 ratio. The combination of CA and MGO also altered its antioxidant activity, at 1:2 ratio CA:MGO antioxidant activity was increased (DPPH and ORAC assays), while no change in antioxidant activity was observed with the TEAC assay.

CA alone and combined with MGO did not cause decreases in cell viability and number evaluated in the SC-1 and Caco-2 cells. MGO increases the sensitivity of cell lines to AAPH induced oxidative damage. CA combinations reduced AAPH-induced oxidative damage. CA partially inhibited the growth of *E. coli* and *B. subtilis* compared to MGO that inhibited bacteria growth by 100%. In combination, no change in the inhibitory properties of MGO was observed. This was confirmed with electron microscopy where the morphology of MGO:CA exposed bacteria was similar to bacteria exposed to MGO.

Limitations and recommendations

In contrast to other studies that measured changes in MGO with adduct formation, in the present study changes in concentration of the original phenolic acid was determined. Using standard methodologies for HPLC analysis of phenolic acids, the adducts could also be detected based on their respective molecular masses. This method can also be used to evaluate the trapping abilities of a wide range of phenolic acids. Honey such as Manuka honey contains both MGO and phenolic acids and flavanoids of various types and in different concentrations. It is unknown if MGO-phenolic acid adducts form in honey and future research will focus on determining if unidentified polyphenol like structures in honey are possibly MGO/polyphenol adducts.

In the reaction mixtures are unreacted MGO, phenolic acids and MGO adducts and as these adducts are not commercially available it is difficult to evaluate specifically the activity of each adduct that forms. Using a semi-separative HPLC methodology as described Pereira *et al.*, (2015) these adducts can be isolated and can then be specifically tested for activity.

The reaction of MGO and phenolic acids in a time dependant manner has been shown (Navarro & Morales, 2015). Future studies should determine the changes in antioxidant content and activity at different times and concentrations, as well as the effect of the selected buffers.

With increasing dosages of MGO alone and in combination with PY, GA and CA, only changes in cell number and viability was observed for SC-1 cells with GA and GA combined with MGO at the highest concentrations. Several studies have shown that MGO causes changes to GSH levels and antioxidant enzyme activity (Table 4.1). Evaluation of GSH levels and antioxidant activity may provide a better indication of the antioxidant status.

With the DCFH-DA assay MGO did not cause oxidative damage in the SC-1 and Caco-2 cell lines. However, with the addition of AAPH measured oxidative damage was greater than AAPH alone. It can then be speculated with the depletion of the antioxidant pathway in the SC-1 and Caco-2 cells, these cells are more susceptible to the oxidative effects of AAPH. This further emphasises the importance of knowing the effect of MGO in these cell lines on the components of the antioxidant pathway.

Trapping of MGO by PY reduced the antibacterial activity of MGO in Gram positive, *B. subtilis* while no effect was observed for GA and CA. A limitation is that this bacteria was used as a model organism and it is not commonly found in wounds. Therefore, future studies should focus on effects in clinically relevant Gram positive wound isolates such as *S. aureus*, *B. cereus* either as plantonic or biofilm cultures. In addition, dosage studies should be undertaken where the MIC of each phenolic acid alone and in combination with MGO can be determined and this will generate more reliable data and also the type of interactions such as additive or synergism can be determined.

Trapping of MGO by phenolic acids was seen in PY and not in CA and GA, This trapping reduced the antioxidant content of PY in TPC. No cytotoxicity resulted with MGO alone and in combination with phenolic acids. Antibacterial activity of MGO was reduced with PY but not with CA and GA. PY is confirmed in other studies and in this study that it traps effectively MGO.

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Appendices

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

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences Research Ethics Committee

22/09/2016

Approval Certificate
New Application

Ethics Reference No.: 347/2016

Title: The trapping of methylglyoxal by phenolic acids: Effect on antioxidant and antibacterial activity

Dear Ms Magalli, Marcelline Magnoumba-Legnanga

The **New Application** as supported by documents specified in your cover letter dated 13/09/2016 for your research received on the 13/09/2016, was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 21/09/2016.

Please note the following about your ethics approval:
- Ethics Approval is valid for 2 years
- Please remember to use your protocol number (**347/2016**) 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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