Antiglycation and antioxidant properties of *Momordica charantia*

A thesis submitted in partial fulfilment of the requirements of the Manchester Metropolitan University for the degree of Doctor of Philosophy

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Abstract

Diabetes mellitus is a multifactorial disorder characterised by hyperglycaemia and leads to complications. These complications are caused by advanced glycation endproducts (AGEs) that form through protein glycation as a consequence of hyperglycaemia. A diversity of plants are utilised worldwide as traditional medications for the treatment of diabetes mellitus, particularly in developing nations. A considerable quantity of literature has been published on *Momordica charantia* (MC) as a potent folk medicine for diabetes mellitus.

The aim of this study was to investigate the antiglycation and antioxidant effects of HWE and CWE of MC on protein glycation. To examine whether MC can inhibit oxidative stress in endothelial cells by studying oxidative stress-related transcription factor parameters and their expression.

Model proteins such as lysozyme and BSA were glycated using sugars (glucose and methylglyoxal) as glycating agents both in the presence and absence of MC extracts. AGE formation and inhibition were monitored by a number of methods. Furthermore, different antioxidant assays were used to study the antioxidant properties of MC. The inhibitory effects of AGEs, glucose, methylglyoxal and MC extracts on proliferation of cultured BAEC were determined in this research. Western blotting was used to examine the potential modulation of the oxidative stress signalling pathways induced by AGEs. Both extracts of MC inhibited the production of AGEs in a dose-dependent fashion and the HWE exhibited the more potent inhibitory effect on AGEs production. Moreover, parameters of oxidative stress including the expression of oxidative stress-related transcription factors were assessed using real-time PCR to study the protective effects of MC.

The expressional inhibition of pro-oxidaive genes and enhancement anti-oidative enzymes could be potent element of vascular complications effect of MC extracts. Thus the aqueous extracts of MC, an edible vegetable, may have therapeutic potential in the management of diabetes mellitus.

Dedication

This thesis is dedicated to Sharavan, Guerrilla and Peshmarga who sacrificed their lives for the freedom of people and soil of Kurdistan.

Declaration

I hereby declare that this study has been completed by myself, and has not been accepted for any degree before and is not currently being submitted in candidature for any degree other than that of PhD of the Manchester Metropolitan University.

Niazi Nazhad

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I would like to thank all my supervisory team of Dr. Nessar Ahmed, Dr. Qiuyu Wang and Dr. Muhammad Saeed Ahmad for their patient guidance, encouragement and advice that they have provided throughout this project.

Abbreviations list

ADA	American Diabetes Asssociation
AG	Aminoguanidine
AGEs	Advanced glycation endproducts
ALEs	Advanced lipoxidation end products
$AlCl_3$	Aluminium trichloride
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BAEC	Bovine aortic endothelial cell
BSA	Bovine serum albumin
CAT	Catalase enzyme
CML	Carboxymethyllysine
CWE	Cold water extract
GA	Gallic acid
DM	Diabetes mellitus
DEAM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECL	Enhanced chemiluminescence
ELISA	Enzyme-linked immunosorbent assay
FBS	Foetal bovine serum

FGF-2	Fibroblast growth factor-2
$FeCl_2$	Ferrous chloride
$FeCl_3$	Ferric chloride
$FeSO_4$	Ferrous sulphate
FRAP	Ferric reducing antioxidant power
GSH	Glutathione
HbA_{1c}	Glycated haemoglobin
HCl	Hydrochloric acid
H_2O_2	Hydrogen peroxide
H_2DCF-DA	2′,7′-Dichlorodihydrofluorescein diacetate
HPLC	High-performance liquid chromatography
H_2SO_4	Sulphuric acid
HWE	Hot water extract
IDDM	Insulin-dependent diabetes mellitus
JAK-2	Janus kinase-2
p-JAK	Phosphorylated Janus kinase 2
kcal	Kilocalories
K_3 Fe (CN) $_6$	Potassium ferricyanide
LDL	Low density lipoprotein
MC	*Momordica charantia*
mM	milli molar

mRNA	Messenger ribonuceleic acid
µM	micro molar
$NaCNBH_3$	Sodium cyanoborohydride
NaCl	Sodium chloride
Na_2CO_3	Sodium carbonate
$NaHCO_3$	Sodium bicarbonate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
Na/K-ATPase	Sodium-potassium pump
NaOH	Sodium hydroxide
NDDG	National diabetes data group
NF-kB	Nuclear factor kappa light chain enhancer of activated B cells
NIDDM	Non-insulin-dependent diabetes mellitus
NO	Nitric oxide
$NO^{\bullet}$	Nitric oxide radical
$O_2^{\bullet}$	Superoxide radical
$OH^{\bullet}$	Hydroxyl radical
PBS	Phosphate buffered saline
PSG	Pencillin, streptomycin and L-glutamine
PKC	Protein kinase C
RAGE	Receptor for advanced glycation endproducts
$ROO^{\bullet}$	Peroxyl radical

ROS	Reactive oxygen species
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SOD	Superoxide dismutase
TCA	Trichloroacetic acid
TEMED	N, N, N′, N′-tetramethylethylenediamine
µl	microlitre
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
WHO	World Health Organization

Publications

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Niazi Nazhad, Nessar Ahmed,Qiuyu Wang, Muhammad Saeed Ahmad, Donghui Liu. Antiglycation activity of *Momordica charantia in vitro*. Oral presentation at Science & Engineering Research Symposium 17 September 2015. Manchester Metropolitan University, UK.

Niazi Nazhad, Nessar Ahmed, Qiuyu Wang, Muhammad Saeed Ahmad, Donghui Liu. Antiglycation and antioxidant of properties *Momordica charantia* (Under preparation)

Chapter 1. General introduction

1. Introduction

Diabetes mellitus (DM) is a metabolic disorder characterised by increased blood glucose concentrations resulting from a lack or partial deficiency of insulin, or insulin resistance. Diabetic patients are susceptible to chronic complications such as nephropathy, retinopathy, atherosclerosis and delayed wound-healing. Hyperglycaemia plays a critical role in the pathogenesis of long-term complications and its toxicity is mediated by increased glycation of proteins. Protein glycation is a simple chemical reaction initiated by a nucleophilic addition reaction between a free amino group from a protein and carbonyl groups of sugars to form freely reversible Schiff bases that subsequently form more stable and relatively irreversible ketoamine or Amadori products. The Amadori product goes through a number of reactions involving dicarbonyl intermediates, such as 3-deoxyglucosones and methylglyoxal, and eventually gives rise to poorly characterised structures called advanced glycation end products (AGEs) (Ahmed, 2005). Glycation causes damage to proteins, lipids and nucleic acids and leads to oxidative stress in chronic conditions (Elosta *et al*, 2012). The accumulation of toxic AGEs can permanently alter the structure and function of proteins as observed in diabetes mellitus, cancer, Alzheimer's disease and inflammatory conditions (Mashilipa *et al*, 2011). The molecular mechanism of oxidative stress leads to diabetic complications are not fully understood. It has been reported that the hyperglycaemia result in generation of ROS, leading to elevated oxidative stress, thus, causing the activation of oxidative-stress signalling pathways, such as NADPH oxidase (NOX family), NF-κB, p38 MAPK, P21, JAK family, JNK/STAT, AGE-RAGE, sorbitol and others. Subsequently leading generation of gene products, such as tissue factor and VEGF, which cause cellular injury and are play key role in long-term of diabetic complications. Therefore, the activation of these pathways appears to enhance insulin resistance and impaired insulin secretion. The location and longevity of endothelial cells mean they are prime targets for hyperglycaemia-induced cellular toxicity. Therefore, endothelial dysfunction may underpin the pathogenesis of diabetic vascular complications (Endemann & Schiffrin, 2004)**.**

Momordica charantia (MC) is used worldwide, especially in Africa and Asia for its valuable medicinal properties. It is used as a fruit juice or leaf tea for several chronic conditions such as diabetes mellitus, colic and hypertension. Several studies have indicated that MC may provide

protection against oxidative stress (Kumar *et al*, 2009; Tupe *et al*, 2014; Umukoro & Ashoro, 2006). Antiglycation agents with antioxidant properties and reduced side effects may provide a therapeutic approach for delaying and preventing diabetic complications (Ramkissoon *et al*, 2012). However, the protective action of MC is still not fully understood. Further studies are needed to understand the antioxidant and antiglycation properties of MC and its protective role in preventing glycation-mediated damage to proteins and endothelial cells.

1.1 Glucose

Glucose is a main energy source for the human body and plays a key role in maintaining health. Adenosine triphosphate (ATP) molecules are a fuel for almost every cellular process, which form as a result of the metabolism of glucose molecules in most cells. A constant level of glucose is required for cells to function (Deng, 2012). Glucose consists of six carbons and has two isomeric forms, acyclic and cyclic, as shown in Figure 1.1. The high stability of glucose is related to its ring structure and this makes it less reactive with amine residues of proteins. Conversely, the presence of free carbonyl residues in the open configuration of glucose accounts for the more reactive form. Furthermore, in addition to glucose, there are other intracellular reducing sugars such as fructose, glucose-6-phosphate (G-6-P), fructose-6-phosphate (F-6-P), galactose, and ribose and their derivatives, that are more reactive and contribute to AGEs formation more than glucose. Moreover, glucose is five times less reactive with amines than galactose; eight times less than fructose; 25 times less than deoxyglucose; and 100 times less than ribose. In this respect, glucose is a very important type of reducing sugar because it is less reactive with protein and therefore contributes less to AGEs formation. Conversely, the body feedback mechanism tightly regulates blood glucose levels in a low range of concentrations (3-6 mM). However, the blood concentration of other more reactive sugars such as fructose, ribose, and methylglyoxal is maintained at much lower (μM) concentrations (Salvayre *et al*, 2009; Takeuchi *et al*, 2010). Glucose is therefore the most abundant type of reducing sugar in the blood.

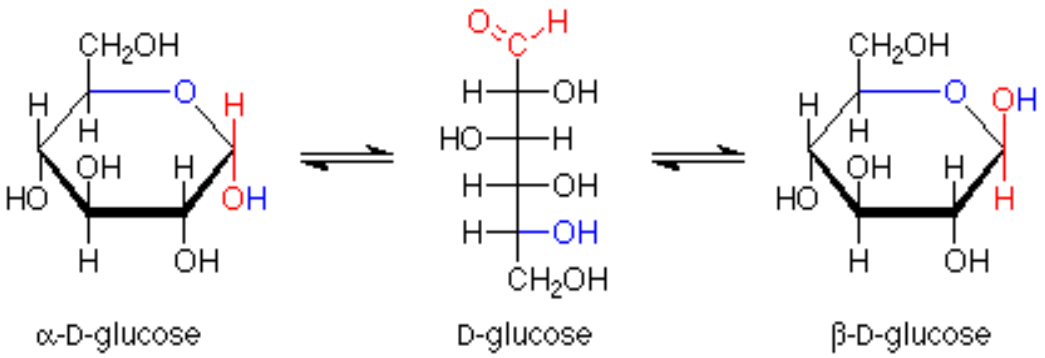


Figure 1.1: Chemical structures of glucose molecules. Acyclic form of D-glucose (centre) and cyclic forms of α-D-glucose (left) and β-D-glucose (right) (Deng, 2012).

1.2 Hyperglycaemia

The level of glucose above normal that is due to inadequate secretion or insufficiency of insulin is defined as hyperglycaemia. Research on clinical and animal models has indicated that excess glucose levels above the baseline are the main cause of metabolic disorder, resulting in diabetic microvascular and macrovascular complications (Verzelloni *et al*, 2011). In humans, insulin and glucagon are important hormones in controlling glucose levels tightly between 3.0 and 7.8 mmol/L. However, initial hyperglycaemic clinical features appear at concentrations of between 15-20 mmol/L (Cox *et al*, 2005; Meijering *et al*, 2006). Hyperglycaemia leads to increased levels of intracellular sugars such glucose, fructose, fructose-3-phosphate (F-3-P), glucose-6-phosphate (G-6-P) and the accumulation of highly reactive dicarbonyl molecules, for example methylglyoxal and glyoxal. It has been known that AGEs form as a result of non-enzymatic reactions between these sugars and their derivatives with other type of molecules (Mashilipa *et al*, 2011). Microvascular and macrovascular complications are associated with diabetes mellitus. The major types of microvascular complications are neuropathy, nephropathy and retinopathy, which are develop in diabetic patients over a period of 10-15 years (Nathan *et al*, 2005). Elevated vascular permeability and prothrombotic conditions, the thickening of basement memberane and reduced blood flow are pathological alterations of microvascular disease (Elosta *et al*, 2012). On the other hand, macrovascular complications play a vital role in a range of pathological changes occurring in most types of blood vessels leading to structural and functional changes. These changes cause endothelial dysfunction, elevated arterial wall stiffness and decreased compliance of the vascular system, thus eventually enhancing

macrovascular complications. One of the most well known macrovascular complications is atherosclerosis, which is believed to cause cardiovascular disorders such as myocardial infarction, peripheral vascular pathologies and increased risk factors of diabetes mellitus. Furthermore, in humans, many organs such as kidney, eye, and blood vessels are dependent on insulin for the production of fuel from glucose, such compartments are more susceptible to the cellular and molecular complications of hyperglycaemia (Verzelloni *et al*, 2011).

1.3 Diabetes mellitus

Diabetes mellitus is a metabolic disorder characterised by long-term hyperglycaemia. When untreated, it may cause severe medical conditions such as chronic hyperglycaemia and disturbances in the metabolism of protein, fats and carbohydrates (Elosta *et al*, 2012). In recent years, the incidence of DM has increased dramatically. Such an increase has occurred in all parts of the world and is believed to be the result of many factors, for instance obesity, the increased intake of fatty food, lack of physical activity, social influence and increasing ageing populations (Romao & Roth, 2008). Moreover, in 2010 the WHO predicted that there will be around 366 million adults suffering from DM in 2030, in contrast, this figure was 171 million in 2007 (WHO, 2007). At present, there are around 344 million people worldwide who have diabetes (WHO, 2013). Additionally, the WHO estimated that DM will be the 7th leading cause of death worldwide in 2030 (WHO, 2011). Generally, DM can be classified into three types; type I and type II are based on the dependence of insulin. It has been reported that both type I and type II are associated with major long-term complications such neuropathy, retinopathy, nephropathy, angiopathy (delay in wound-healing), vascular diseases and diabetic foot and digestive insufficiencies (Ahmed *et al*, 2004).

Type I DM, formerly known as insulin-dependent diabetes mellitus (IDDM), or juvenile diabetes, is recognised by the lack of insulin secretion and results from the autoimmune destruction pancreatic beta cells. Approximately, 10% of people with diabetes have this type of diabetes mellitus (WHO, 2013).

Type II DM, formerly known as a non-insulin-dependent diabetes mellitus (NIDDM), is caused by a combination of insulin insufficiency and/or insulin resistance (Rolo & Palmeira, 2006). At present, type II DM represents for about 90% of people with diabetes worldwide (WHO, 2013).

The third type is known as gestational diabetes mellitus occurs in pregnancy. This form is described as any degree of glucose intolerance, even if the patient has pre-existing diabetic symptoms (Nakata *et al*, 2013).

1.4 Maillard reaction

This is a chemical reaction between amines of protiens and carbonyl compounds of reducing sugars. The importance of the Maillard reaction was not recognised until the mid 1970s when the relation between diabetic complications and glycated haemoglobin A1c (HbA1c) was established. For the first time, in 1980, the term glycation products was used by scientists instead of the Maillard reaction. One of the most important sources of amines in biological systems are the primary amino groups of free amino acids, the ε-amino group of lysine and arginine residues of proteins and peptides. In contrast, reducing sugars such as glucose, ribose, galactose, and fructose and their derivatives, are the prominent sources of carbonyl compounds donating to the Maillard reaction (Ahmad *et al*, 2007).

1.5 Biochemistry of protein glycation

Protein glycation is a series of complex reactions between the free amino groups of proteins and the carbonyl groups of a reducing sugar. The reaction generally includes three stages named initiation, propagation and an advanced stage. Moreover, the reaction rate is very slow because no enzymatic catalyst contributes, and usually it requires several days to several weeks to complete.

1.5.1 Protein glycation: Initiation stage

The first step of protein glycation is initiated by the nucleophilic addition reaction between amino groups of proteins, lipids and nucleic acids, with carbonyl groups of reducing sugars such as glucose to produce a freely reversible Schiff base such as glycosaylamine (Mashilipa *et al*, 2011). This reaction needs hours to occur. Once this product is formed, it rearranges itself further to form a more stable structure called an Amadori product or ketomine like haemoglobin A1c or 1-amino-α-deoxyketose as illustrated in Figure 1.2. The Amadori products need days to form and, once formed, they are stable and almost irreversibe (Marchetti, 2009; Mashilipa *et al*, 2011).

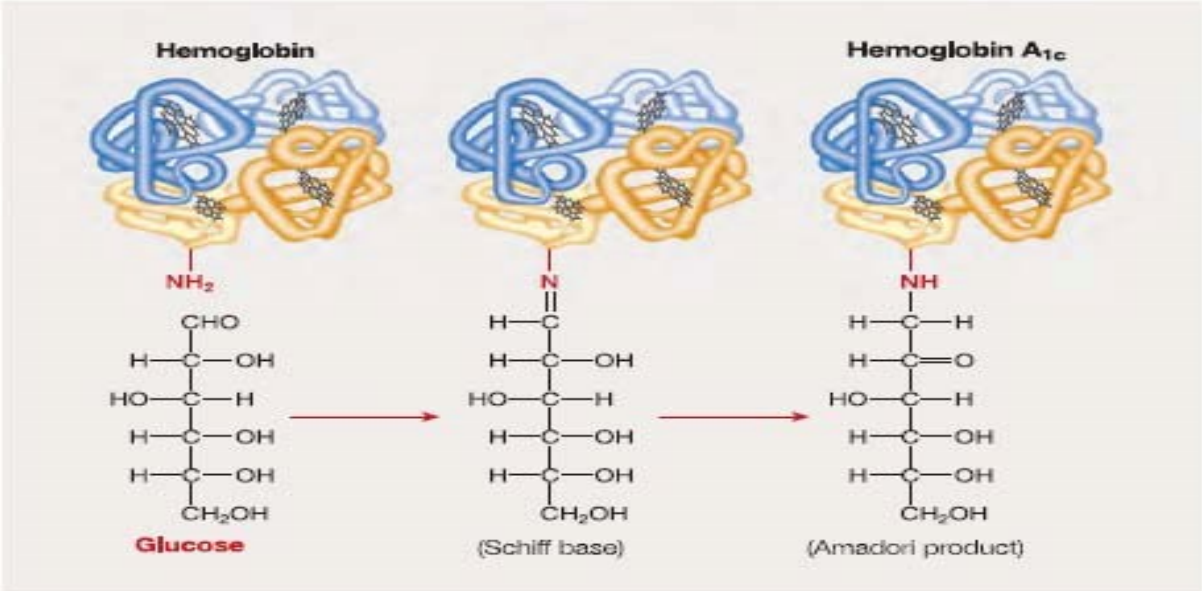


Figure 1.2: Formation of glycated haemoglobin A1c (HbA1c) (Marchetti, 2009)

1.5.2 Protein glycation: Propagation stage

Amadori products undergo a number of chemical rearrangements, dehydration, degradation and oxidation reactions to produce other amino or carbonyl compounds which may act with other amino molecules (Mashilipa *et al*, 2011). These post-Amadori products react at higher rates than the original sugar from which they are derived. Glyoxal, methylglyoxal and 3-deoxyglucosone (3-DG) are the most well known and potent types of post-Amadori products in the glycation reaction (Elosta *et al*, 2012).

1.5.3 Protein glycation: Advanced stage

The advanced stage of protein glycation is characterised by formation of AGEs molecules, which starts with the reaction of post-Amadori products with the free amino groups of proteins. Similar to an intermediate stage of protein glycation, the post- Amadori products within this stage undergo further degradation, dehydration, condensation, oxidations and cyclisation reactions within the amino-carbonyl compounds to create different types of AGEs such as fluorescent or non-fluorescent and cross-linked or non-cross-linked forms (Mashilipa *et al*, 2011).

1.5.4 Autoxidative glycation (Wolf pathway)

Autoxidative glycation is characterised by a non-enzymatic reaction between the α-hydroxycarbonyl group of reducing sugars and oxygen molecules. Subsequently,

α- oxoaldehydes and hydrogen peroxide are regarded as main products of this reaction. It has been reported that free radicals play a vital role in this process and the reaction is started by the enolisation of the initial monocarbohydrates (Ahmed, 2005; Ahmad *et al*, 2007). Consequently, the enediol formed is dehydrated to a dicarbonyl compound and other types of AGEs. Within the above reaction, a number of additional AGEs and dicarbonyl molecules are formed. Hence, erythrose or erythitol split from glucose molecules to provide more reactive reducing sugars such as glyoxal and 3-deoxyglucosone. Eventually, these products react with other production of methylglyoxal and glyceraldehyde (Giacco & Brownlee, 2010) The following reactions illustrate some steps of autoxidative glycation as shown in Figure 1.3

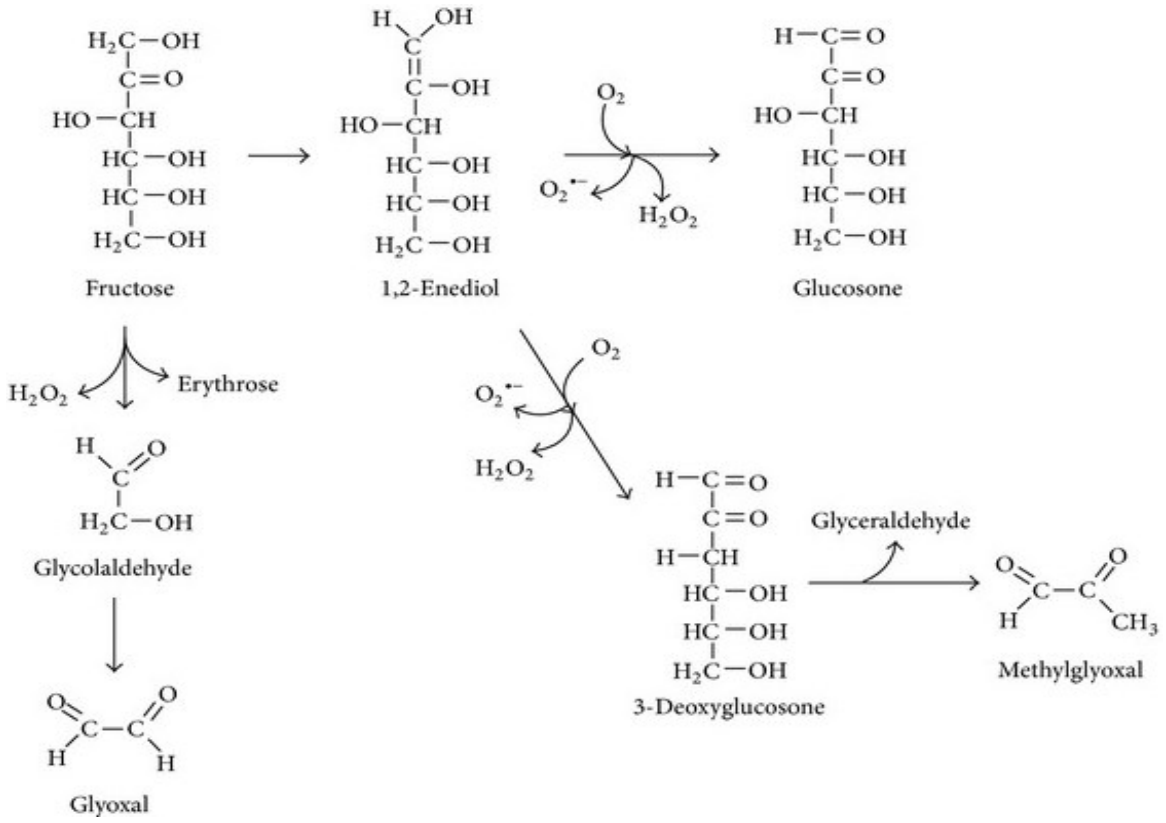


Figure 1.3: Autoxidative glycation (Wolff pathway) (Semchyshyn HM, 2013).

1.5.5 Schiff base fragmentation (Namiki pathway)

Previous research has indicated that there is another pathway for the production of AGEs, prior to the formation of Amadori products. Fragmentation of the Schiff base may take place to produce dicarbonyl molecules; hence, this leads to the production of free radical species as shown in Figure 1.4. Moreover, glyoxal and methylglyoxal are formed via Schiff base fragmentation through the Namiki pathway (Elosta *et al*, 2012).

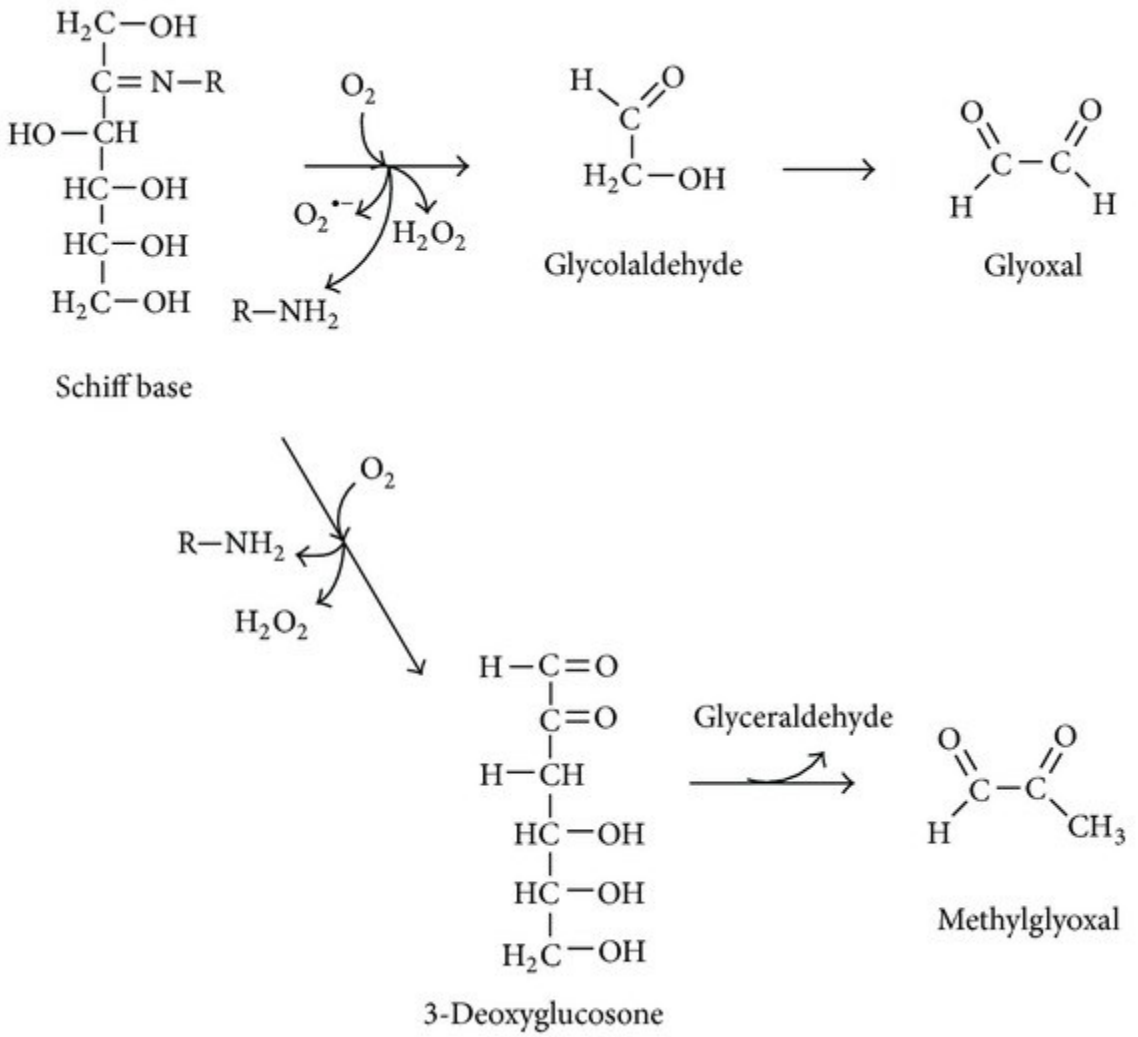


Figure 1.4: Oxidative Schiff base fragmentation (Namiki pathway) (Semchyshyn HM, 2013).

1.5.6 Glycoxidation

Autoxidation of Amadori products, commonly known as glycoxidation products, defines the characteristic products formed by consecutive glycation and oxidation reactions (Giacco & Brownlee, 2010). The free carbonyl group in the enediol form of glucose in the reacting medium may undergo oxidation in a transition metal-dependent reaction to an enediol radical anion. Hence, these products change to superoxide anion radicals through the production of ketoaldedydes. Superoxide anion dismutase produces H_2O_2 and in the presence of oxygen species produces free radical species like hydroxyl radicals ($OH^{\bullet}$) and dicarbonyl compounds (Ahmad *et al*, 2007). This process is defined as glucose autoxidation (Dean, 1987) and it is outlined in Figure 1.5. Consequently, the glycoxidation reaction of Amadori products generates

AGEs which are believed to play a key role in diabetic complications (Giacco & Brownlee, 2010).

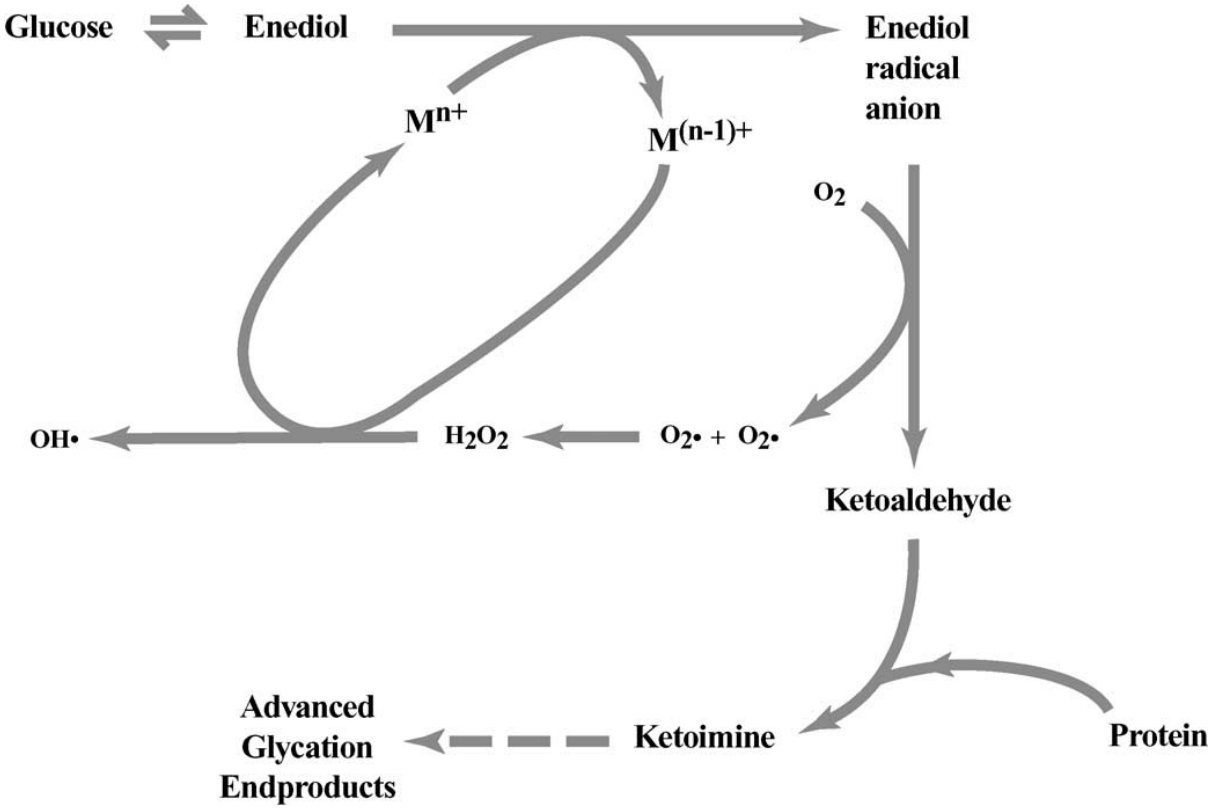


Figure 1.5: Metal-catalysed autoxidation of glucose molecules to protein-reactive dicarbonyls is paralled by the production of superoxide (O^{-}_{2}). Superoxide free radicals can dismutalse to hydrogen peroxide (H_2O_2), then are changed to reactive hydroxyl radicals in the presence of transition metals (Ahmed, 2005).

1.6 Factors influencing protein glycation

The first step of the non-enzymatic reaction between free carbonyl reducing sugars and the amino residues of proteins are primarily dependent on several factors such as pH, metal ions, oxygen and the electrophilicity of the sugar carbonyl carbon (Ahmed, 2007; Mashilipa *et al*, 2011). Therefore, temperature, water activity and the percentage of the reducing sugar in the open ring form may also have a direct influence on the rate and extent of the Amadori rearrangement (Elosta *et al*, 2012). Subsequent studies indicated that protein glycation is influenced by the presence of adjacent histidine residues, carboxylate residues and negatively charged ions, for example phosphates, present in the reaction medium (Ahmed, 2005). Several studies have highlighted that the production of glycated proteins *in vivo* is dependent on the degree and duration of hyperglycaemia, the half-life of the protein and the inherent reactivity of particular amino residues (Smit & Lutgers, 2004).

1.7 Types of AGEs

AGEs are a large family of complex heterogeneous molecules that consist of both fluorescent and non-fluorescent residues characterised by yellow-brown pigmentation and their capability to produce cross-links between free amino groups of proteins (Ramasamy *et al*, 2005). However, all types of AGEs can be divided into four classes according to their chemical and physical properties (Ahmad *et al*, 2007). Biochemical and immunohistochemical studies have proposed that carboxymethyllysine (CML) modifications of proteins are predominant in AGEs that accumulate *in vivo* (Basta *et al*, 2004).

1.7.1 Fluorescent cross-linked AGEs

The fluorescent cross-linked AGEs are the minor AGE molecules, which account for only 1% or less of the total cross-linking molecules. Certain fluorescent cross-linked AGEs, for instance, pentosidine, vesperlysine A- B- C and crossline (Elosta *et a*l, 2012), have been recognised under physiological conditions *in vitro*. Examples of these types of AGEs are shown in Figure 1.6A.

1.7.2 Non-fluorescent cross-linked AGEs

Non-fluorescent cross-linked AGEs are believed to be major types of AGEs structures and possess a vital role in protein cross-linking *in vivo*. For instance, glyoxal-lysine dimers (GOLD)and methyl-glyoxal-lysine dimers (MOLD) are common types of non-fluorescent cross-linked AGEs (Ahmed, 2005; Elosta *et a*l, 2012). Examples of non-fluorescent cross-linked AGEs are illustrated in Figure 1.6B

1.7.3 Fluorescent non-cross-linked AGEs

Similarly to cross-linked AGEs which inhance the function and structure of proteins, a range of fluorescent non-cross-linked AGEs have been discovered in the blood of diabetic patients. Furthermore, argpyrimidine is a typical example of AGEs structure belonging to this family of fluorescent non-cross-linked AGEs and are demonstrated in Figure 1.6C (Elosta *et a*l, 2012; Mashilipa *et al*, 2011).

1.7.4 Non-fluorescent non-cross-linked AGEs

In addition to other types of AGEs there is another group of AGEs called non-fluorescent non-cross-linked AGEs which arise from protein glycation under physiological conditions. Carboxymethyllysine (CML), carboxyethyllysine (CEL), pyrraline and imidazolone are the common types of this class of AGEs that are shown in Figure 1.6D (Elosta *et a*l, 2012).

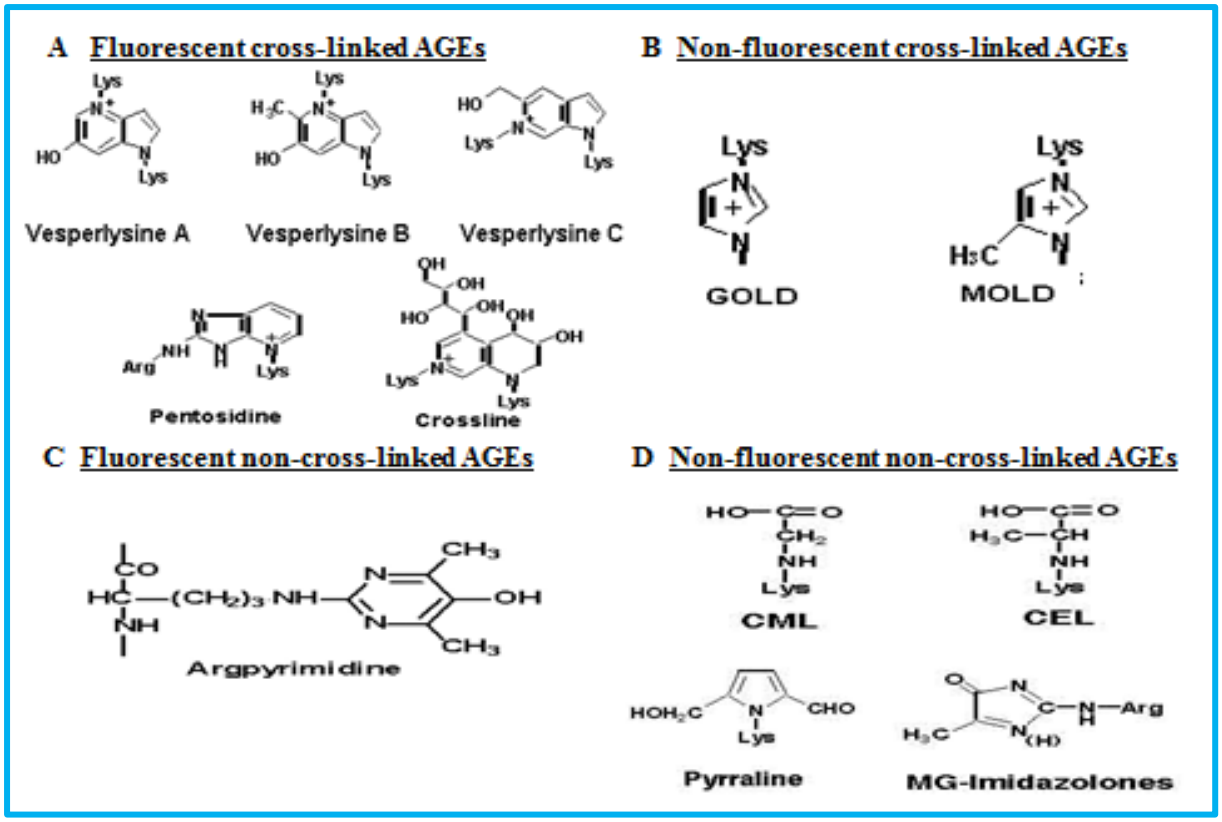


Figure 1.6: Chemical structures of different types of AGEs:
(A) Fluorescent cross-linked AGEs, (B) Non-fluorescent cross-linked AGEs
(C) Fluorescent non-cross-linked AGEs, (D) Non-fluorescent non-cross-linked AGEs.

1.8 Intercellular protein glycation and AGE formation

The oxidation of Amadori products and lipid peroxidation lead to the generation of AGE molecules. Several studies have indicated that, along with glucose as a main precursor for AGE formation, other reducing sugars are involved as being more reactive than glucose (Ahmad *et al*, 2007). Moreover, the more reactive reducing sugars such methylglyoxal form via non-oxidative glyceraldehyde-3-phosphate fragmentation and produce the most reactive AGEs in the intercellular compartment (Brownlee, 2005). In regard to these findings, further studies were carried out using the AGE inhibitors benfotiamine and thiamine. Benfotiamine is a lipid-soluble thiamine precursor obtaining higher bioavailability than thiamine and, reduces the severity of the complications which are associated with diabetes such neuropathy, nephropathy, retinopathy and angiopathy by preventing the production of AGEs (Balakumar *et al*, 2010). Exposure of the endothelial cells to glucose for 7 days resulted in alleviating AGEs levels 14 fold. This increase most likely results from glycation by more highly reactive intercellular reducing sugars such as glucose-6-phosphate, methylglyoxal and fructose (Ahmed, 2005).

1.9 Major exogenous sources of AGEs formation

One of the greatest exogenous sources of highly reactive AGEs is a complex diet that is rich in proteins, fatty substances and carbohydrates. Certain type of foods and dietary factors directly affect production of AGEs. Heating is an important factor in food processing, and plays a key role in increasing the formation of AGEs. In addition, the production of AGEs in food during cooking depends particularly on other factors, for instance the ingredients, temperature and the duration of heat application. Hence, foods containing high levels of fat and prepared at high temperature under dry conditions form high levels of AGEs (Goldberg *et al*, 2004; Vlassara *et al*, 2008). It has been reported that tobacco smoking is recognised as a serious, worldwide public health concern and is accounted as a major exogenous source of AGE production. Previous studies have demonstrated that consequential increases in serum concentrations of AGEs have been detected in diabetic smokers as compared with non-smokers, irrespective of diabetes (Fujioka & Shibamoto, 2006). Tobacco leaves naturally contain a variety of chemical substances and sugars such as glucose, fructose and sucrose. Cigarette smoke produces highly toxic materials such as glycotoxins, which are absorbed from the lungs into blood vessels and accelerate the production of AGEs. In addition, high concentrations of glyoxal and methylglyoxal are produced from burning cigarettes. Methylglyoxal forms as a result of the thermal decomposition of saccharides, which are considered as essential precursors of smoking-associated AGEs (Talhout *et al*, 2006).

1.10 Biological effects of AGEs

The modification and aggregation of different proteins may generate several pathological processes, which lead directly to tissue damage. Thus, accelerated AGE formation or decreased degradation causes the accumulation of AGEs in tissues (Bohlender *et al*, 2005). AGEs cause tissue injury which comes from a variety of mechanisms such as aggregation and precipitation of proteins, cross-link production of AGEs, prevention of particular functions of proteins, the formation of free radical molecules, interaction with specific cellular receptors called RAGE (receptors for glycation end products), and intercellular glycation of proteins (Rojas & Morales, 2004).

1.11 Receptor for advanced glycation endproducts

The receptor for advanced glycated endproducts known as (RAGE), belongs to an immunoglobulin super family, and possesses a mass of 45-kDa. A number of researchers have

reported that the RAGE gene is situated on chromosome six and is located between class II and III of the histocompatibility complex genes (Hudson & Schmidt, 2004; Srikanth *et al*, 2011). RAGE is a transmembrane receptor consisting of 394 amino acids, with a single hydrophobic transmembrane domain of 19 amino acids, and a short cytoplasmic tail of 43 amino acids. The extracellular part of RAGE contains of one the V-type of immunoglobulin domains, followed by two C-type immunoglobulin domains. Furthermore, structure function studies have illustrated that the V-domain is vital for ligand binding. On the contrary, the cytosolic tail is critical for RAGE-mediated intercellular signalling (Srikanth *et al*, 2011). There is another form of RAGE receptor, not of full length, lacks the cystolic tail located inside the membrane is a dominant-negative form called DN-RAGE, and not essential in RAGE–mediated intercellular signalling as shown in Figure 1.7 (Hallam *et al*, 2010). RAGE is highly conserved in all species, present in a wide variety of tissues, particularly in the heart, lung, and skeletal muscle. A considerable number of studies have investigated AGE-binding protein receptors. These studies have identified several AGE receptors, including lactoferrin, oligosaccharyl transferase complex protein 48 (AGE-R1), 80K-H protein (AGE-R2), galectine-3 (AGE-3), lysozyme, SRA, CD-36 and receptors for AGE (RAGE), which are the most important signal transducers for AGEs (Bohlender *et al*, 2005; Hudson & Schmidt, 2004). Moreover, RAGE is localised in different cell types, including endothelial cells, macrophages, smooth muscle cells, mesangial cells and epithelial cells, lymphocytes, monocytes, neurons and was originally extracted from bovine lung endothelium for its ability to bind AGE molecules (Basta, 2008).

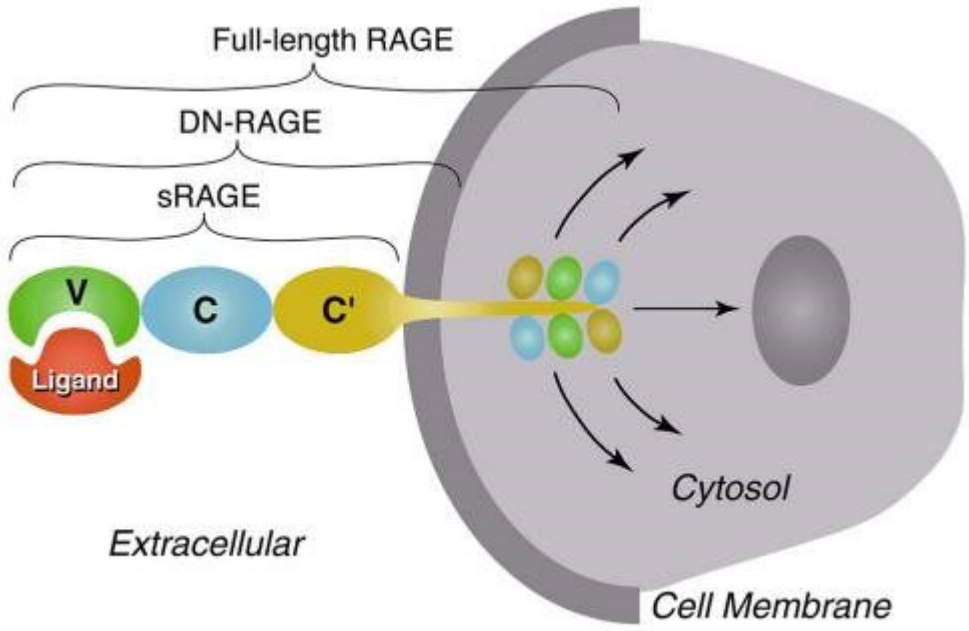


Figure 1.7: Structure of RAGE. The extracellular part of RAGE contains of one the V-type of immunoglobulin domains, followed by two C-type immunoglobulin domains, the cystolic tail located inside the membrane is a dominant-negative form called DN-RAGE (Hallam *et al*, 2010 .

1.12 Diabetic mellitus and oxidative-stress signalling pathways

A considerable amount of evidence reported that hyperglycaemia causes production of AGE, which play a vital role in generation of ROS, ultimately leading to elevated oxidative stress in several tissues (Elosta *et al*, 2012). ROS causes injury to cell components such as proteins, lipids and nucleic acids (DNA and RNA). Thus, natural endogenous antioxidant enzymes such as Catalase (CAT), Superoxide dismutase (SOD) and Glutathione peroxidase (GPx) are available within cells to engulf ROS and these defences systems are vital for maintaining normal cellular functions. When the endogenous antioxidant system fails to provide proper response to restore cellular redox balance, oxidative-stress ensures. The major result is the generation of gene products that lead cellular injury and are significantly responsible for the complications of diabetes mellitus (Stefanie *et al*, 2012).

AGE-RAGE interaction leads to the generation of a variety of biological effects such as elevated secretion of cytokinase, tumour necrosis factor-α (TNF-α), interleukin-1α (1L-1α) and interleukin-6 (IL-6α) (Bierhaus *et al*, 2005; Elosta *et al*, 2012). The most important biological effects of AGEs binding to RAGE is the formation of intercellular ROS. The first step of ROS

production is triggered by the activation of NADPH-oxidase system (NOX family including NOX1, 2, 3, 4, 5 and DOX1 and DOX2) (Frey *et al*, 2009). Nevertheless, glucose-metabolising pathway consisting the enzymes aldose reductase and sorbitol dehydrogenase that is discovered in organs that develop diabetic complications. Many studies have indicated that production of ROS and the increase in oxidative stress are significant factors in generating signal transduction and changes in gene expression (Cho *et al*, 2013). Furthermore, the transcription factor NF-κB is accounted as a major intercellular target of hyperglycaemia and oxidative stress (Nigel *et al*, 2009).

In addition, AGE generation is improved in the presence of hyperglycaemia and oxidative stress. AGE-RAGE interaction stimulates a various number of signal transduction pathways including p21 ras, mitogen activated protein kinasen (MAPK) such as p38, extracellular regulated (ERK)-1/2 and c-Jun N-terminal kinase (JNK), Cdc42/rac, and Jak/Stat pathways as demonstrated in Figure 1.8 (Cho *et al*, 2013). The consequence is the generation of gene products, such as vascular endothelial growth factor (VEGF), Vascular cell adhesion molecule-1 (VCAM-1) and others, which cause cellular injury and are significantly responsible for the long term complications associated with diabetic mellitus (Basta *et al*, 2004; Hudson & Schmidt, 2004). ROS is an important component in oxidative stress and plays a key role in diabetic complications (Rojas & Morales, 2004).

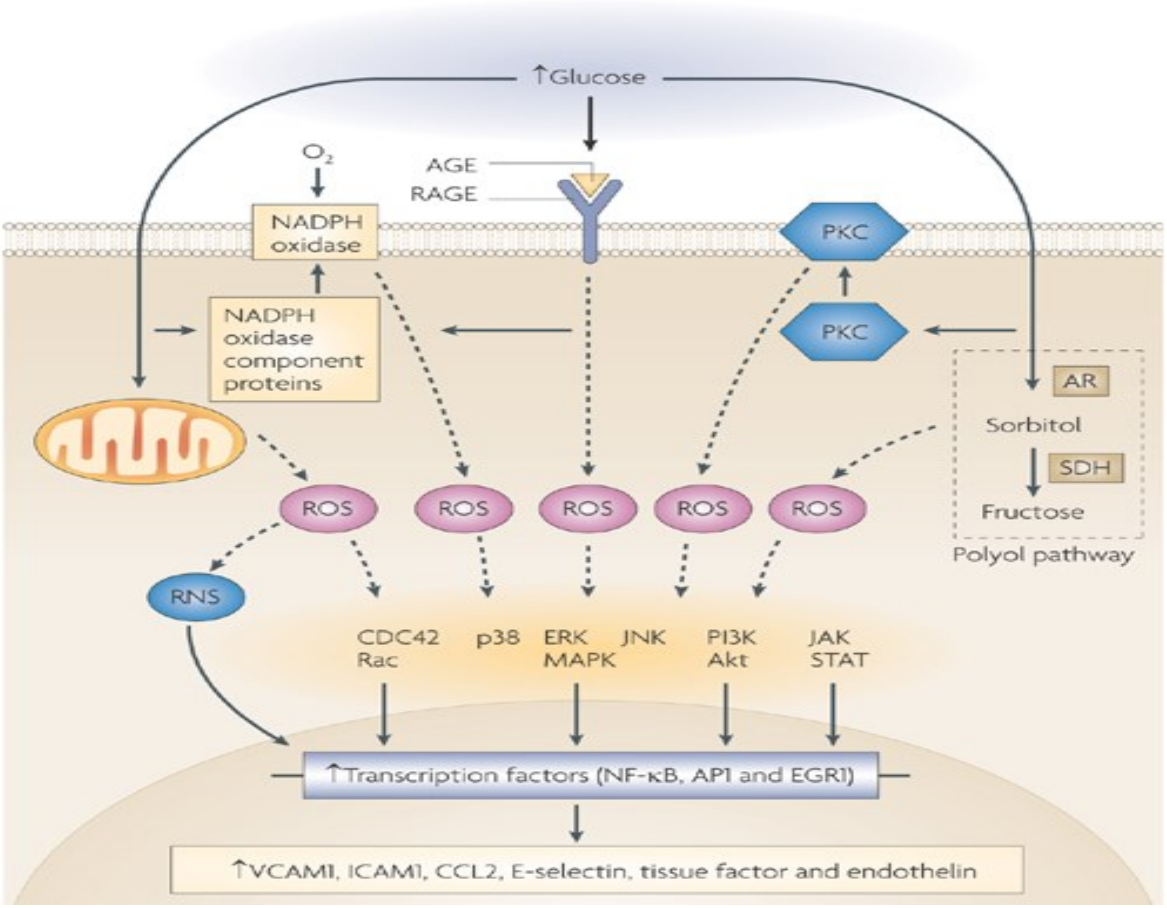


Figure 1.8: Signal transduction pathways activated by AGE-RAGE interaction. Activation of extracellular and cystolic domains of RAGE by AGEs mediates increased the production of ROS by different signalling pathways (Nigel *et al*, 2009).

1.13 Glycation and oxidative stress

The adverse effects of oxidative stress on human health have received serious attention. There is a strong relation between the formation of AGEs and oxidative stress (Miguel, 2010). The most important chemical reactions occurring in every cell are the oxidation and reduction of molecules. The results of these reactions are the formation of free radicals, such as superoxide anion radicals ($O_2^{\bullet-}$), hydroxyl radicals ($OH^{\bullet}$), singlet oxygen ($^1O_2$), hypochlorous acid (HOCl), peroxyl radicals ($ROO^{\bullet}$), alkoxyl radicals ($RO^{\bullet}$) and non-free radical molecules such as hydrogen peroxide (H_2O_2) which are able to react with cell components such as proteins, lipids, and nucleic acids (Rains *et al*, 2013). Eventually, these interactions lead to changes in the structure and function of the cells, create oxidative stress, and may cause several chronic diseases such as atherosclerosis, Alzheimer's disease, chronic inflammation and diabetes (Nigel *et al*, 2009) . Glycated proteins play a vital role in the enhancement of oxidative stress

in diabetes and form approximately 50-fold more free radicals than non-glycated proteins (Pandey *et al*, 2010). Both ROS and reactive nitrogen species (RNS) are classed as free radicals. They are formed as a consequence of normal cellular reactions. Hence, free radicals are not only produced as a result of normal metabolism, but also form as a consequence of exposure to toxic agents, environmental pollutants, drugs, radiation, natural food constituents, smoking and inflammatory disease (Jones *et al*, 2008).

1.14 Antioxidant enzymes and oxidative stress

ROS are considered a major factor of oxidative stress, which exist in all cells in balance with antioxidant compounds. In a normal cell, there is an appropriate pro-oxidant–antioxidant balance. Oxidative stress occurs when this balance is disrupted due to the reduction of antioxidants or elevated accumulation of ROS or both (Pandey & Rizvi, 2010). The biological effects are controlled *in vivo* by different enzymatic and non-enzymatic antioxidant defence systems. Antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase, and non-enzyme antioxidants including ascorbic acid (vitamin C), α-tocopherol (vitamin E), glutathione, uric acid, carotenoids, flavonoids, catechins and ferritin are important components of the antioxidant system against oxidative stress (Krishnaiah *et al*, 2011). Furthermore, any chemical compound capable of forming potentially toxic oxygen species are also known as pro-oxidants. Antioxidants consist of enzymes and molecules that may act as potent physical barriers to inhibit ROS production or ROS transfer to vital biological sites of action such as the cell membrane. Therefore, they act as chemical traps that engulf energy and electrons, quenching ROS activity (Miguel, 2010).

1.15 Diabetic complications and AGEs

Macrovascular and microvascular are recognised as main types of diabetic complications. Despite a considerable amount of literature on this topic, it is still unclear what the exact cause of most diabetic complications is. However, chronic exposure to hyperglycaemia via elevated formation and accumulation of AGEs plays a key role in the progress of diabetic complications. Similarly, reduced AGE formation through intensive glycaemic management may decrease the risk vascular complications in diabetic patients (Ahmed & Thornalley, 2007; O'Sullivan & Dinneen, 2009).

1.15.1 AGEs and microvascular complications

Generally, microvascular complications can be defined as dysfunctional alterations occurring in the microvascular bed. However, retinopathy, neuropathy and nephropathy are the most common type of microangiopathy (Peppa & Vlassara, 2005), and it takes 10-15 years to develop such complications in patients suffering from diabetes. Additionally, microangiopathy is characterised by increased vascular permeability and a prothrombotic state, the thickening of basement membranes and decreasing blood flow (Huebschmann *et al*, 2006). Notably, there is no effective therapy for microangiopathy complications associated with diabetes mellitus (Ahmed & Thornalley, 2007).

1.15.1.1 Diabetic retinopathy

Diabetic retinopathy is one of the commonest microangiopathy complications in diabetes mellitus and it can cause loss of vision on a worldwide scale. The occurrence of retinopathy increases with the length of existence of diabetes mellitus. After 20 years of diabetes, nearly all patients with type I diabetes and more than 60% of patients with type II diabetes prove to have some degree of retinopathy. A large number of *in vivo* and *in vitro* longitudinal studies involving diabetic retinopathy have illustrated that increased intercellular and extracellular AGEs during diabetes can play a key role in diabetic retinopathy development and progression (Anitha *et al*, 2008; Sato *et al*, 2006). Furthermore, the elevated AGE production can generate abnormal proliferative reactions in retinal microvascular endothelial cells, and subsequently cause abnormal expression of endothelial nitric oxide synthase (eNOS) (Giacco & Brownlee, 2010). Additionally, AGE production displays an inhibitory response on pericyte growth, thereby causing a loss of pericytes, which is the earliest histological hallmark of diabetic retinopathy. This, in turn, causes basement membrane thickening which significantly contributes to the breakdown of the inner blood-retinal barrier (Stitt & Curtis, 2005). Neural dysfunction and depletion may occur in diabetic retinopathy as a result of the production and accumulation of AGEs (Anitha *et al*, 2003). It has been reported that growth factors, for example VEGF, growth hormone and transforming growth factor beta have also been (Fowler, 2008). In recent years, there has been a study in animal models that reported that suppression of VEGF formation is accompanied by decreased progression of retinopathy (Fowler, 2011). postulated to play a pivotal role in the progression of diabetic retinopathy. Increases in the production of VEGF in diabetic neuropathy may be related to the response to hypoxia

1.15.1.2 Diabetic cataract

Hyperglycaemia and the duration of diabetes are considered main risk factors for the development of cataracts; diabetic patients suffer from cataracts 10 years earlier than the healthy individual (Gul *et al*, 2008). AGEs precursors such methylglyoxal, play a pivotal role in cataract formation. Long-lived proteins such as lens crystallins, are more likely to be prone to glycation (Fowler *et al*, 2011). Glycation of lens crystallins may result in protein cross-linking, oxidation and the production of the high molecular weight aggregates responsible for cataracts (Cheng *et al*, 2006; Linetsky *et al*, 2008). Moreover, glycation of channel proteins and the sodium potassium pump (Na/K-ATPase) may significantly contribute towards cataract production in diabetes (Gallicchio & Bach, 2010).

1.15.1.3 Diabetic nephropathy

Diabetic nephropathy is the main cause of end stage renal disease, where a three-fold elevation in the accumulation of AGEs is indicated, and is responsible for a significantly increased morbidity and mortality in patients with diabetic mellitus (Makita *et al*, 1991; Yamagishi *et al*, 2007). It occurs in 40-50% of all diabetic patients (Wolf, 2004). Diabetic nephropathy is associated with complications such as coagulation of glomerular and tubular basement membranes, thickening and mesangial matrix expansion, which eventually leads to vascular occlusion, microvascular impairment and fibrotic conversion, thus resulting in glomerulosclerosis (Coughlan *et al*, 2005). The kidney is a key target for AGEs-mediated damage, and also is a major site of clearance of AGEs (Goh & Cooper, 2008). In addition, diabetic nephropathy involves large concentrations of AGEs and these can be related to reduced clearance in the kidney rather than elevated formation by glycation of proteins (Monnier *et al*, 2005). Recently, *in vivo* studies have shown that higher levels of pentosdine and CML are detected in diabetic patients' renal tissues with or without end-stage renal disorder (Beisswenger *et al*, 2013; Hashimoto *et al*, 2010).

1.15.1.4 Diabetic neuropathy

More than 50% of all patients with diabetes suffer from neuropathy complications, which are the cause of significant morbidity and mortality (Duby *et al*, 2004). Diabetic neuropathy results in continuous damage to the peripheral sensory and autonomic nervous system (Beisswenger *et al*, 2013). The production of AGEs is an important biochemical pathway which contributes to diabetic neuropathy (Fowler, 2008). Recent evidence has suggested that AGEs-modified peripheral nerve myelin is vulnerable to phagocytosis by macrophages, which results in

segmental demyelination. Consequently, the alteration of main axonal cytoskeletal proteins, for instance tubulin, neurofilaments and actin by AGEs, leads to axonal degeneration and damaged axonal transport in diabetic neuropathy (Sugimoto *et al*, 2008). Furthermore, AGEs alter the expression levels of the inducible form of nitric oxide synthase (iNOS), and in turn, this may reduce nerve blood flow and cause hypoxia in the peripheral nerves (Wada & Yagihashi, 2005). Previous research has indicated that interactions between AGEs and RAGE contribute to endoneural vascular dysfunction, resulting in microangiopathy in the peripheral nerves, although their precise role remains unknown (Huijberts *et al*, 2008).

1.15.2 AGEs and macrovascular complications

Macrovascular complications are associated with a series of pathological alterations influencing major blood vessels and resulting in structural and functional abnormalities. It has been reported that the glycation of wall structures and functional alterations generates endothelial dysfunction and thickening of artery walls or reduces vascular compliance. This results in structural amendments. Moreover, atherosclerosis is counted as the greatest risk factor leading to diabetic macrovascular complications such as stroke, myocardial infarction and peripheral vascular disorders (Rahman *et al*, 2007).

1.15.2.1 Diabetic atherosclerosis

Atherosclerosis appears to be a multifactorial disorder in origin, resulting from excessive accumulation of lipids in the vascular wall, which limits blood flow. Currently, it is the root cause of the biggest killer worldwide. Several studies thus far have linked AGE production with atherosclerosis development in diabetic patients (Huijberts *et al*, 2008; Mallika *et al*, 2007). Conversely, AGE-RAGE interaction may also cause increased endothelial impairment, elevated plaque destabilisation, neointimal proliferation and delayed wound healing (Guo & DiPietro, 2010; Hallam *et al*, 2010). Moreover, AGEs provoke vasoconstriction via quenching of NO (Basta *et al*, 2004; Pandolfi & De Filippis, 2007). It has been suggested that AGE–RAGE interaction may play a central role in the development of atherosclerosis in diabetic patients (Jandeleit-Dahm & Cooper, 2008; Soro-Paavonen *et al*, 2008). Additionally, activation of NF-kB and activator protein-1 transcription factors in vascular wall cells by AGEs leads to the elevated expression of a multiple of atherosclerosis-related genes, including endothelin-1, VCAM-1, inflammatory cytokines and PAL-1(Laakso, 2010).

1.16 Measurement of AGEs

AGEs are recognised as complex molecules in nature and their detection in tissue and serum has been difficult (Smit & Lutgers, 2004). To date, AGEs measurement is limited to investigate laboratories, and there is no universal accepted assay for measuring AGEs. Several assays have been used qualitatively and quantitatively to examine the level of AGEs. Fluorescence spectroscopy is reckoned to be a main method for detection of fluorescent AGEs *in vitro* and *in vivo* (Vigneshwaran *et al*, 2005). The fluorescence measurement of AGEs was used because of the easy to reproduce data by incubating proteins and reducing sugar such as glucose in order to explore its occurrence in diabetes (Monner *et al*, 2005). However, this technique has limitations because it is only an indicator of the trend of AGEs and does not provide any information to recognise the AGEs structurally (Giardino *et al*, 1994). The use of matrix–assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) has successfully been utilized to examine AGEs (Lapolla *et al*, 2005). This is a rapid and cost effective method with benefits of soft ionisation, excellent sensitivity; high resolution and minimum sample work up. MALDI-TOF-MS, peptide mapping is an important technique to measure specific protein glycation products *in vitro* and *in vivo* (Ahmed & Thornalley, 2007; Kislinger *et al*, 2004; Niwa, 2006). Nevertheless, separation assay methods, for example SDS-PAGE, are utilised during the course of glycation research (Ahmad *et al*, 2007; Xie *et al*, 2011). This is a common and cost effective technique which easily detects oligomerisation of glycated protein. Moreover, immunochemical techniques such as ELISA using polyclonal and monoclonal antibodies specific to defined AGEs structures, allow highly sensitive and quantitative estimations of AGEs (Elosta *et al*, 2012).

1.17 Medicinal plants and diabetes mellitus

The World Health Organization (WHO) has estimated that 80% of the earth's inhabitants rely on traditional medicine for their primary health care needs, and most of this therapy involves the use of plant extracts and their active components (WHO, 2013). Traditional medicines have been used for treating chronic disorders such diabetes since ancient times; they are derived mainly from medicinal plants, minerals and organic matter. According to the WHO, approximately 150 species out of 21,000 plants are commercially available for medicinal purposes in the world (Joseph & Jini, 2013). A recent report has indicated that approximately 30% of patients with diabetes mellitus use traditional medicines (Raman *et al*, 2012). A large amount of medicinal plants are currently under extensive study and analysis *in vivo*, but only a

small portion of these studies have successfully entered clinical trials. Nevertheless, none of these plant extracts have so far been approved for clinical use. Recently, a considerable number of studies have identified that complimentary medicine in combination with antiglycation and antioxidant nutrients is believed to be a safe and simple complement to traditional medicines and it is hoped they may prevent and target diabetic complications (Ahmad *et al*, 2007; Babu *et al*, 2006). For example, tomato paste showed strong inhibitory activity against glycation (Kiho *et al*, 2004) and autoxidative reactions (Cervantes-Laurean *et al*, 2006). In addition, other medicinal plants containing polyphenolic molecules such as in soy sauces (Mashilipa *et al*, 2011), *Luobuma* tea (Yokozawa & Nakagawa, 2004), garcinol from *Garacina indica* (Yamaguchi *et al*, 2000) and crisilineol from *Thymus vulgaris* (Roby *et al*, 2013) have been identified to be potent inhibitors of AGE production. Nevertheless, a range of other antioxidant compounds has been shown to have antiglycation properties *in vitro*. For instance, the extract of green tea, is composed of a large portion of tannins (flavonoidas) (Babu *et al*, 2007), garlic (*Allium sativum*), which contains large amounts of S-allyl cysteine (Ahmad *et al*, 2007) as is curcumin isolated from turmeric (*Curcuma longa*) (Qadir *et al*, 2016). A considerable number of natural compounds such as caffeic acid, chlorogenic acid, epigallocatechin-3-gallate, capsaicin and hesperidin, have shown AGEs inhibitory effects (Popova *et al*, 2010).

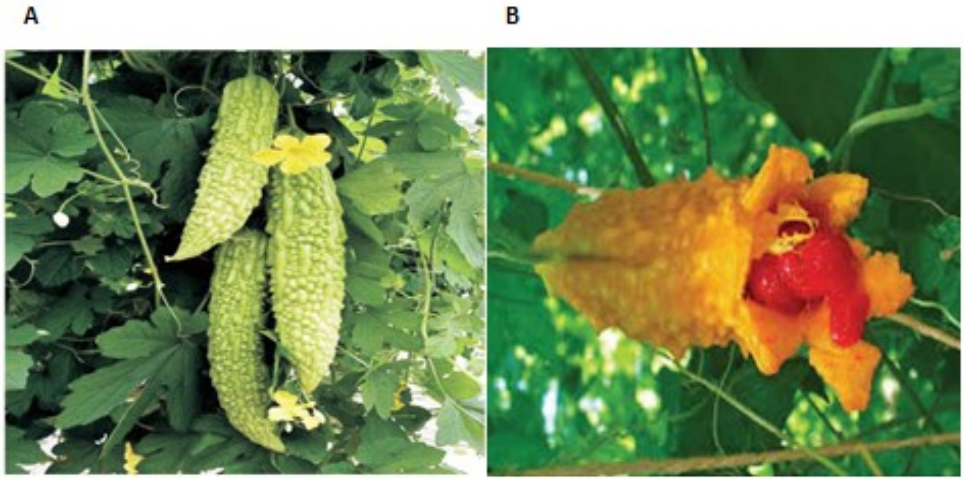
1.18 Description of *Momordica charantia*

Momordica charantia also known as Karela or bitter melon, is characterised by a slender flowering annual vine, and belongs to the *Cucurbitaceae* family. I *Momordica charantia* is amongst the popular medicinal plants currently used for alleviating diabetes worldwide. It is often referred to as food medicine, and its use is supported by modern scientific evidence (Joseph & Jini, 2013). MC is counted as a rich source of natural antioxidants, which are safe, inexpensive and are bioactive.t is a green vegetable, widely grown in all tropical areas of the world. This plant can grow up to 4-5 m in length, and bears a fruit with a warty surface. The immature, emerald green fruit changes to orange-yellow when ripe as demonstrated in Figure 1.9. MC possesses bitter-tasting fruit, stems, leaves and seeds (Joseph & Jini, 2013). Furthermore, MC possesses medicinal properties such as antimicrobial, antihelminthic, anticancerous, antimutagenic, abortifacient, antifertility and antidiabetic properties. Different parts of MC have different calorific values, for example, leaves produce 213.26 kcal/100g, fruit 241.66 kcal/100g and seeds 176.61 kcal/100g, respectively (Bakare *et al*, 2010).

Figure 1.9: *Momordica charantia* plant.

A- Immature green fruit and flower of (Joseph & Jini, 2013)

B- Fully ripe fruit and seeds (Cao *et al*, 2014)



In scientific terms, the plant is to referred as:

a- Kingdom: Plantae
b- Division: Plantae
c- Order (family): Cucurbitales
d- Genus: *Momordica*
e- Species: *M. charantia* L
f- Duration: Annual
g- Synonyms: *Momordica chinenus*, *Momordica elegans*, *Momordica indica*, *Momordica operculata*, *Momordica sinenuns* and *Silyos fauriei.*

1.19 Phytochemical constituents of *Momordica charantia.*

MC possesses about 230 compounds as shown in Table 1.1. The most important property of this plant for humans and animals is its antioxidant activity. This antioxidant property of MC is related to the mixture of steroidal saponins named charantins, insulin-like peptides, triterpene, steroids, lipids, phenolics and alkaloids which are present mainly in the fruit of MC. Subsequently, the fruit of the plant has shown the most effective anti-hyperglycaemic properties (Paul & Raychaudhuri, 2010). However, MC fruits contain high amounts of different vitamins such as vitamin C, vitamin E, vitamins B1, B2, B3 and B9 (folate) and also a high density of minerals including potassium, calcium, zinc, magnesium and iron. It also contains

large amounts of dietary fibre. Conversely, several glycosides separated from the fruit and stem of MC are classified under the genera of cucurbitane triterpenoids. Subsequently, the possible anti-hyperglycaemic mechanism of MC is related to four different triterpenoids that have AMP-activated protein kinase activity (Joseph & Jini, 2013). Nonetheless, the potent antioxidant activity of MC is partially due to phenolics, flavonoids, flavones, terpenes, anthraquinones and glucosinolates, which all possess a bitter taste (Joseph & Jini, 2013; Snee *et al*, 2011). Polyphenolic compounds, which have been shown to be more effective antioxidants *in vitro* than others, are synthetic antioxidants such as tocopherols and ascorbate. This property of polyphenolics is related to their free radical scavenging activity. The antioxidant abilities of these active compounds is derived from three different properties. Firstly, they possess high reactivity as hydrogen or electron donors. Secondly, polyphenol-derived radicals stabilise and delocalise unpaired electrons. Thirdly, they chelate transition metal ions (Tupe *et al*, 2014). Antidiabetic effects of MC have been examined in cell culture, animal models and human studies (Hui *et al*, 2009).

Table 1.1: The major phytochemicals and constituents in different parts, body, leaves, fruits and seeds, respectively of *Momordica charantia*.

Plant source	Phytochemicals present	References
Plant body	Momorcharins, momordenol, momordicilin, momordicins, momordicinin, momordin, momordolol, charantin, charine, cryptoxanthin, cucurbitins, cucurbitacins, cucurbitanes, cycloartenols, diosgenin, elaeostearic acids, erythrodiol, galacturonic acids, gentisic acid, goyaglycosides, goyasaponins and multiflorenol	(Orlovskaya & Chelombitko, 2007; Manoharan *et al*, 2014)
Plant leaves	Glycosides, saponins, alkaloids, fixed oils, triterpenes, proteins and steroids	(Hui *et al*, 2009)
Plant fruit	Momorcharins, momordicine, charantin, polypeptide- p insulin, ascorbigee, amino acids, aspartic acid, serine, glutamic acid, threonine, glutamic acid, threonine, alanine and g-amino butyric acid. Other constituents include pipecolic acid, luteolin and a number of fatty acids such as lauric, myristic, palmitic, palmitoleic, stearic, oleic, linoleic, linolenic acid	(Manoharan *et al*, 2014)
Plant seeds	Urease, amino acids: valine, threonine methionine, isoleucine, lectin, leucine, phenylalanine, vicine.	(Leung *et al*, 2009; Orlovskaya & Chelombitko, 2007)

1.20 Possible pharmacological actions of *Momordica charantia*.

Antiglycation and antioxidant properties of MC are derived from its phytochemical constituents. This activity could be exerted via different physiological and biochemical processes as shown Figure 1.10. Briefly, MC has some activity in the regulation of glucose and lipid metabolism, which has been tested in diabetic animals and diabetic patients. The mode of action is not yet fully understood. MC can be utilised as an alternative medicine for the management of diabetic complications. Moreover, the therapeutic efficacy of MC requires it to be evaluated in clinical trials with a large sample size (Cefalu *et al*, 2008).

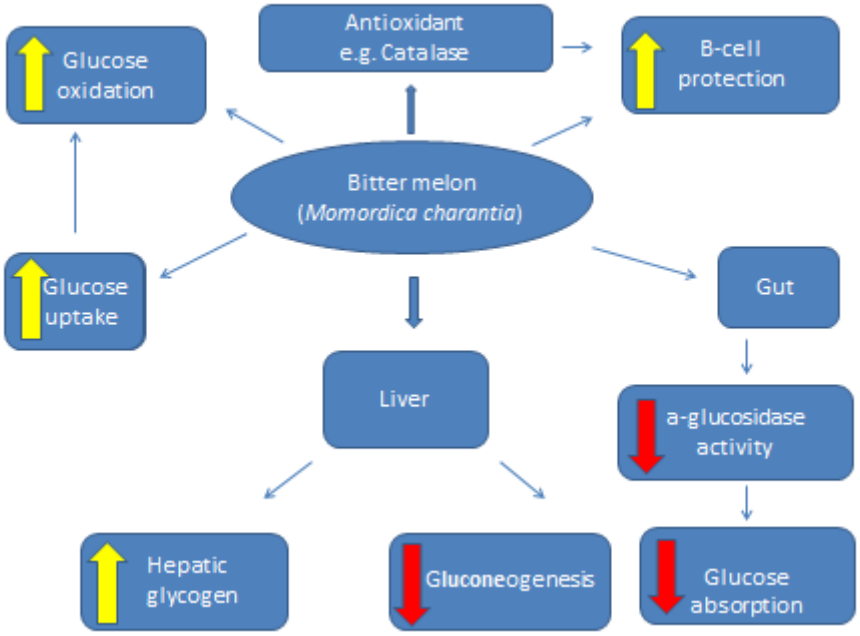


Figure 1.10: Possible mechanism of *Momordica charantia* in the reduction of blood Glucose (Prabhakar & Mukesh, 2011)

1.21 Toxicity of *Momordica charantia*.

It has been reported that MC is safe, with no signs and symptoms of nephrotoxicity, hepatotoxicity, any adverse reaction on food intake, growth organ weights or haematological parameters *in vivo* when it given orally in low doses up to 60 days (Joseph *et al*, 2013). Nevertheless, large numbers of clinical trials have indicated decreased toxicity of all parts of MC when administrated orally (Kumar *et al*, 2010). On the contrary, toxicity and even death, in laboratory animals has been reported when the extract was administered in high doses in other ways rather than orally (Joseph *et al*, 2013). Vicine is an active chemical compound contained within the seeds of MC. It can trigger symptoms in susceptible individuals with

glucose-6-phosphate dehydrogenase deficiency (Leung *et al*, 2009). Previous research has reported that vicine could causes hypoglycaemic coma and convulsions in children, decreased fertility in mice, a favism-like syndrome, elevations in gamma-glutamyltransferase and alkaline phosphatase levels in animals, and headache (Kumar *et al*, 2010). MC can sometimes cause heartburn and ulcers and should not be taken during pregnancy.

1.22 Aims

- To determine whether MC can inhibit the formation of AGEs *in vitro* .
- To determine the antioxidant properties of MC and its active ingredients *in vitro*.
- To determine whether MC can inhibit oxidative stress in endothelial cells by studying oxidative stress-related transcription factor parameters and their expression.

Chapter 2. Materials and methods

2.1 Materials

- 24-well, 48-welland 96-well plates (Scientific Laboratory Supplies, UK)
- 2-Mercaptoethanol (Sigma, UK)
- Acetic acid (Fisher, UK)
- Acrylamide/Bis solution 40% (Bio-Rad Laboratories, Germany).
- Aminoguanidine (Sigma, UK)
- Ammonium persulphate (Sigma, UK)
- Antibiotics: Penicillin and Streptomycin solution containing L-glutamine (PSG) in 0.9% NaCl (Sigma, UK)
- Ascorbic acid (Sigma, UK)
- Blotting papers (Schleicher and Schuell, Germany)
- Bovine aortic endothelial cells, secondary cell line (Cell and Molecular Biology Research Laboratory, Manchester Metropolitan University, UK)
- Bovine serum albumin (Sigma, UK)
- Bromophenol blue (Serva, Germany)
- Chloroform (Sigma, UK)
- ColorBurst™ Electrophoresis Marker for SDS-PAGE (Sigma, UK)
- Coomassie brilliant blue (Sigma, UK)
- Copper sulphate (Fluka, UK)
- Cryotubes (Scientific Laboratory Supplies, UK)
- Detoxi-gel endotoxin removal columns (Thermo Scientific, USA)
- D-glucose (BDH, UK)

- Dialysis tube (Visking, UK)
- 2′,7′-Dichlorodihydrofluorescein diacetate (Sigma, UK)
- Dulbecco's modified Eagle's medium (Lonza SPRL, Belgium)
- ECL1 and ECL2 kits (Amersham Biosciences, UK).
- Eppendorf tubes (Scientific Laboratory Supplies, UK)
- Ethanol (Fisher, UK)
- E-toxate kit for endotoxin measurement (Sigma, UK)
- Ferric chloride ($FeCl_3$) (Sigma- Aldrich, UK)
- Ferrous chloride ($FeCl_2$) (Sigma- Aldrich, UK)
- Ferrous sulphate ($FeSO_4$) (Sigma- Aldrich, UK)
- Fibroblast growth factor-basic (R & D Systems, USA)
- Foetal bovine serum (Lonza, Belgium)
- Gallic acid (Sigma- Aldrich, UK)
- Glacial acetic acid (Fisher, UK)
- Glycine (BDH, UK)
- Glycerol (BDH, UK)
- Hydrochloric acid (Sigma, UK)
- Isopropanol (Sigma, UK)
- Lysozyme (Sigma, UK)
- Mannitol (Sigma, UK)
- 2-Mercaptoethanol (Sigma, UK)
- Methanol (Fisher, UK)
- Methylglyoxal (Sigma, UK)

- *Momordica charantia* – bitter melon (Local market, Manchester-UK)
- Muslin cloth (Local cloth shop, Chorlton, Manchester, UK)
- N, N, N′, N′-tetramethylethylenediamine (TEMED) (Sigma, UK)
- Nitrocellulose membrane (Scientific Laboratory Supplies, UK)
- Quinine sulphate (Sigma- Aldrich, UK)
- Parafilm (UK)
- Phosphate buffer saline (Lonza, UK)
- Plate sealers (R & D Systems, USA)
- Potassium ferricyanide (Fluka, UK)
- Potassium persulphate (Sigma, UK)
- Potassium phosphate mono- and di-basic anhydrous (Sigma, UK)
- Protein molecular weight marker for SDS-PAGE (Sigma, UK)
- Razor blades (Kratos Analytical, UK)
- Ribose (BDH, UK)
- Skimmed milk powder (Local store, Manchester, UK)
- Sodium acetate (Fluka, UK)
- Sodium azide (Sigma, UK)
- Sodium bicarbonate (Sigma, UK)
- Sodium chloride (Sigma, UK)
- Sodium cyanoborohydride (Fluka, UK)
- Sodium dodecyl sulphate (BDH, UK)
- Sulphuric acid (Sigma-Aldrich, UK)
- Tissue culture flasks (T-25, and T-75) (Scientific Laboratory Supplies, UK)

- Trichloroacetic acid (Sigma, UK)
- Tris (hydroxymethyl) methylamine (BDH, UK)
- Trypsin-10X (Sigma, UK)
- Tween 20 solution (Sigma, UK)
- Trolox, (Fluka, UK)

2.2 Equipment and software

- Adobe illustrator CS4
- Adobe Photoshop 7.0
- Analytical balance (Sartorius Machatronics Ltd, UK)
- Automated cell counter (Bio-Rad, UK)
- Autovortex mixer SA1 (Stuart Scientific Co, UK)
- Analytical balance (Sartorius Machatronics Ltd, UK)
- Blender (Kenwood Limited, UK)
- Centrifuge S415D (Eppendorf, Germany)
- Class II microbiological safety cabinet (Walker Safety Cabinet Ltd, UK)
- CO_2 incubator for cell culture use (Lab Impex Research, UK)
- Cross power 500 for electrophoresis (Atto, Japan).
- Digital multi channel pipettes (Eppendorf, Germany)
- Dual gel casting electrophoresis chambers (Atto, Japan)
- Eppendorf Centrifuges 5415 D (Eppendorf, Germany)
- Fuji S2 Pro camera
- G Box Chem HR 16 (gel documentation and analysis system) (Syngene, UK)
- Gene tool image analyzer (Syngene, UK)
- Grant-bio shaker POS-300 (Grant Instruments, UK)
- Ice maker (P & S Refrigeration, UK)
- Image J software analyser (free on line software)

- Inverted phase contrast microscope (Nikon TMS, Japan
- Laboratory freezer (Scientific Laboratory Supplies, UK)
- Laboratory fridge (Scientific Laboratory, UK)
- Laboratory pH/mV/temperature meter AGB-75 (Medical Scientific Instruments, England).
- LTE IP 30 incubator for glycation samples (Scientific Laboratory Supplier, UK)
- Luminescence spectrometer model LS 30 (Perkin Elmer LAS Ltd, UK).
- Magnetic stirrer hotplate (Stuart Scientific Co, UK)
- Microplate reader, 96-well (Spectramax, Finland)
- Microsoft office Excel 2003 (Microsoft, USA)
- Rotator shaker R100 (Luckman Ltd, UK)
- Sodium dodecyl sulphate-polyacrylamide gel electrophoresis tank (mini-Protean® 3 apparatus) (Bio-Rad Laboratories, Germany).
- Statistical Package for the Social Sciences 16.0 for Windows (SPSS) (SPSS Inc., Chicago, USA)
- Syngene G Box (Bio-imaging system) (Syngene, UK)
- G Box Chem HR 16 (gel documentation and analysis system) (Syngene, UK)
- Trans-blot SD semi-dry transfer cell (Bio-Rad Laboratories, Germany).
- Ultrospec 2000 UV–VIS spectrophotometer (Pharmacia Biotech Ltd, UK)
- Water bath (Grant Instruments Ltd, UK)
- Water de-ionizer (Millipore, UK)

2.3 Solutions

- Solution A: Acrylamide (29.2 g) and N, N´-methylene-bis-acrylamide (0.8 g) were dissolved in 100 ml of distilled water.

- Solution D: ammonium persulphate (100 mg) was dissolved in 1ml of distilled water. the solution was freshly prepared before use.

- BSA (1 mg/ml) standard solution for the Bradford protein assay: Bovine serum albumin (20 mg) was dissolved in 20 ml of distilled water. Freshly made, 30 minutes prior the experiment.

- Blocking buffer (1% BSA-TBST): BSA (1g) was dissolved in 100 ml of Tris-buffered saline and tween-20 (TBST). The pH was adjusted to 7.4.

- Destaining solution for SDS-PAGE analysis: Methanol (250 ml) was dissolved in 70 ml of acetic acid and the volume was made up to 1 L with distilled water.

- Developing solution for Western blotting membrane: ECL_1 solution (1 ml) was mixed with (1 ml) of ECL_2 solution and the mixture was applied to the membrane.

- Electrode buffer (running buffer) for SDS-PAGE and Western blotting analysis: Tris-base (12.02 g), SDS (4 g) and 57.68 g of glycine were dissolved in 2 L of distilled water. The buffer was stored at room temperature.

- Blocking buffer 5% milk as used in Western blotting analysis: Skimmed milk (5 g) was dissolved in 100 ml of TBST buffer. The pH was adjusted to 7.4.

- Sample buffer for used in Western blotting: Tris-base (1.51 g), 20 ml of glycerol, 4 g of SDS, 10 ml of 2-mercaptoethanol and 0.004 g of bromophenol blue were dissolved in 100 ml of distilled water. The solution was filtered and the pH adjusted to 6.8 and stored at -20°C.

- Running buffer: Tris-base (1.5 g), glycine (7.2 g) and 0.5g of SDS were dissolved in 500 ml of distilled water.

- Solution C: Tris-HCl buffer 0.5 M, pH 6.8; Tris-hydroxymethyl aminomethane (6.1 g), 0.4 g of SDS and 4.2 ml of HCl were dissolved in distilled water, made up to 100 ml. The pH was adjusted to 6.8 and the solution bottle was covered with aluminium foil and stored in a fridge.

- Solution B: Tris-HCl buffer (1.5 M), pH 8.8; Tris (Tris-hydroxymethyl amino methane) (18.2 g), 0.4 g of SDS and 2 ml of HCl were dissolved in distilled water. The pH was adjusted to 8.8 and the solution was stored in a room temperature.

- Sample treatment buffer: Sodium dodecyl sulphate (0.1 g), and 0.1 ml of 2-mercaptoethanol were added to 1 ml of solution C and 2 ml of glycerine and the volume was made up to 10 ml with distilled water. The sample treatment buffer was stored at room temperature.

- Separation gel (12.5%) for Western blotting analysis: Separating solution (2.5 ml) was added to 4.2 ml of distilled water and mixed with 3.3 ml of 40% cold acrylamide solution. Then, 100 µl of ammonium persulphate (10%) was added to the mixture. Gel polymerization was initiated by adding 10 µl of TEMED and allowed to polymerize for 10 – 15 minutes.

- 0.1 M and 0.2 M sodium phosphate buffer solution (pH 7.4): the acid component of the buffer (sodium dihydrogen phosphate) and the basic component (sodium hydroxide) were weighed out in a certain ratio according to the Henderson Hasselbalch equation. The desired volume was then accordingly adjusted with distilled water and the solution was mixed. The pH meter was calibrated at pH 7.0 and the pH of the buffer was adjusted to 7.4. Sodium azide (3 mM) was added to the mixture to prevent any bacterial growth. The buffer was stored at 4 °C.

- Stacking solution used for Western blotting analysis: Tris-base (15 g) and 1 g of SDS were dissolved in distilled water. The pH was adjusted to 6.8 with HCl. The final volume of the buffer was made up to 250 ml with distilled water.

- Staining solution for SDS-PAGE analysis: Coomassie brilliant blue (2.5 g) was dissolved in 500 ml of methanol and 100ml of acetic acid. The volume was made up to 1 L with distilled water and then filtered. The staining solution was stored at room temperature.

- TBST buffer: Tris-base (2.422 g), 16.36 g of NaCl, and 2 ml of Tween 20 were dissolved in 2 L of distilled water. The pH was adjusted to 7.4 with HCl.

- Towbin buffer used for Western blotting analysis: Tris-base (1.51 g), 7.2 g of glycine and 0.167 g of SDS were dissolved in 400 ml distilled water and 75 ml of methanol. The pH was adjusted to 8.3. The final volume of the buffer was made up to 500 ml with distilled water.

- Tracking dye solution for SDS-PAGE analysis: Bromophenol blue (1 mg) was added to 0.1 ml of glycerine and mixed with 0.9 ml of distilled water.

- Reconstitution of FGF-2: Sterile phosphate- buffered saline was added to the vial in order to prepare a working stock solution (≥ 100 g/ml). The carrier-free protein was immediately used upon reconstitution to avoid loss in activity. FGF-2 was stored at -20 °C.

2.4 Methods

2.4.1 Preparation of *Momordica charantia* extracts

The fresh plant MC was purchased from a local Asian market (Manchester, England) in February 2013. The extraction method in this study was based on a method used previously (Manoharan *et al*, 2014). Briefly, whole plant (fruit pulp and seed) were used in this extraction. The whole plant was washed thoroughly with deionized water. Approximately 100 g of the clean plant was weighed and then cut into small pieces and mixed with 100 ml of deionized water, then blended for 5 minutes at low speed, and blended again for another 5 minutes at high speed. The green supernatant made up to 200 ml with deionized water then blended again for a few seconds. Firstly, 100 ml of the sample was sonicated at 35°C for 25 minutes, and then the solution filtered by muslin cloth, followed by centrifugation for 30 minutes at 9000 x g. After that, the supernatant was transferred into a 250 conical flask, and then evaporated under air pressure until the supernatant become a very thick substance (concentrated) that was for CWE of MC. The preparation for HWE in addition to above steps, 100 ml 100 ml of the sample was incubated for two hours at 67°C, then sonicated at 35°C for 25 minutes and filtered by muslin cloth (Manoharan *et al*, 2014). Both samples of concentrated extracts were freeze dried and kept at -80ºC for future use.

2.4.2 Glycation of proteins *in vitro*

The detection of glycated proteins was based on the methods described previously (Ahmad *et al*, 2007). In short, either BSA or lysozyme (10 mg/ml) were incubated with different concentrations of sugars (glucose, ribose or methylglyoxal) in 0.1 M Na phosphate buffer containing 3mM sodium azide, pH 7.4 at 37 °C ± different concentration of *Momordica charantia* extracts for a defined period of time. Control sample were incubated under the same conditions without the addition of sugars or extracts. After the incubation period all samples were kept at -20ºC for future use.

2.4.3 Protein dialysis

Samples were dialyzed to remove unbound sugars, prior to protein estimation. A 10 cm length of dialysis tubing was boiled for two minutes in distilled H_2O with a few drops of chloroform to prevent microbial contamination. The tube was removed from the boiling water, and then washed with distilled water. The glycated protein samples were defrosted to room temperature

and loaded into dialysis tubing, and then dialyzed against 4 L of distilled water at 4°C with 4-5 changes for two days; the dialysis was carried out by stirring the tube samples with frequent changes of the buffer until the equilibrium was reached. The samples were then removed from the dialysis tube and stored at -20ºC for future use.

2.4.4 Endotoxin removal

The Detoxi-gel resin column underwent regeneration through wash cycles with five resin-bed volumes of 1% sodium deoxycholate, and subsequently by 3-5 resin-bed volumes of basal DMEM. Bovine serum albumin-AGEs were diluted in DMEM containing red phenol (used a tracking dye) and added to the column. The flow ceased when the samples filled the resin-bed from top to bottom. The column was for 60 minutes at room temperature prior to samples being collected. In order to achieve greater efficiency with a gravity-flow column, the samples emerging from the column after the void volume were collected.

2.4.5 Measurement of endotoxin content

Glycated proteins (BSA-AGEs) were examined for endotoxin content using E-toxate kit. A 100 µl of MC extracts, water and endotoxin standards were added to the bottom of test tubes. Consequently, 100 µl of E-toxate working solution was added to each tube by inserting a pipette to just above the contents, and the lysate was then allowed to flow down the inside of the tube. The tubes were mixed gently and covered with foil or parafilm, and incubated for 1 hour at 37 °C. Following incubation, the tubes were gently inverted to observe evidence of gelation. The formation of a hard gel was considered to be a positive test. All other result including soft gels, turbidity, and increase in viscosity or clear liquid were considered to be negative tests. Notably, the amount of endotoxin level (EU/ml) was calculated using the following equation:

Endotoxin (EU/ml) = 1/ H x L
Where L is the lowest concentration of endotoxin standared found positive and H is the highest dilution of sample (+ ve).

2.4.6 Determination of protein concentration by Bio-Rad protein assay.

BSA (1 mg/ml) stock solution was diluted with distilled water to obtain the concentrations of 0, 5, 10, 20, 40, 60, 80 and 100 µg/ml for standards. Different sample extracts were prepared in a ratio of (1:10) where 10 µl of each sample diluted with 90 µl of distilled water. Bio-Rad solution was used as a dye reagent in a ratio of 1:5 in distilled water, then 2 ml of this solution

was added to each extract and standarsd. The absorbance of the protein solutions were measured at 570 nm and a standard curve generated. All values were expressed as µg/ml. An example of standard curve established for protein estimation is shown in Figure 2.1)

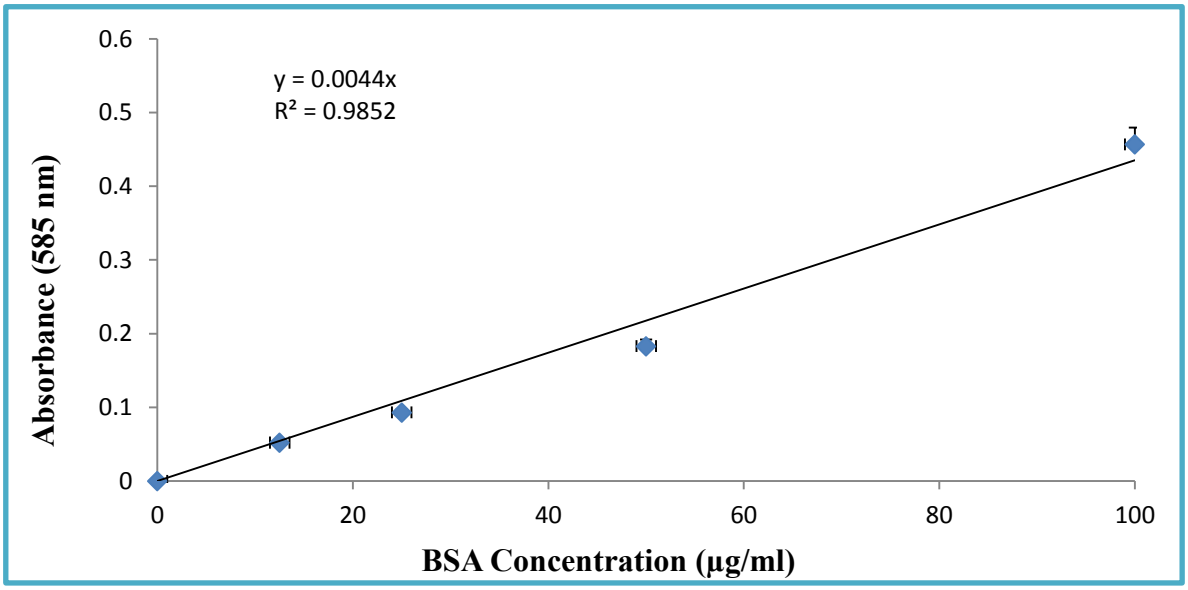


Figure 2.1: Calibration graph for protein estimation using the Bio-Rad method. Each value illustrates the mean ± SD (n=3).

2.4.7 Measurement of cross-linked AGEs by SDS-PAGE

Cross-linking of glycated proteins was determined by the extent of dimer and polymer formation in protein samples removed from cross-linking assays carried out in the presence MC extracts. This was assessed by SDS-PAGE using 10% polyacrylamide gels for lysozyme according to an established method (Xie *et al*, 2011). The glass plate sandwiches were assembled with a gaskets and clips in place. The separating gel consisting of 10% w/v for BSA and 15% w/v for lysozyme separation was introduced and left to polymerise for 1 hour at room temperature. Then the stacking gel (4.5 % w/v) was introduced, combs quickly inserted and allowed to polymerise for up to 30 minutes. 5µl of ColorBurst® electrophoresis marker was loaded in the first well of each gel and 3µl of tracking dye loaded in the rest of the wells. Then 10µl of appropriate control was loaded in the second well and 10µl of treated samples loaded in each of the remaining wells. Electrophoresis was carried out at 75V until the blue band reached the separating gel (approximately after 45 minutes). Then voltage was increased to 150V and left for approximately 60 minutes. The gels were stained with coomassie brilliant blue for 90-120 minutes. This was followed by a series of distaining washes on an orbital shaker until the background cleared and was ready for photographs to be taken. The gel images were

analysed using G Box Chem HR16. Bands were compared within the same gel. Integrated Density (I.D) of bands was measured and analysed and the percentage inhibition of cross-linked AGEs was calculated using the following formula:

% inhibition = (I.D without inhibitor – I.D with inhibitor) / I.D without inhibitor x 100

2.4.8 Measurements of fluorescent AGEs

The formation of AGEs was assessed by their characteristic fluorescence emission spectra at 420 nm after excitation at 350 nm. Glycated samples were thawed at room temperature and 0.2 ml of each sample was diluted in 1.8 ml of distilled water to give a solution with a final concentration of 1 mg/ml. Fluorescent measurements were carried out on a fluorimeter. Fluorescence intensity standards were used to calibrate and monitor the performance of the instrument and calibrated against quinine sulphate (1 ng/ml) in H_2SO_4. Fluorescence of AGEs was expressed in arbitrary units (AU) per mg of protein.

2.4.9 Detection of AGEs using ELISA

Cell Bio labs AGEs ELISA Kit was used for the detection and quantitation of AGEs protein adducts. AGE-BSA was obtained by incubating BSA(10 mg / ml) at 37 °C with ribose (0.5 M) in sodium phosphate buffer (0.1M, pH 7.4) containing 3 mM sodium azide for defined period of time (Mashilip *et al*, 2013). Dialysis and protein estimation of the AGE-BSA product was obtained. The glycated samples were diluted to a final concentration of 10 µg/ml. 100 µl of each sample was incubated for two hours at 37°C. Each glycated sample and AGE-BSA standards were assayed in duplicate or triplicate. After incubation 250 µl of PBS buffer was used twice for washing the 96-well plate to remove the protein samples. A 200 µl of assay diluents (blocking solution) was used to block the remaining protein binding sites in each coated well then incubated for 24 hours at 4°C on an orbital shaker. The plate was washed twice with (0.1M, pH 7.4) 250 µl of washing buffer. 100 µl of diluted secondary antibody-HRP conjugate was then added to each well and incubated for 1 hour at 37°C on an orbital shaker, followed by washing the wells, five times with washing buffer. A volume of 100 µl substrate solution, warmed to room temperature, was added to each well and incubated at 37°C for half an hour. The enzymatic reaction was stopped by adding 100 µl of stop solution to each well. The absorbance at 450 nm was measured immediately using a microplate reader.

2.4.10 Measurement of total phenolic compounds

The amount of total phenolic compounds in MC extracts were measured based on the method reported by Miliauskas *et al*, 2004 using Folin-Ciocalteu reagent with little modification. Briefly, 1 ml aliquots of the ethanolic HWE and CWE of MC (10 g/l) were mixed with 5 ml of Folin-Ciocalteau reagent (diluted 10-fold), then, 5 ml of 10 % Na_2CO_3 solution was added, and allowed to stand for 1 hour at room temperature. The standard curve was prepared using 0–0.3 mg/ml ethanolic gallic acid solutions. The absorbance detected at 760 nm and the results were expressed as mg/ml of gallic acid equivalents. Calibration standard is shown in Figure 2.2. A phenolic content in MC extracts in gallic acid equivalents was calculated as described below:

C= c* v/m

Where: C total content of phenolic compounds, mg/g plant extracts, in GAE

c: the concentration of gallic acid established from the calibration curve (mg/ml)

v: the volume of extracts (ml)

m: the weight of pure plant extracts (g)

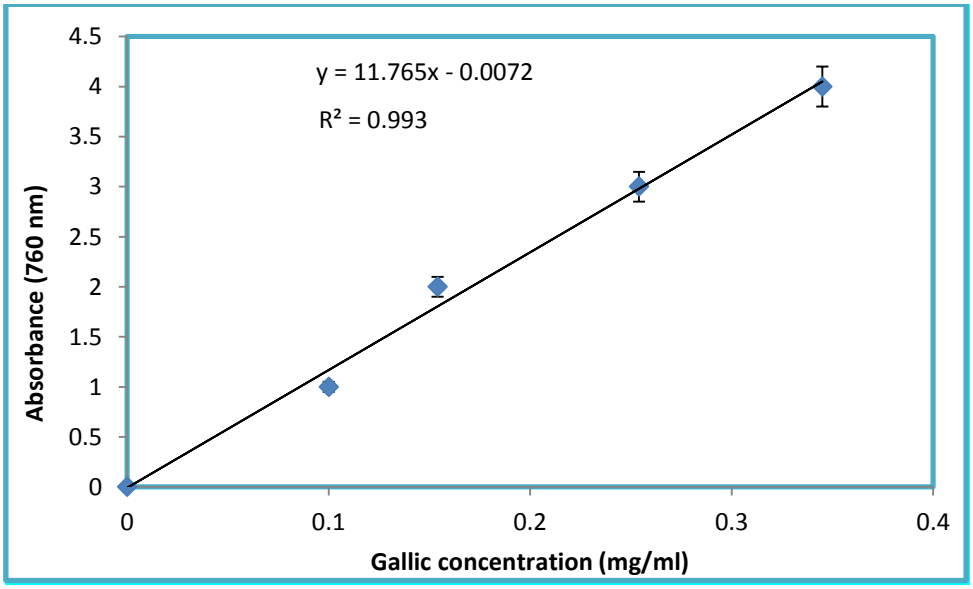


Figure 2.2: Standard curve using ethanolic gallic acid solution. Gallic acid (0-0.3 mg/ml) was used as a standard solution for phenolic contents. Each value represents the mean ± SD (n=3).

2.4.11 Measurement of ferric ion reducing power

The antioxidant activity of MC extracts was tested by a ferric ion reducing antioxidant power assay (FRAP) as described previously (Thaipong *et al*, 2006). 1 ml of different concentrations of MC extracts were mixed with 1 ml of methanol (50%), followed by 2.5 ml of phosphate buffer and 2.5 ml of 1% potassium ferricyanide $K_3Fe(CN)_6$. The mixture was incubated at 50 ºC for 30 minutes. Then, 2.5 ml of 10% trichloroacetic acid (TCA) was added to the mixture and the samples were centrifuged at 2900 x g for 10 minutes, after which 2.5 ml of the upper layer of supernatant was taken from each sample tube, and mixed with 2.5 ml of deionised water and 0.5 ml of 0.1% ferric chloride solution ($FeCl_3$). The absorbance was measured at 700 nm. Distilled water (dH_2O) mixed with FRAP reagents was used as a blank. In addition, different concentrations of vitamin C (ascorbic acid) were used as a positive control. All the analyses were carried out in triplicates and mean values were taken.

2.4.12 Measurement of cupric ion reducing/antioxidant capacity

The cupric ions (Cu^{2+}) reducing ability of MC extract was measured according to modified method described before (Apak *et al*, 2007). 1ml of different concentrations of HWE or CWE of MC were mixed with 1 ml $CuCl_2$ solution (0.01M), 1 ml ethanoic neocuproine solution (0.0075 M), 1 ml CH_3COONH_4 buffer (1M), and 0.6 ml of distilled was made up to a final volume of 4.6 ml in each tube. The tubes were kept at room temperature for 30 minutes, then the absorbance was measured at 450nm. Distilled water (dH_2O) mixed with CUPRAC reagents was used as a blank instead of the extracts. Different concentrations of vitamin C (ascorbic acid) were used as a positive control. All the analyses were carried out in triplicates and average values were taken.

2.4.13 Measuring superoxide dismutase activity

Superoxide dismutase enzyme activity of the sample was calculated using superoxide dismutase assay kit from (Cayman chemical) according to the manufacturer's instructions. BAEC (3 x 10^5/ml) were seeded into a 6-well plate, in 2 ml of complete culture medium (10% FBS) DEME. When they were confluent (80-90%), medium was discarded, and cells were washed with PBS. Fresh complete culture medium (10% FBS) was added to each well. Combination of HWE and CWE of MC (90 ug/ml) with BSA-AGE (10 µg/ml), methylglyoxal (5 mM), glucose (25 mM), were added to the flasks, Trolox (10 mM) was used as positive control. Control wells treated with DMEM supplemented with 10% FBS were included in the

experiments. The contents of the plate were gently mixed to avoid possible dislodging of cells and incubated in a humidified atmosphere of 95% air and 5% CO_2, at a temperature of 37°C for a further 24 hours. After the incubation, cells were washed three times with PBS. Cell lysis buffer (0.4 ml) was added to each wells and kept on ice. Gently the plate were shaken gently on ice for a few minutes, cells were scraped using a cell scraper. The total cells lysates were transferred to cold Eppendorf tubes on ice, sonicated for two seconds and stored at -80 ºC until used.

The equation below, which obtained from the linear regression of the standard curve substituting the linearized rate (LR) for each samples. One unit is defined as the amount of enzyme required to display 50% dismutation of the superoxide radical.

SOD (U/ml) = [(sample LR – intercept / slope) x 0.23ml / 0.01ml] x sample dilution

2.4.14 Measuring catalase activity

Catalase enzyme activity of the sample was calculated using catalase assay kit from (Cayman chemical) according to the manufacturer's instructions. BAEC (3 x 10^5/ml) were seeded into a 6-well plate, in 2 ml of complete culture medium (10% FBS) DEME. When they were confluent (80-90%), medium was discarded, and cells were washed with PBS. Fresh complete culture medium (10% FBS) was added to each well. Combination of MC extracts (90 µg/ml) with BSA-AGE (10 µg/ml), methylglyoxal (5 mM), glucose (25 mM), were added to the flasks, Trolox (10 mM) was used as positive control. Control wells treated with DMEM supplemented with 10% FBS were included in the experiments. The contents of the plate were gently mixed to avoid possible disloding of cells and incubated in a humidified atmosphere of 95% air and 5% CO_2, at a temperature of 37°C for a further 24 hours. After incubation, cells were washed three times with PBS. Cell lysis buffer (0.4 ml) was added to each wells and kept on ice. The plate was shaken gently on ice for a few minutes, cells were scraped using a cell scraper. The total cells lysates were transferred to cold Eppendorf tubes on ice, sonicated and stored at -80 ºC until used.

Notably, to calculate formaldehyde concentration of the samples the equation obtained from the linear regression of the standard curve was used by substituting corrected absorbance values for each sample.

Formaldehyde (µM) = [sample absorbance – (y- intercept) / slope] x 0.17 ml / 0.02 ml]

Conversely, the CAT activity of the samples was obtained by using the following equation. One unit is defined as the amount of enzyme that will lead to the production of 1.0 nmol of formaldehyde per minutes at 25 ºC.

CAT Activity = µM of sample / 20 min. x Sample dilution = nmol/min/ml

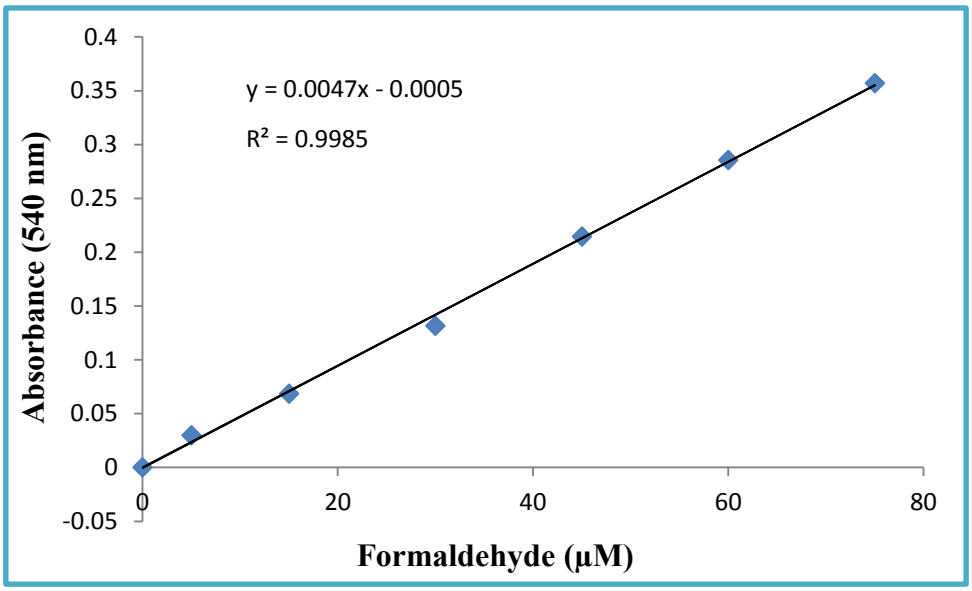


Figure 2.3: Standard curve using formaldehyde solution.

2.4.15 Measuring reactive oxygen species

Antioxidant capacity of MC extracts against reactive oxygen species was tested according to the method previously (Brimson & Tencomnao, 2011). Prior to the experiment the dye 2′,7′-Dichlorodihydrofluorescei diacetate (H_2DCF-DA) stock solution was prepared by dissolving 100 mg of the dye powder into anhydrous DMSO. Later this stock solution was further diluted to a final concentration of 0.5 mM and kept as 100 µl aliquots in a – 20 ºC freezer. To avoid dye degradation by light and air, the stock solution and aliquots were prepared in the dark. For each experiment, a new aliquot was used. BAEC (3 x 10^5/ml) were seeded into a 6-well plate, in 2 ml of complete culture medium (10% FBS) DEME. When they were confluent (80-90%), medium was discarded, and cells were washed with PBS. Fresh complete culture medium (10% FBS) was added to each well. Combination of HWE and CWE of MC (90 µg/ml) with BSA-AGE (10 µg/ml), methylglyoxal (5 mM), glucose (25 mM), were added to the flasks, Trolox (10 mM) were used as positive control. Control wells treated with DMEM supplemented with 10% FBS were included. The contents of the plate were gently mixed to avoid possible dislodging of cells and incubated in a humidified atmosphere of 95% air and 5% CO_2, at a

temperature of 37°C for a further 24 hours. After the incubation time, cells were washed three times with PBS. Cell lysis buffer 0.4 ml was added to each wells and kept on ice. Gently the plate was shaken gently on ice for a few minutes, cells were scraped using a cell scraper. The total cells lysates were transferred to cold Eppendorf tubes on ice, sonicated for two seconds and stored at -80 ºC until used.

2.4.16 Thawing of cells

Frozen cell cryotubes were taken from liquid nitrogen, and were accordingly cleaned with 70% ethanol. Inside the tubes, there was a build-up of pressure, which was released inside a laminar flow hood. Subsequently, the frozen cells were gently defrosted at a temperature of 37°C. The cell suspension was transferred to a universal tube containing a pre-warmed complete medium. The suspension was centrifuged at 1300 rpm for 5 minutes, after that the supernatant was discarded and the cell pellet re-immersed in 1 ml of fresh, (10% FBS) DEME. Subsequently, cells were seeded in T-75 flasks and incubated at 37°C.

2.4.17 Heat inactivation of foetal bovine serum

Frozen FBS was incubated in a water bath (at a temperature of 37°C) until the serum was thawed, and subsequently incubaed for 30-45 minutes in a 56°C water bath. Notably, every 10 minutes, the bottle would be turned. Following this period, the bottle was taken out of the water and allowed to settle until it reached room temperature. The heat-inactivated serum was aliquoted into 50 ml Falcon tubes and stored at - 20°C.

2.4.18 Cell culture of BAEC

Bovine aortic endothelial cells (BAEC) were generously provided by Dr. Donghui Liu (Manchester Metropolitan University's Cell and Molecular Biology Research Laboratory). The endothelial cells were characterised by the presence of the von Willebrand factor. Bovine aortic endothelial cells were cultured routinely in T-75 flasks; these containers had been previously coated with 0.1% gelatine. On a weekly basis, cells were sub-cultured using DMEM contains 5.1 mM glucose; this was further complemented with 15% FBS, 200 mM L-glutamine, 10,000 U/ml penicillin and 10 mg/ml streptomycin. During this period, the culture flasks were incubated in a humidified atmosphere of 95% air and 5% CO_2, at a temperature of 37°C. After confluence had been achieved, the BAEC culture was increased; this was achieved by taking part of the freezing cells, as well as by increasing the flask number (sub-culture). Passage levels 6–14 was used throughout the final experiments. For sub-culturing of cells the seeding of

bovine aortic endothelial cells (1.5 x 10^5 – 5 x 10^5) was carried out using of 12 ml of 15% DMEM in T-75 flasks. Incubation was implemented in a CO_2 incubator for a period of 24 hours. After 24-hours, it was replaced with a fresh complete medium. Trypsinization of cells was started when the cell density reached confluence state by removing the medium and discarding it. Using 10 ml of sterile PBS, the cells were washed in triplicate, and incubated with 5–10 ml of 1 X trypsin solution for 2-5 minutes. When cell detachment was achieved, 5 ml of complete medium was added to the flask to counteract the function trypsin. The cell suspension was centrifuged at 1300 rpm for 5 minutes. Upon discarding the supernatant, the pellet was re-immersed in the fresh medium (1ml) on ice, and then the number of cells per ml was counted. Freezing medium was prepared using of 90% FBS and 10% DMSO. Cold FBS (9 ml) was taken, to which 1 ml of DMSO was added. Freezing medium was mixed and stored at a temperature of 4°C. Fresh freezing medium was prepared when required for use. Freezing of cells was achieved by using two cryotubes with an estimated 3.5 x 10^5 cells per cryotubes. They were taken from the same confluent flask and stored at −20°C for a period of half an hour. Subsequently, they were either stored overnight at −80°C. The cells were subsequently moved into liquid nitrogen for long-term storage.

2.4.19 Cell counting

Cells were counted using a Coulter counter. Isotonic solution (10 ml) was mixed with 50 µl of cell suspension in a counting chamber, and counted in triplicate. During this stage of counting, there was also the calculation of the dilution factor. Suspended cells were frozen in liquid nitrogen or otherwise sub-cultured. Importantly, when noting a cell concentration in 1ml, this is calculated as follows:

Cell concentration / ml = cell count x dilution factor

2.4.20 Assessment of cell proliferation using the Coulter counter

BAECs (2.5x10^4 cells/ml) in 0.5 ml of culture medium (15% FBS) were seeded in 24-well plates and allowed to adhere for approximately 4 hours. The complete medium was discarded and the cells were washed gently three times with PBS. Fresh media supplemented with 2.5% FBS was added to each well. BSA-AGE (10 µg/ml), sugars such as glucose (25, 50, 75 and 100 mM), methylglyoxal (1, 2, 4 and 5 mM), and MC extracts at low concentrations of (15, 30, 60 and 90 µl /ml), and at high concentrations of (0.1, 0.2, 0.4, 0.6, 0.8 and 1 mg /ml) were added to the wells. Growth factors such as FGF-2 (25 ng/ml) were used as positive control.

Control wells treated with DMEM supplemented with 10% FBS were included. The contents of the plate were gently mixed to avoid possible dislodging of cells and incubated in a humidified atmosphere of 95% air and 5% CO_2, at a temperature of 37°C for a further 48 hours. Cells were washed three times with PBS and trypsinized. The number of cells was counted using a Coulter counter. Each experiment was carried out in triplicate, and repeated at least three times independently.

2.4.21 Assessment of cell viability using automated cell counter

BAECs ($2.5x10^4$ cells/ml) in 0.5 ml of culture medium (10% FBS) were seeded in 24-well plates and allowed to adhere for approximately 4 hours. The complete medium was discarded and the cells were washed gently with PBS three times. Fresh media supplemented with 2.5% FBS was added to each well. BSA-AGE, sugars and MC extracts at various concentrations were added to the wells. Growth factors such as FGF-2 (25 ng/ml) was used as a positive control. Control wells treated with DMEM supplemented with 15% FBS were also included. The contents of the plate were gently mixed to avoid possible dislodging of cells and incubated in a humidified atmosphere of 95% air and 5% CO_2, at a temperature of 37°C for a further 72 hours. Cells were washed three times with PBS and trypsinized. Cells (10 µl) were mixed with 10 µl of trypan blue. The number of cells was counted using the automated cell counter. Each experiment was carried out in triplicate, and repeated at least three times independently.

2.4.22 Cell lysis for Western blot

BAECs (3×10^5/ml) were seeded into a 6-well plate, in 2 ml of complete culture medium (10% FBS) DEME. When they were confluent (80-90%), medium was discarded, and cells were washed with PBS. Fresh complete culture medium (10% FBS) was added to each well. MC extracts (90 µg/ml) with BSA-AGE (10 µg/ml) or with methylglygoxal (5 mM) or with glucose (25 mM) were added to the flasks, respectively. Control wells treated with DMEM supplemented with 10% FBS were included in the experiments. The contents of the plate were gently mixed to avoid possible dislodging of cells and incubated in a humidified atmosphere of 95% air and 5% CO_2, at a temperature of 37°C for a further 24 hours. After the incubation time, cells were washed three times with PBS. Cell lysis buffer 0.4 ml was added to each wells and kept on ice. Gently the plate was shaken on ice for a few minutes, cells were scraped using a cell scraper. The total cells lysates were transferred to cold Eppendorf tubes on ice, sonicated for two seconds and stored at -80 ºC until used

2.4.23 Western blot

The acrylamide gel was prepared by mixing 3.3 ml of 40% bis-acrylamide with 4.2 ml distilled water and 2.5 ml separating buffer in a universal tube. 100 µl of 10% ammonium persulphate solution was added followed by 10 µl TEMED solutions and the gel left to polymerise for 20 minutes. A few drops of isopropanol were added on top of the gel. Isopropanol was removed after 15 minutes and rinsed with plenty of distilled water. As much as possible, distilled water was removed by filter paper and then the stacking solution was prepared in a second universal tube, by combining 1.45 ml of 40% bis-acrylamide with 6.1 ml distilled water, and 2.5 ml stacking buffer. As before, 100 µl APS was added followed by 10 µl TEMED solution and the gel left to polymerise for a minimum of 20 minutes. Slowly the combs, clamps, and gaskets were then removed taking care not to damage the wells and the gel plates inserted into the electrophoresis chamber. The chamber was subsequently filled with reservoir buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3). A total of 500 ml-electrode buffer was used to fill the tank, in the space between the two sets of glass plates. Protein samples, sample buffer and molecular weight marker were taken out from the freezer and warmed to room temperature. The protein concentration of each sample was determined by Bio-Rad protein assay to facilitate equal protein loading/well. Equal amounts of protein sample and sample buffer were mixed in Eppendorf tube and boiled for 10 minutes, then sample buffer (20 µl), molecular weight marker (8 µl) and the protein sample from plaques (20 µl) were gently loaded. Sample buffer was added to the first and last well and in the second well molecular weight marker was added. Samples containing 15 µg protein up to 20 µl solution, along with pre-stained molecular weight markers, were separated by SDS-PAGE (10% w/v) for 45 minutes at 60V (when samples were in stacking gel) and switched to 200V for separation until the dye, bromophenol blue, reached the bottom of the separation gel.

2.4.24 Blotting the gel

Two nitrocellulose membranes and 12 pieces of blotting paper were soaking for two minutes in towbin's buffer. Stacking gels were removed from the separation gels and discarded. The gels were sandwiched separately. The sandwich was assembled in an electro blotter for each gel as follows: Three pieces of blotting paper, nitrocellulose membranes, gel and at the top three pieces of blotting papers. Any bubbles within the sandwiches were removed by rolling a clean 5 ml tip over the sandwich. Proteins were transferred to the membrane at 40 mA/gel for one hour.

2.4.25 Blocking the nitrocellulose membranes

Membranes were blocked with 1% BSA-TBS tween (pH 7.4) for 1 hour at room temperature on a rotating shaker. The blocking buffer was discarded and membranes were incubated separately in 12 ml of primary antibody solution (1:1000) dilution such as rabbit polyclonal anti-actin, p21, pJAK-2, NOX4, NOX5, RAGE and goat polyclonal antibodies to total JAK at 4^0C. After overnight incubation on a rotating shaker, primary antibody solutions were discarded and membranes were washed five times in TBS-tween buffered saline and tween-20 (TBS-tween) for 8 minutes each at room temperature on a rotating shaker. Membranes were incubated for 1 hour at room temperature in 12 ml of goat anti-rabbit or rabbit anti-goat horseradish peroxidase-conjugated secondary antibody diluted in TBS-tween containing 5% (w/v) de-fatted milk (1:1000) for actin, NOX4, NOX5, p.JAK-2, total JAK, p21, and RAGE, respectively. After incubation, secondary antibody solutions were discarded and membranes were washed five times in TBS-tween for 8 minutes each at room temperature on a rotating shaker.

2.4.26 Developing the nitrocellulose membranes

In a dark room, membranes were immersed in enhanced chemiluminescence (ECL) solution. To prepare the ECL solution, 1ml of (Lominol enhancer) solution was added to 1ml of (Peroxide) solution and kept in the dark room for five minutes. Once the ECL solution was prepared it was then poured on to the membranes for one minute, left in the dark room and excess reagent was drained off, and membranes were quickly wrapped in cellophane films and kept in a box. The box was taken to the G-Box and the sample was measured as a chemiluminescent sample, the intensities of bands on the membranes were quantified by image J analysis software. The results were semi-quantitative and compared to the control.

2.4.27 RNA extraction

BAEC ($5x10^5$ cells/ml) in 10 ml of culture medium (10% FBS) were seeded in T.75 (60 mm) flask, and allowed to adhere for approximately 24 hours. The complete medium was discarded and the cells were washed gently three times with PBS. Fresh complete medium 10% FBS was added to each each flask. Combination of BSA-AGE (10 μg/ml), HWE and CWE of MC (90 μg/ml) were added to the flasks. Control wells treated with DMEM supplemented with 10% FBS were included in the experiments. The contents of the plate were gently mixed to avoid possible dislodging of cells and incubated in a humidified atmosphere of 95% air and 5% CO_2,

at a temperature of 37°C for a further 48 hours. Cells were washed three times with PBS. The cells were kept in -80 ºC for later use. Each experiment was carried out in triplicate, and repeated at least three times independently. Cells were removed from the freezer, placed in RNA extraction cabinet, allowed to thaw for two minutes in room temperature, then 4ml TRIzol reagent was added directly to the cells in the culture flask. The homogenized samples were incubated for 5 minutes at room temperature. 1 ml of the homogenise sample was transferred into Eppendorf tube, and 0.2 ml of chloroform added into each tube. Tubes were shaken vigorously by hand for 15-20 seconds, incubated for 2-3 minutes at room temperature. Samples were centrifuged at 12,000 x g for 15 minutes at 15 ºC, after the mixture separated into a lower red phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. Notably, RNA remained exclusively in the aqueous phase, which accounted for 50% of total volume. The aqueous phase of the samples were removed from the tube by angling the tube at 45º and pipetting the solution out, caution was taken to avoid drowning any of the interphase or organic layer into the pipette when the aqueous phase was removed. The aqueous phase was transferred into a new tube. A volume of 0.5 ml of 100% of isopropanol was added to the aqueous phase, the mixture was incubated at room temperature for 10 minutes, and centrifuged at 12,000 x g for 10 minutes at 4 ºC. RNA extracts appeared as a gel-like pellet on the side and bottom of the tube. The pellet was washed in 1 ml of 75% ethanol, and was vortexed briefly, centrifuged at 7500 x g for 5 minutes at 4 ºC , then discarded the solution. The RNA pellet was air dried for 5-10 minutes, and resuspended in 15 ul of RNase-free water by passing the solution up and down several times through a pipette tip. The solution was stored at -70 ºC.

2.4.28 RNA quantification

The concentration of RNA was determined using NanoDrop 2000. RNA purity was measured using the ratio of the absorbance at 260 nm and 280 nm. A value between 1.8 and 2.0 was considered as pure RNA.

2.4.29 Complementary DNA Synthesis

Extracted RNA was reverse transcribed into cDNA using ImProm-II^{TM} Reverse Transcription system (Promega, UK) according to manufacturer's instructions. In brief, 1µg of total RNA, 1 µl oligo $(dT)_{15}$ primer (0.5 U/µl), 4 µl of 25 mM $MgCl_2$, 4 µl of 5 X reaction buffer (pH 8.5), 1µl of 10 mM dNTP mix, 0.5 µl of 2500 U/ml recombinant ribonuclease inhibitor (Rnasin) and 1µl of 50 U/ml reverse transcriptase were combined in nuclease-free water to a final volume of 20 µl per reaction. The reaction programme included denaturing at 70 ºC for 5 min

(primers/template was denatured), followed by annealing at 25ºC for 5 min, extension at 42 ºC for 1 h, and incubation at 70 ºC for 15 min (to inactivate reverse transcriptase) in a thermal cycler. The synthesized cDNA was stored at -20 ºC.

2.4.30 Primer design

The primers were designed using software Primer3. All sequences of primers were compared with GeneBank® using the Basic Local Alignment Search Tool (BLAST) program, which is available at the National Centre for Biotechnology Information (NCBI). Table 2.1 shows the sequences of primers used for RT-PCR.

Table 2.1: The sequence of primers used for RT-PCR analysis

Gene symbol	Forward Primer (5' → 3')	Revers Primer (5' → 3')
ACTB	5'-TAGTTTCGTGAATGCCGCAG-3'	5'-CAAAGTTCTGCATCGCCACT-3'
GPx	5'-CAAGGAGATCGAAGGCTTGC-3'	5'-CGTAGTTGACAGCAGGCATC-3'
HOX B13	5'-GCATCCCTTTACCCTGACCT-3'	5'-GGAACTGGCGAATACTGCTG-3'
NOX1	5'-TGCTTATCTGGCTCTCCCTG-3'	5'-GCATCTCGTGGAGGTAGTGA-3'
NOX4	5'-TGCCCAAGGAGAAGCTAGAC-3'	5'-ATCTCGGAGCTCCTCGAAAG-3'
NOX5	5'-CAAGAAGCCGAGTGAGGAGG-3'	5'-TTGAGGTTCTGGTCACACGA-3'
P22 phox	5'-CGCACCTCCTCAACTTCTTC-3'	5'-CAGCGTTGTTCTTGCCATCT-3'
P47 phox	5'-CAGGGACGCTTACAGATTGC-3'	5'-CATGCTCCCACACGTCAATC-3'
CAT	5'-CAGTGGTCAGGACATCAGGT-3'	5'-CATTCTTCCAGTCAACCGGG-3'
Mn-SOD	5'-CATGCTCCCACACGTCAATC-3'	5'-CCCTGGAGAAGAGCTACGAG-3'

2.4.31 Statistical analysis

Statistical analysis was performed using Microsoft Office Excel 2009. The results of each experiment are expressed as mean ± standard deviation. Data were analysed for statistical significance using Student *t*-test for comparison between two groups and one way ANOVA for more than two groups. All analysis were performed using PRISM software. Variations with p values ≤ 0.05 were considered statistically significant.

Chapter 3. Antiglycation properties of *Momordica charantia* extracts

3.1 Introduction

Reducing sugars such as glucose can react non-enzymatically with the free amino residues of proteins, fatty acids and nucleic acids via a series of reactions generating reversible Schiff base dicarbonyl compounds (Huang *et al*, 2008). The products of this reaction undergo further reactions to become irreversibly cross-linked, heterogeneous fluorescent compounds called AGEs (Chang *et al*, 2011). AGEs are a complex class of molecules generated from protein glycation under the potent influence of oxidative or carbonyl stress (Smit & Lutgers, 2004). High levels of glucose in the body cause more AGE production (Hsu & Zimmer, 2010). Dicarbonyl compounds such methylglyoxal are generated such as an intermediate of protein glycation and is a potent precursor of AGE production and found at high levels in blood or tissues from diabetic subjects (Marchitti P, 2009). Furthermore, prolonged exposure to hyperglycaemia, impaired glucose metabolism and oxidative stress leads to accumulation of methylglyoxal, hence this induce serious toxic effects to varieties of cells such as endothelial, epithelial, macrophage and neuronal cells (de Arriba *et al*, 2007). Glycation of protein is a spontaneous process depending *in vivo* on the degree and duration of hyperglycemia (Schmitt *et al*, 2005). AGEs accumulate gradually and can permanently change protein formation and function (Elosta *et al*, 2012). AGEs accumulate in great extents in proteins with long half live like extracellular collagen, lens crystalline, laminin and fibronectin, hence this leads to changing protein forms and bio-chemical properties (Thornalley *et al*, 1999; Verzelloni *et al*, 2011). The production and accumulation of AGEs has been demonstrated to play a central role in the pathogenesis of diabetic complications (Jack & Wright, 2012). Conversely, in order to inhibit diabetic complications, inhibitors of AGE production have an essential role in future therapy. It has been suggested that the metal catalysed oxidation reactions play a key role in stimulating the rate of AGEs generation (Chen *et al*, 2011). Therefore, compounds with antiglycation and antioxidant activities may delay the process of AGE production by inhibiting further oxidation of metal-catalyzed glucose oxidation (Chen *et al*, 2011; Mukherjee *et al*, 2012).

Beside synthetic agents, recent attention has focused on the advantages of medicinal plants with both antiglycation and antioxidant activities. Synthetic compounds and medicinal plants have been estimated as inhibitors against the production of AGEs, since the synthetic AGEs

inhibitors may be divided into three groups which are carbonyl trapping compounds which attenuate carbonyl stress, metal ion chelators , which reduce glycation and cross-link breakers that reverse AGE cross-linking (Reddy & Beyaz, 2006). Medicinal plants have been recognised to be relatively safe for human use and many plant products have been examined for their ability to inhibit AGEs. In this respect, medicinal plants with wide broad properties could be promising tools with at least disadvantage effects and as superior alternative to synthetic medicines.

Momordica charantia also known as bitter melon is one of the world's ancient medicinal plants especially in tropical countries, which has been used for the management of several conditions such as ulcers and diabetes. A considerable number of studies have investigated the anti-hyperglycaemic properties (Joseph & Jini, 2013), and anti-diabetic properties of MC (Singh *et al*, 2011). Animal and human studies suggest that the plant extracts of MC possess antihyperglycaemic effects. The active compounds within MC are either fat-soluble or water-soluble compounds, since lectin, vicine and insulin-like polypeptide and glycoside are water soluble, and are more soluble in HWE than in CWE of MC extracts (Dans *et al*, 2007). Water has been used in this study to extracts active ingredients from MC, since the solvent is safe, cheap and affordable and for its bioavailability (kumar *et al*, 2010).

Advantage of hot water over cold extract is hot water increases the solubility of the MC compounds affected by the increase of the extraction temperature, hence increasing in mass transfer. Under these environments, water viscosity is decreased, resulting a better penetration of the solvent extract in the matrix of the plant cells, subsequently increasing the extracting rate (Budrat & Shotipruk, 2009). In contrast with other methods of extraction hot water was regarded to possess good extraction efficiency and precision (Ong *et al*, 2006). Antiglycation activity of medicinal plants may be related to their polyphenolic content (Ardestani & Yazdanparast, 2007; Ho *et al*, 2010; Hsieh *et al*, 2007). However, MC extracts may express their antiglycation activity through inhibition of the post-Amadori phase, where free radical such as superoxide anion and metal ions are involved. Chelating and free radical species scavenging activities are important for inhibition of AGEs. Previous study has indicated that MC extract inhibit AGEs formation in diabetic rats (Chaturvedi & George, 2010). However, comparative studies between HWE and CWE of MC on cross-linked AGEs have not been reported before**.**

3.2 Aim and objectives

The aim of this study was to investigate and compare the effects of MC extracts on the production of AGEs *in vitro*.

The objectives were:

- To assess and compare the effects of different concentrations of reducing sugars and different times of incubations on fluorescent and cross-linked AGEs *in vitro*.
- To examine and compare the ability of MC extracts to inhibit the production cross-linked AGEs *in vitro*.
- To study and validate the effect of MC extracts on the production of AGEs *in vitro* using ELISA techniques.

3.3 Methods

Lysozyme was glycated by incubating with different concentrations of sugars with / or without MC extracts as described in section 2.4.2. Crosslinked AGEs were examined as described in section 2.4.7. The production of fluorescent AGEs were investigated by their characteristic fluorescence as mentioned in section 2.4.8. AGEs was detected using ELISA as mentioned in section 2.4.9.

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3.4 Results

3.4.1 Effect of different glucose concentrations on the formation of AGEs *in vitro*.

Glucose was incubated with lysozyme, cross-linked AGEs were formed that caused production of dimers having a molecular weight of approximately 28.7 kDa as indicated by molecular weight markers as shown in Figure 3.1, lane a. The effect of glucose on cross-linked AGEs formation are clearly visible by their ability to reduce electrophoretic mobility and increase the intensity of the dimer bands in a dose-dependent manner as shown in Figure 3.1, lane c to h.

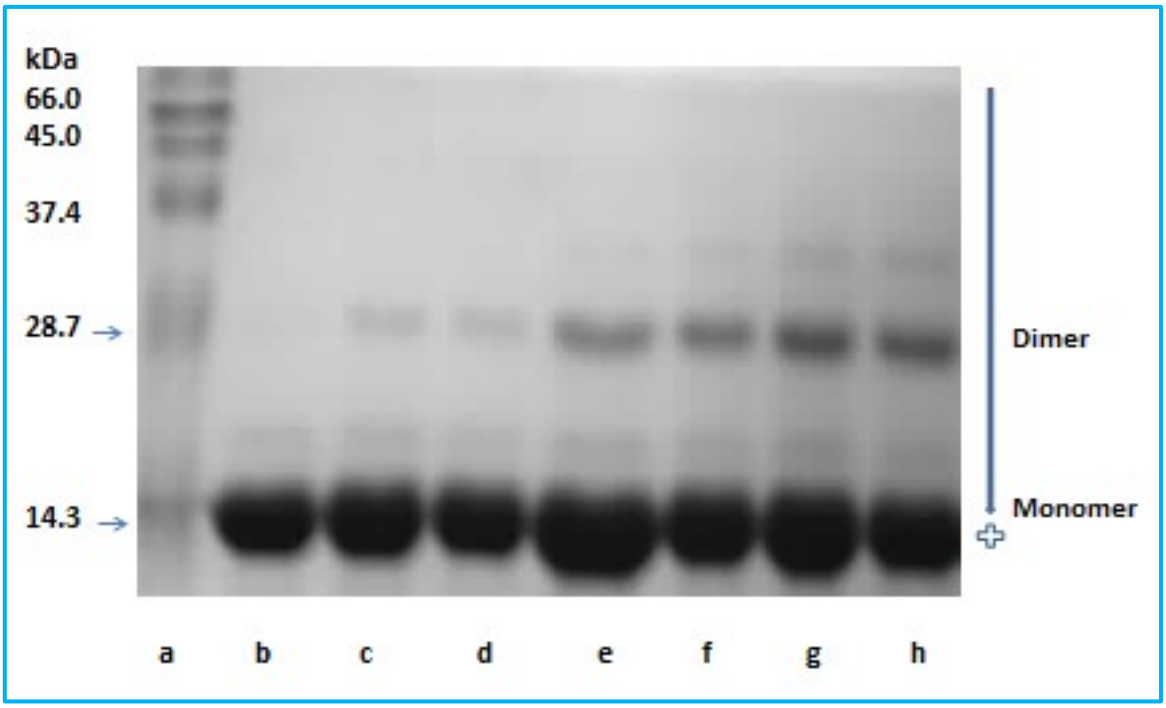


Figure 3.1: Gel showing the effect of different glucose concentrations on the formation of AGE. SDS-PAGE gel showing lysozyme (10 mg/ml) incubated alone (lane b) or in the presence of (c) 0.1 , (d) 0.2 , (e) 0.4 , (f) 0.6 , (g) 0.8 and (h) 1 M glucose in 0.1 M sodium phosphate buffer (pH 7.4) at 37 °C for 35 days. The result is a representative figure of three independent experiments.

Incubation with glucose showed a significant ($p< 0.05$) increase in cross-linked AGEs in comparison with the control as shown in Figure 3.2. This effect also depended on the dose of glucose. The cross-linked AGE production was more than 27-fold greater when lysozyme was incubated for 35 days with 1 M glucose as compared to the control and 9 -fold greater when 0.1 M glucose was used.

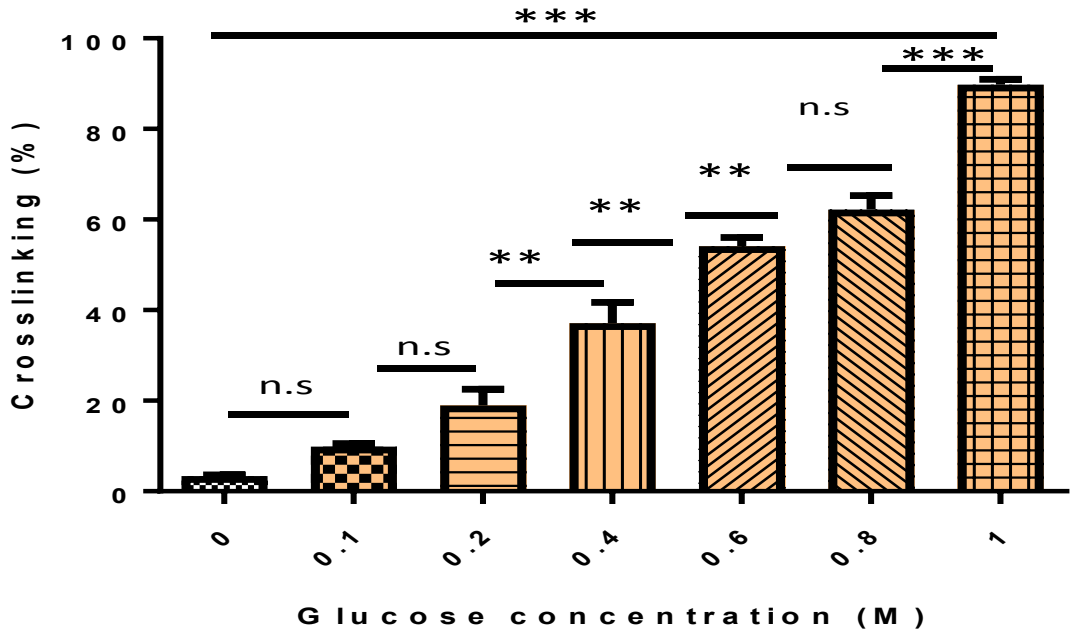


Figure 3.2: Effect of different glucose concentrations on the formation of AGE. The scan of SDS-PAGE gel shows the effect of different concentrations of glucose on formation of AGE in 0.1 M sodium phosphate buffer pH 7.4 at 37 °C for 35 days. Each value represents the mean ± SD (n = 3), where (n.s =non-significant), ** $p < 0.001$ and *** $p < 0.0001$ v.s control (Lysozyme).

3.4.2 Effect of period of incubation of glucose on the formation of AGE *in vitro*.

An increasing quantity of cross-linked AGEs were produced when lysozyme was allowed to react with glucose for different incubation periods. The control sample contained lysozyme only Figure 3.3, lane a. Decreased electrophoretic mobility with higher M_r and increased dimer and trimer intensity as compared to control was observed with varying incubation periods of glucose as demonstrated in Figure 3.3, lane b to i.

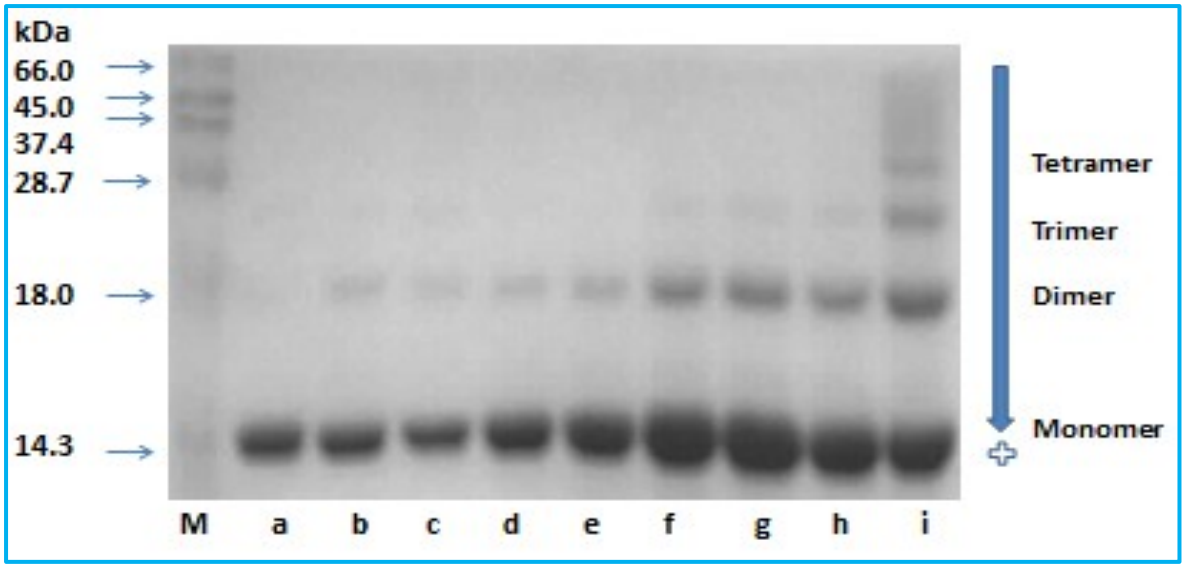


Figure 3.3: Gel showing the effect of different incubation times of glucose on AGE formation. SDS-PAGE gel showing lysozyme (10 mg/ml) incubated alone (lane a) or in glucose (0.5 M) for different incubation time (b) 0 , (c) 7 , (d) 14 , (e) 21 , (f) 28 , (g) 35 , (h) 42 and (i) 49 days in 0.1 M sodium phosphate buffer of pH 7.4 at 37 °C for 49 days. The result is a representative figure of at least three independent experiments.

Formation of cross-linked AGEs was significantly ($p < 0.05$) increased in comparison with the control sample and this effect depends on time of incubation. The production of cross-linked AGEs was more than 60 fold greater when lysozyme was incubated with glucose for 49 days as compared to control, and 4.7 fold greater than when incubated for 0 days as shown in Figure 3.4

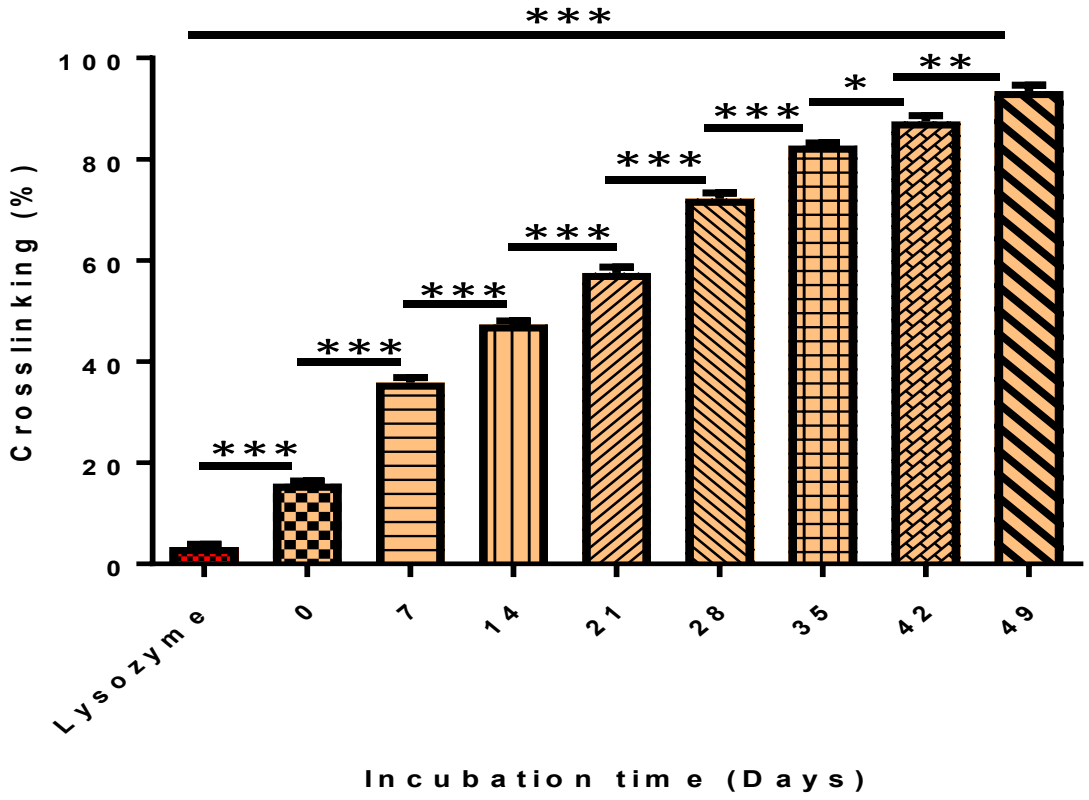


Figure 3.4: Effect of different incubation times of glucose on the formation of AGE. The scan of SDS-PAGE gel shows the effect of lysozyme (10 mg/ml) with different incubation times in 0.1 M sodium phosphate buffer, pH 7.4 at 37 °C for 7 weeks. Each value represents the mean ± SD (n=3), * $p < 0.05$, * * $p < 0.001$ and *** $p < 0.0001$ v.s control (lysozyme).

3.4.3 Effect of HWE of *Momordica charantia* on glucose-induced AGEs

Lysozyme incubated in the presence of glucose produces sufficient cross-linked AGEs. The effect HWE of MC on cross-linked AGEs formation are clearly visible by their ability to reduce electrophoretic mobility and the intensity of the dimer bands in a dose-dependent manner as shown in Figure 3.5.

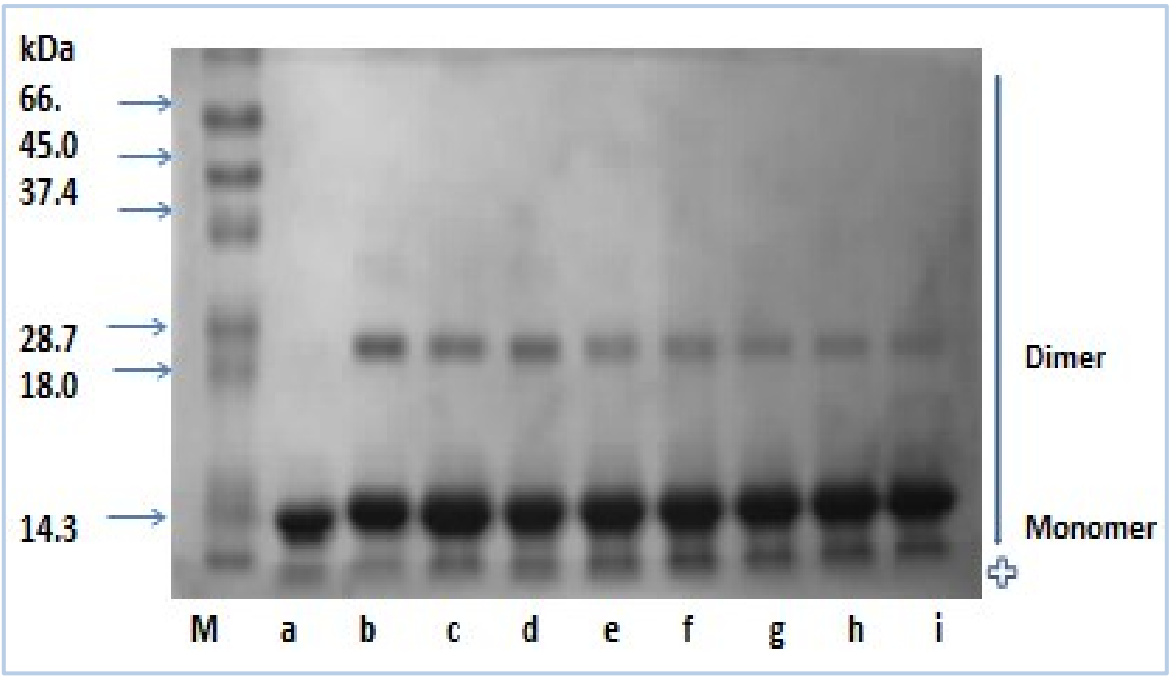


Figure 3.5: Gel showing the effect of HWE of MC on glucose-induced AGEs. SDS-PAGE gel showing Lysozyme (10 mg/ml) incubated without glucose (lane a) or in the presence of 0.5 M glucose (lane b) in 0.1 M sodium phosphate buffer pH 7.4 at 37 °C for 35 days. The inhibition of dimer production of glucose-lysozyme AGEs was determined using different concentrations of HWE of MC lane (c) 7, (d) 14, (e) 30, (f) 45, (g) 60, (h) 75 and (i) 90 mg/ml. The result is a represent of at least three independent experiments.

Production of cross-linked AGEs *in vitro* was significantly ($p < 0.05$) inhibited by MC extracts as compared to the control sample and this inhibition was dependent on MC extract concentrations as maximum (77%) inhibition was observed in the sample with 90 mg/ml of MC extracts as in Figure 3.6.

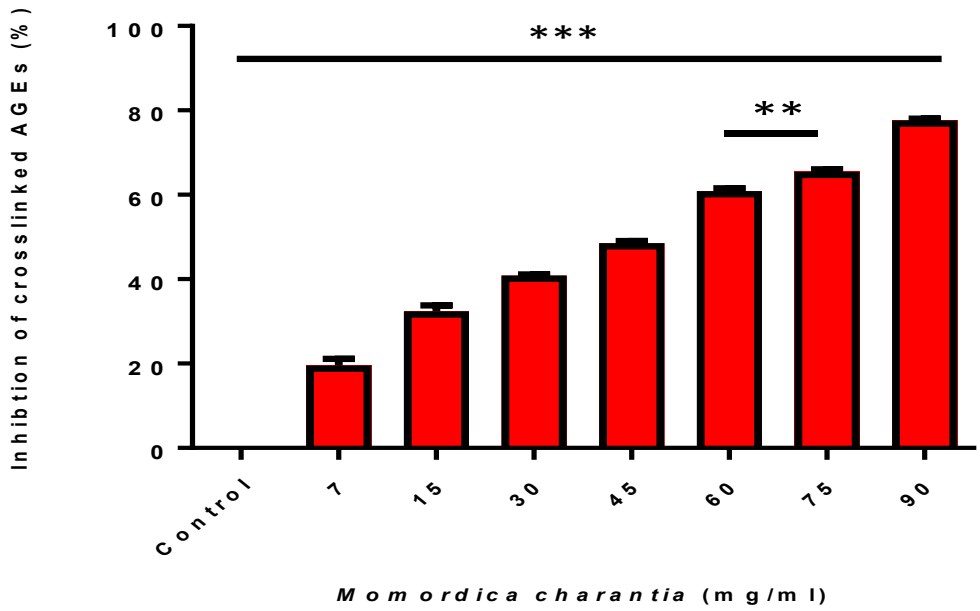


Figure 3.6: Effect of HWE of MC on AGE formation. The scan of SDS-PAGE gel shows the effect of lysozyme (10mg/ml) incubated in the presence of glucose (0.5 M) produces cross-linked AGEs. The glycation mixture was incubated with different concentration of HWE of MC for 35 days and percentage inhibition of glucose-induced AGE production. Each value represented the mean ± SD (n=3), ** $p < 0.001$, *** $p < 0.001$ v.s control.

3.4.4 Effect of CWE of *Momordica charantia* on glucose-induced AGEs.

Lysozyme incubated in the presence of glucose produces sufficient cross-linked AGEs. The effect of CWE of MC on cross-linked AGEs formation are clearly visible by their ability to decrease intensity of the dimer bands in a dose-dependent manner as illustrated in Figure 3.7. These results showed that inhibition of cross-linked AGEs formation by CWE of MC was dose-dependent.

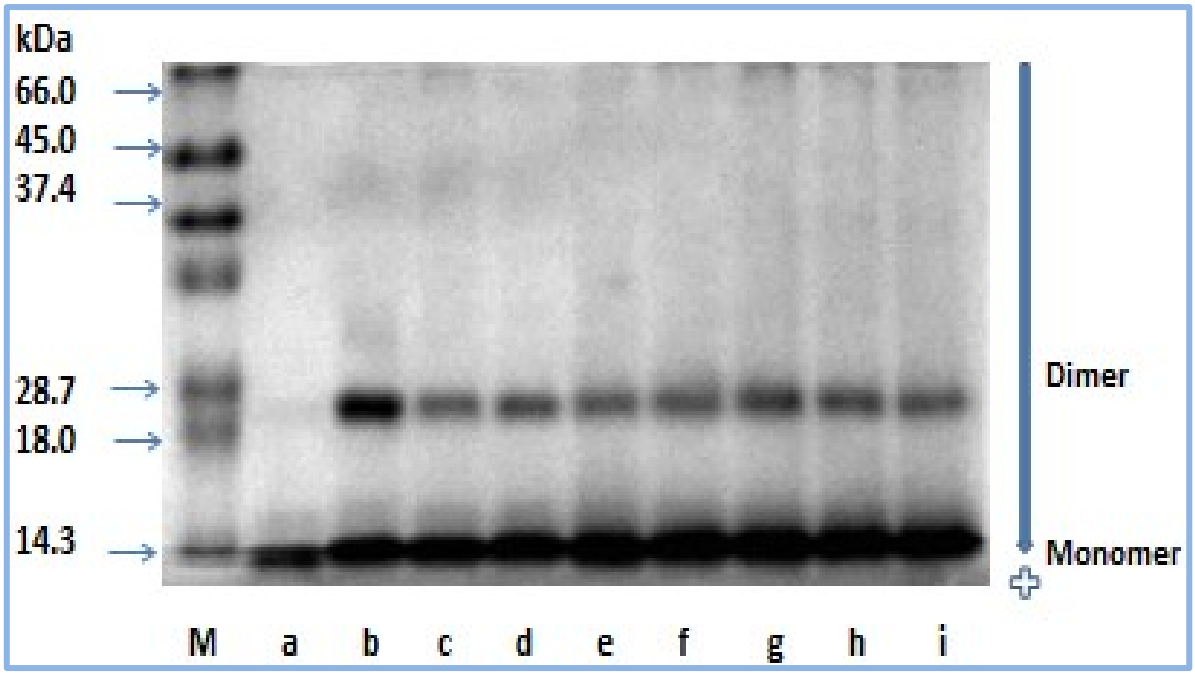


Figure 3.7: Gel showing the effect of CWE of MC on glucose-induced AGEs. SDS-PAGE gel showing lysozyme (10 mg/ml) incubated without glucose (lane a) or in the presence of 0.5 M glucose (lane b) in 0.1 M sodium phosphate buffer pH 7.4 at 37 °C for 35 days. The inhibition of dimer formation was determined using different concentrations of CWE of MC lane (c) 7, (d) 14, (e) 30, (f) 45, (g) 60, (h) 75 and (i) 90 mg/ml. The result is a representative figure of at least three independent experiments.

Production of cross-linked AGE *in vitro* was significantly ($p < 0.05$) inhibited by MC extracts as compared to the control sample and this inhibition was dependent on MC extract concentrations as maximum (41%) inhibition was observed in the sample with 90 mg/ml of MC extracts as in Figure 3.8

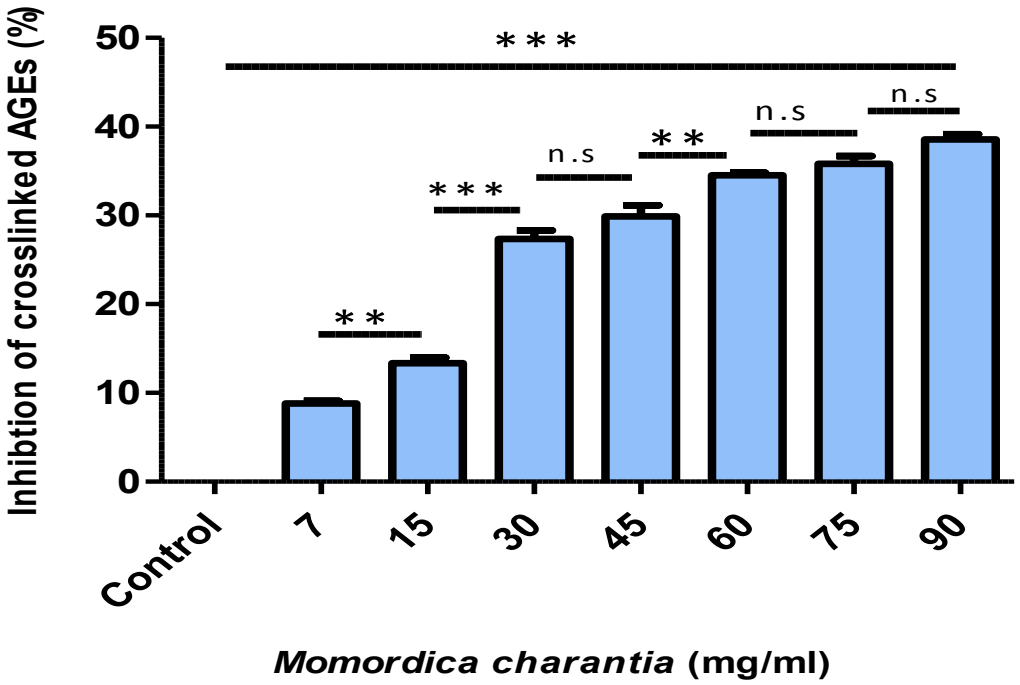


Figure 3.8: Effect of CWE of MC on glucose-induced AGE formation. The scan of SDS-PAGE gel shows the effect lysozyme (10mg/ml) incubated in the presence of glucose (0.5 M) produces cross-linked AGEs. The glycation mixture was incubated with different concentration of CWE of MC for 35 days. The percentage inhibition of AGE production was investigated utilizing different concentration of CWE of MC. Each value represented the mean ± SD (n=3), where n.s = non-significant , * * $p < 0.001$ and *** $p < 0.0001$ v.s control.

3.4.5 Effect of HWE of *Momordica charantia* on methylglyoxal-induced AGEs.

The reaction between methylglyoxal and lysozyme results in formation of cross-linked AGEs after 7 days of incubation. The monomers, dimers, trimers and tetramers of the cross-linked AGEs were inhibited substantially depending on the concentration of HWE of MC as shown in Figure 3.9. The reduced electrophoretic mobility of cross-linked AGEs with higher molecular weights were demonstrated in lanes b-i compared to the control lane a. The effect of different concentrations of HWE of MC clearly showed a reduction in the intensity of the dimer bands, which was used to measure the percentage of inhibition.

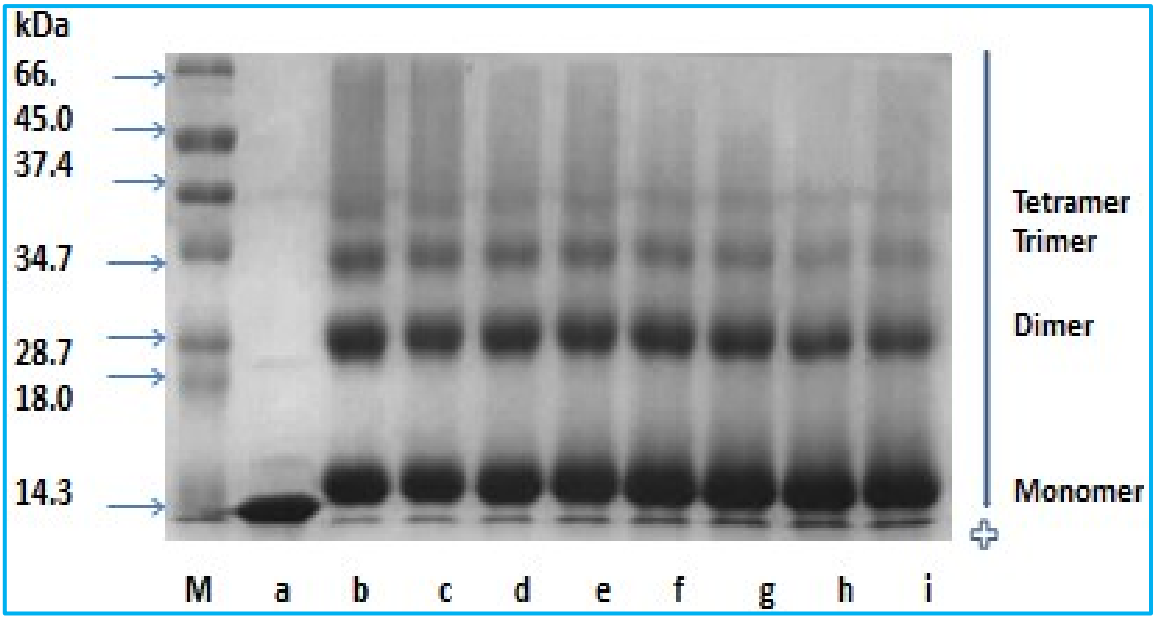


Figure 3.9: Gel showing the effect of HWE of MC on methylglyoxal-induced AGEs. SDS-PAGE gel showing lysozyme (10 mg/ml) incubated alone (lane a) or in the presence of 0.1 M methylglyoxal (lane b) and the effect of different concentrations (c) 7, (d) 14, (e) 30, (f) 45, (g) 60, (h) 75 and (i) 90 mg/ml of HWE of MC in 0.1 M sodium phosphate buffer pH 7.4 at 37 °C for 7 days. The result is a representative figure of at least three independent experiments.

Production of cross-linked AGE *in vitro* was significantly ($p < 0.05$) inhibited by MC extracts as compared to the control sample and this inhibition was dependent on MC extract concentrations as maximum (73%) inhibition was observed in the sample with 90 mg/ml of MC extracts as in Figure 3.10

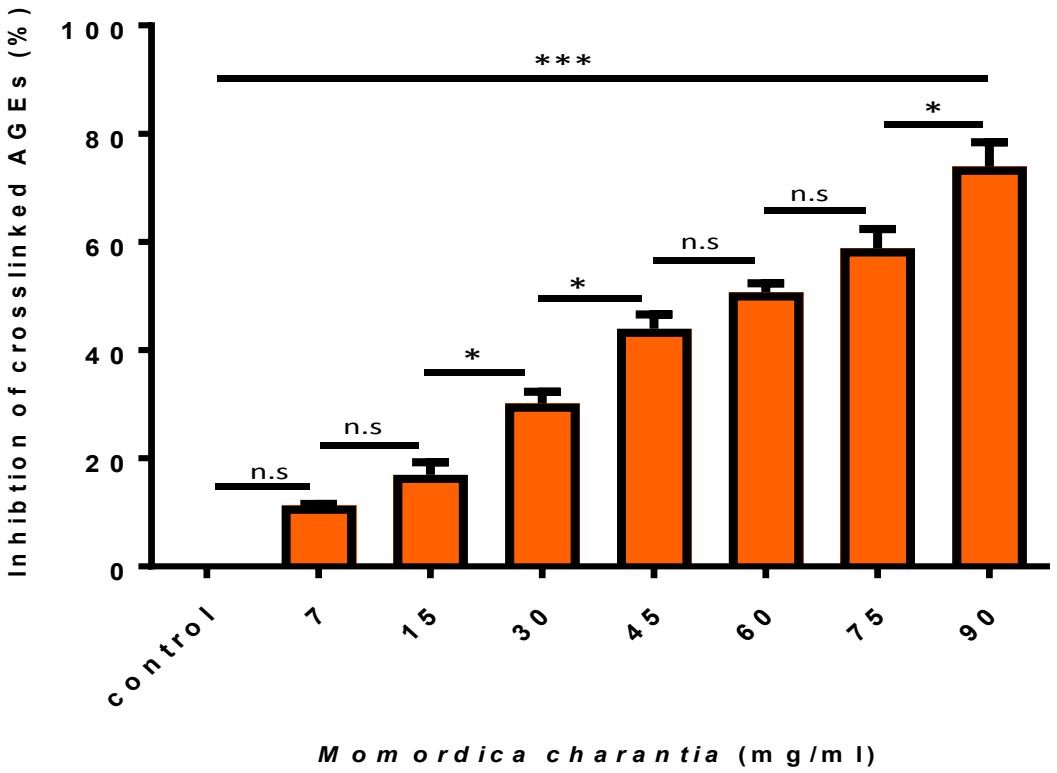


Figure 3.10: Effect of HWE of MC on methylglyoxal-induced AGE formation. The scan of SDS-PAGE gel shows the effect lysozyme (10mg/ml) incubated in the presence of methylglyoxal (0.1 M) produces cross-linked AGEs. The glycation mixture was incubated with different concentration of HWE of MC for 7 days. Each value represented the mean ± SD (n = 3), where n.s = non-significant , * $p < 0.05$ and *** $p < 0.0001$ v.s control.

3.4.6 Effect of CWE of *Momordica charantia* on methylglyoxal-induced AGEs.

The reaction between methylglyoxal and lysozyme results in formation of cross-linked AGEs after 7 days of incubation. The monomers, dimers, trimers and tetramers of the cross-linked AGEs were inhibited substantially depending on the concentration of CWE of MC as shown in Figure 3.11. The reduced electrophoretic mobility of cross-linked AGEs with higher molecular weights were demonstrated in lanes c-i compared to the control lane a. The effect of different concentrations of CWE of MC clearly showed a reduction in the intensity of the dimer bands, which was used to measure the percentage of inhibition.

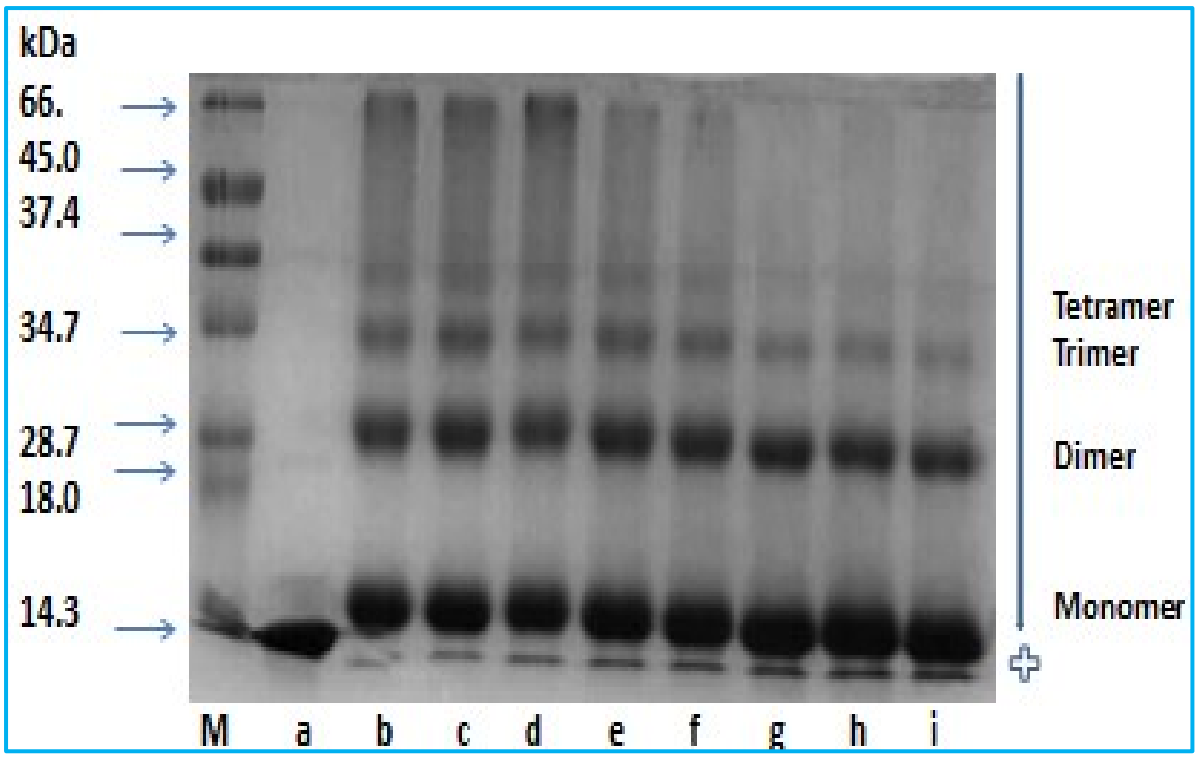


Figure 3.11: Gel showing the effect of CWE of MC on methylglyoxal-induced AGEs. SDS-PAGE gel showing lysozyme (10 mg/ml) incubated alone (lane a) or in the presence of 0.1 M methylglyoxal (lane b) and the effect of different concentrations (c) 7, (d) 14, (e) 30, (f) 45, (g) 60, (h) 75 and (i) 90 mg/ml of CWE of MC in 0.1 M sodium phosphate buffer pH 7.4 at 37 °C for 7 days. The result is a representative figure of at least three independent experiments.

Production of cross-linked AGE *in vitro* was significantly ($p < 0.05$) inhibited by MC extracts as compared to the control sample and this inhibition was dependent on MC extract concentrations as optimum (62%) inhibition was observed in the sample with 90 mg/ml of CWE of MC extracts as shown in Figure 3.12.

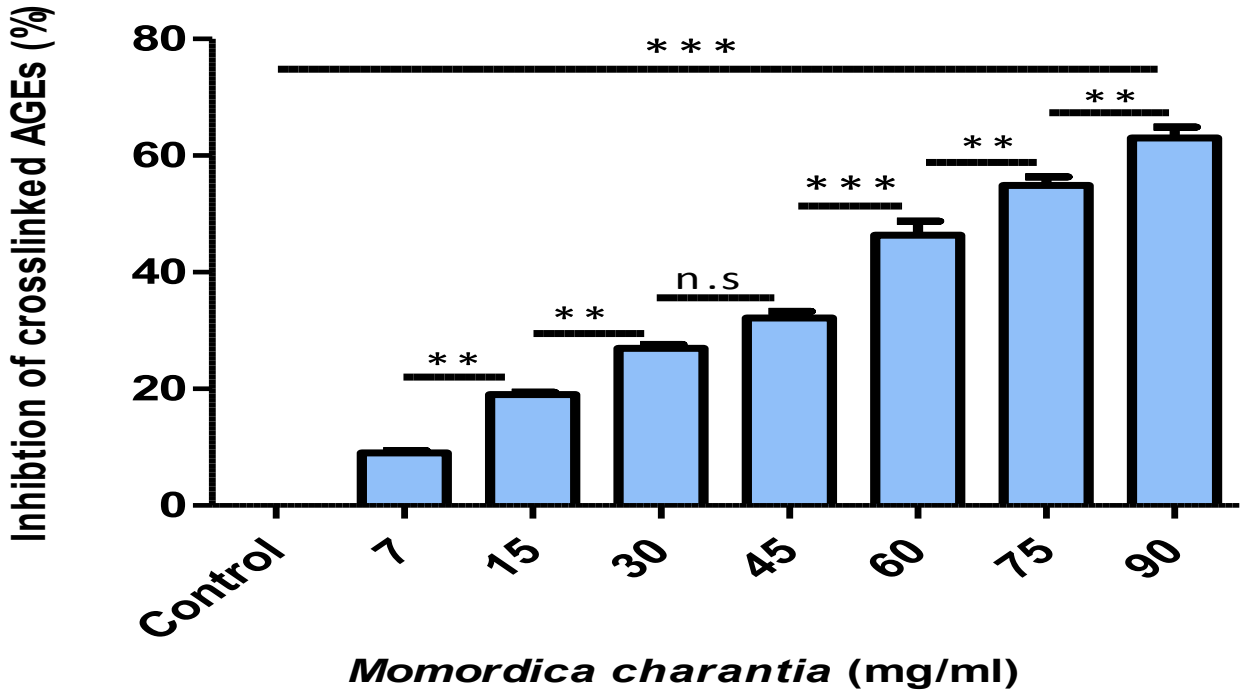


Figure 3.12: Effect of CWE of MC on methylglyoxal-induced AGE formation. The scan of SDS-PAGE gel shows the effect of lysozyme (10 mg/ml) incubated in the presence of methylglyoxal (0.1 M) produces cross-linked AGEs. The glycation mixture was incubated with different concentration of CWE of MC for 7 days. Each value represented the mean ± SD (n = 3), where n.s = non-significant , * * $p < 0.001$ and *** $p < 0.0001$ v.s control.

3.4.7 Effect of different glucose concentrations on fluorescent AGE formation *in vitro*

Figure 3.13 demonstrates the effect of different concentrations of glucose on the fluorescence AGE production over 35 days. Subsequent analysis was made by using 1 M glucose. In contrast with the control, the intensity of the fluorescence of lysozyme incubated with glucose showed a significant increase ($p < 0.01$). The effect determined for lysozyme being incubated for 35 days with 1 M glucose was more than 11.2 fold greater as compared to control and 5 fold greater than the effect observed in case of 0.1 M glucose.

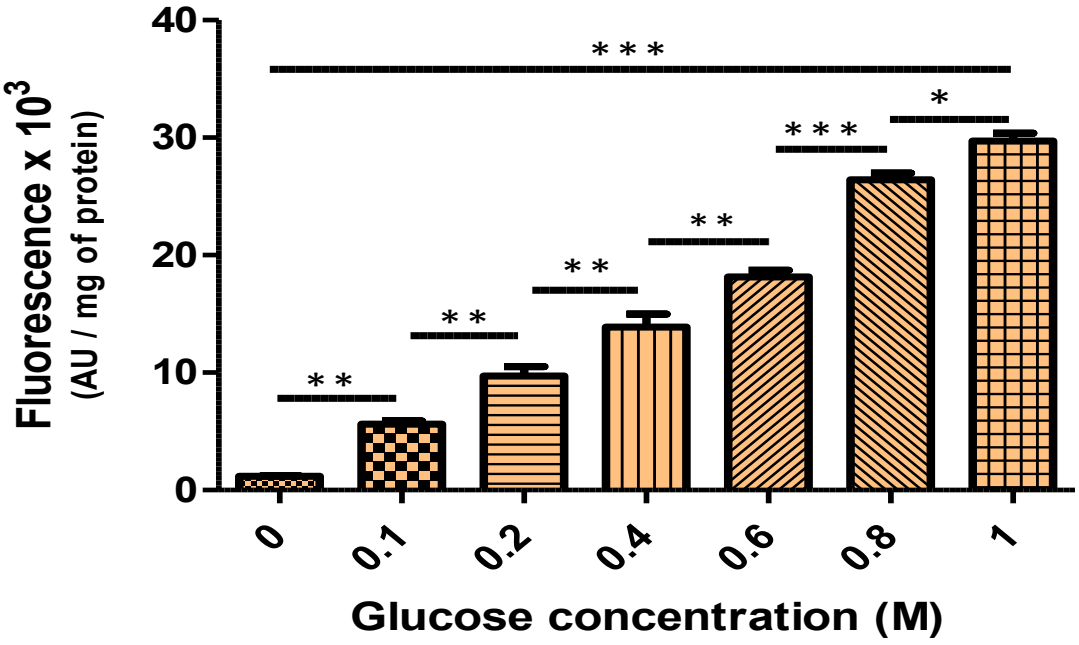


Figure 3.13: Effect of different glucose concentrations on the formation of fluorescent AGE. The graph shows effect of different concentrations of glucose on fluorescent AGEs formation. Lysozyme (10 mg/ml) was incubated with different concentrations of glucose in 0.1 M sodium phosphate buffer pH 7.4 at 37 °C for 35 days. Each value represents the mean ± SD (n = 3), * $p < 0.05$, * * $p < 0.001$ and *** $p < 0.0001$ v.s control (lysozyme).

3.4.8 Effect of different incubation time of glucose on the formation of fluorescent AGE *in vitro.*

Lysozyme incubated with glucose for 35 days showed a significantly ($p<0.01$) higher fluorescence level than that detected within the control sample without glucose. Formation of fluorescent AGEs is time-dependent. Fluorescence obtained in case of lysozyme incubated with glucose for 35 days was 6.8 fold greater compared to control and 3.7 fold greater than the starting time of incubation as shown in Figure 3.14

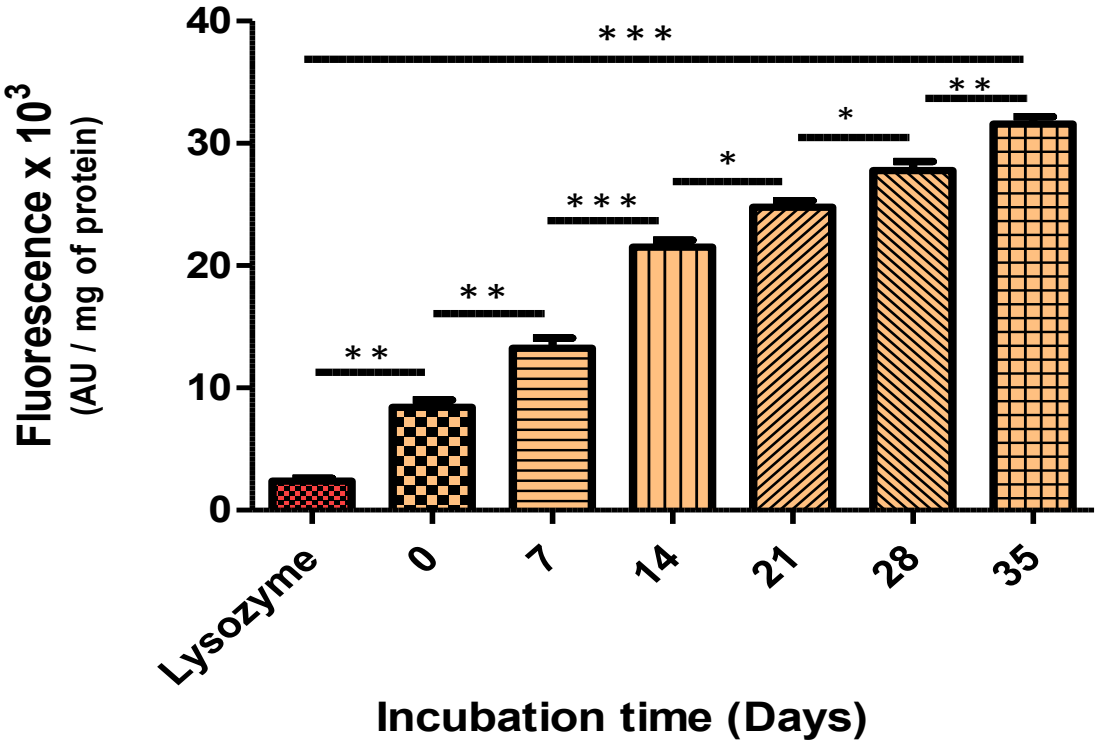


Figure 3.14: Effect of incubation time of glucose on the formation of fluorescent AGE. The graph shows the effect of different incubation times on fluorescent AGEs formation. Lysozyme (10 mg/ml) was incubated with 1 M glucose in 0.1 M sodium phosphate buffer pH 7.4 at 37 °C for 35 days. Each value represents the mean ± SD (n = 3), *p< 0.05 **p< 0.001 ***p< 0.0001 v.s control (lysozyme).

3.4.9 Effect of low concentration of HWE of *Momordica charantia* on glucose-induced AGEs

The effects HWE of MC on cross-linked AGEs formation are clearly visible by their ability to decrease electrophoretic mobility and intensity of the dimer bands as shown in Figure 3.15. These results showed that the inhibition of cross-linked AGEs production by HWE of MC was dose dependent in manner.

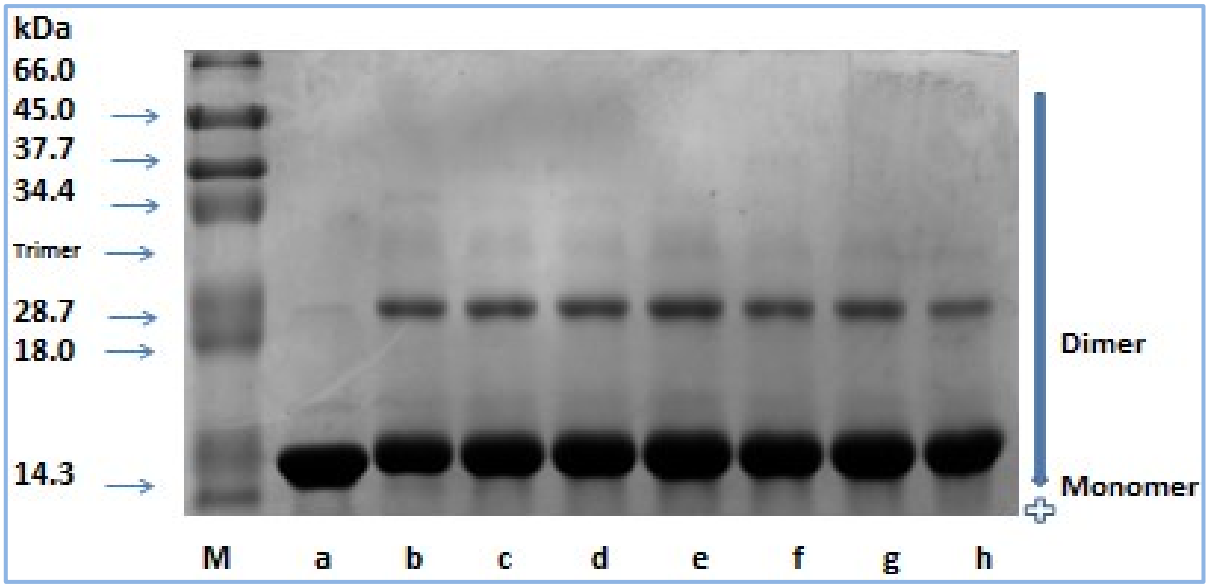


Figure 3.15: Gel showing the effect of low concentration of HWE of MC on the formation of AGEs. SDS-PAGE gel showing lysozyme (10 mg/ml) incubated alone (lane a) or in the presence of 50m M glucose (lane b) and the effect of different concentrations (c) 1, (d) 2, (e) 3, (f) 4, (g) 5, and (h) 7.5 mg/ml of HWE of MC in 0.1 M sodium phosphate buffer pH 7.4 at 37 °C for 12 weeks.

Formation of cross-linked AGE *in vitro* was significantly ($p < 0.05$) inhibited by MC extracts as compared to the control sample and this inhibition was dependent on MC extract concentrations as optimum (58.54%) inhibition was observed in the sample with 7.5 mg/ml of HWE of MC extracts as in Figure 3.16.

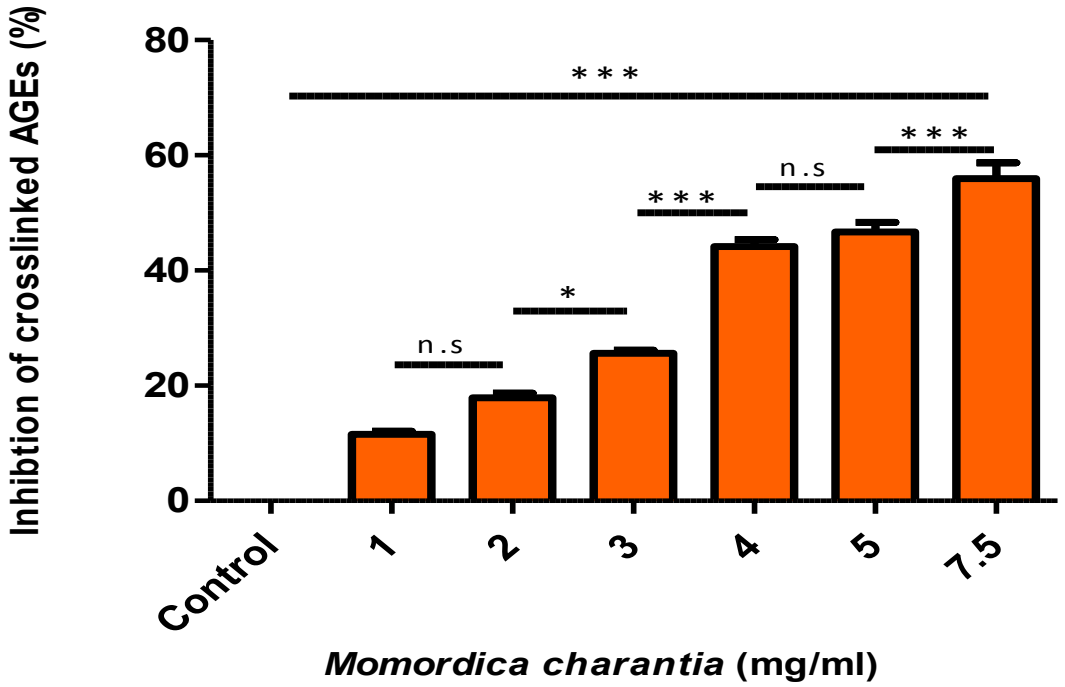


Figure 3.16: Effect of low concentration of HWE of MC on the formation of AGEs. The scan of SDS-PAGE gel shows the effect of different concentrations of HWE of MC on fluorescent AGE formation. Lysozyme (10 mg/ml) was incubated with 50 mM glucose alone or in presence of different concentrations of HWE of MC in 0.1 M sodium phosphate buffer pH 7.4 at 37 °C for 12 weeks. Each value represents the mean ± SD (n = 3), where n.s =(non-significant), *$p < 0.05$, ***$p < 0.0001$ v. control (lysozyme + glucose).

3.4.10 Effect of low concentration of CWE of *Momordica charantia* on glucose-induced AGEs.

The effects CWE of MC on cross-linked AGEs formation are clearly visible by their ability to decrease electrophoretic mobility and intensity of the dimer bands as demonstrated in Figure 3.17. These results showed that the inhibition of cross-linked AGEs production by HWE of MC was dose dependent in manner.

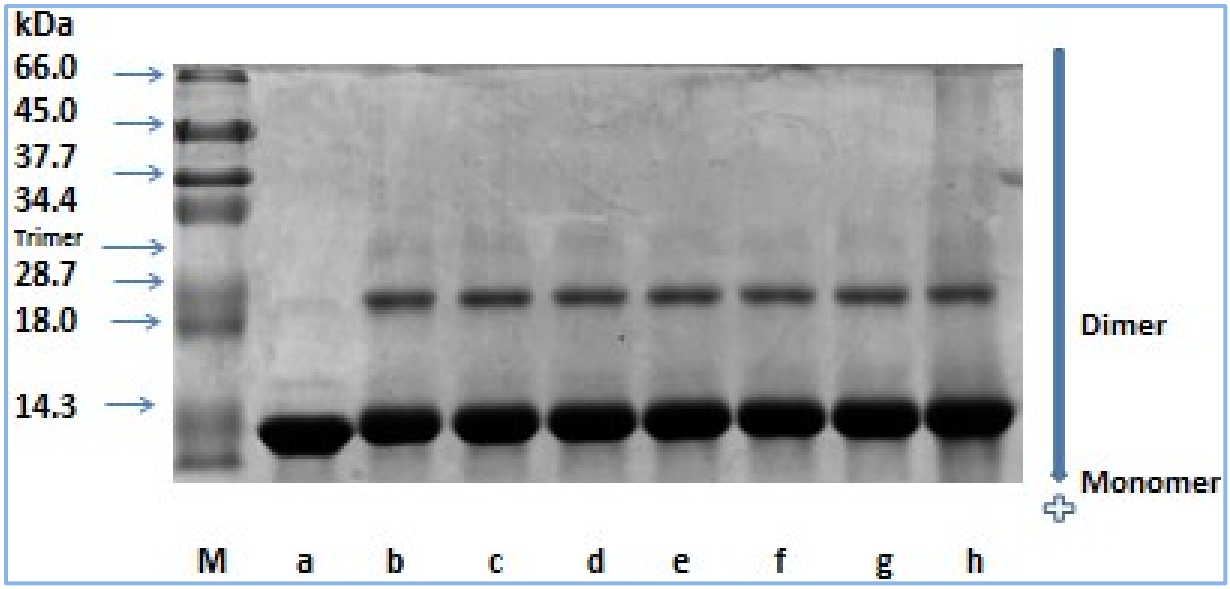


Figure 3.17: Gel showing the effect of low concentration of CWE of MC on the formation of AGE. SDS-PAGE gel showing lysozyme (10 mg/ml) incubated alone (lane a) or in the presence of 50m M glucose (lane b) and the effect of different concentrations (c) 1, (d) 2, (e) 3, (f) 4, (g) 5, and (h) 7.5 mg/ml of CWE of MC in 0.1 M sodium phosphate buffer pH 7.4 at 37 °C for 12 weeks.

Formation of cross-linked AGE *in vitro* was significantly ($p < 0.05$) inhibited by MC extracts as compared to the control sample and this inhibition was dependent on MC extract concentrations as optimum (29.54%) inhibition was observed in the sample with 7.5 mg/ml of HWE of MC extracts as shown in Figure 3.18

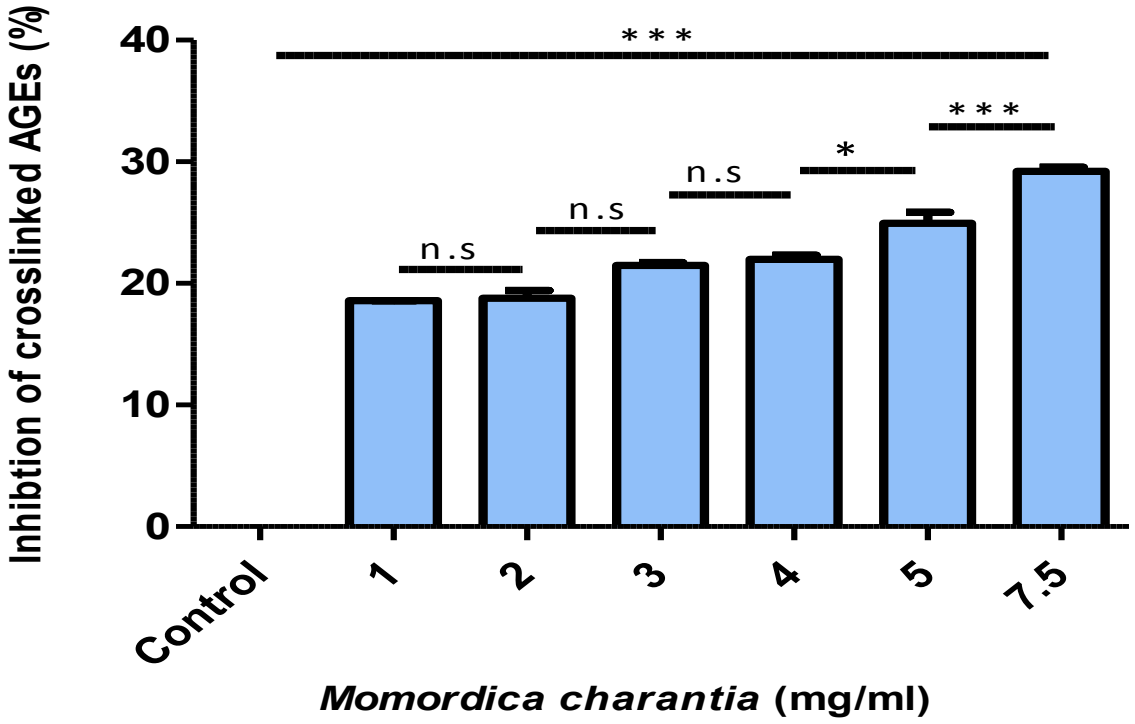


Figure 3.18: Effect of low concentration of CWE of MC on the formation of AGE. The scan of SDS-PAGE gel shows the effect of different concentrations of CWE of MC on fluorescent AGEs formation. Lysozyme (10 mg/ml) was incubated with 50 mM glucose alone or in presence of different concentrations of CWE of MC in 0.1 M sodium phosphate buffer pH 7.4 at 37 °C for 12 weeks. Each value represents the mean ± SD (n = 3), where n.s = (non-significant), *$p < 0.05$ and ***$p < 0.0001$ v.control (lysozyme +glucose).

3.4.11 Effect of low concentration of HWE of *Momordica charantia* on the formation of fluorescent AGE *in vitro*.

The glycation mixture was incubated with different concentrations of HWE of MC for 12 weeks. The results showed that inhibition of fluorescent cross-linked AGEs formation by CWE of MC was dose-dependent in manner.

Formation of cross-linked AGE *in vitro* was significantly ($p < 0.05$) inhibited by MC extracts as compared to the control sample and this inhibition was dependent on MC extract concentrations as optimum (40 %) inhibition was observed in the sample with 7.5 mg/ml of HWE of MC extracts as shown in Figure 3.19

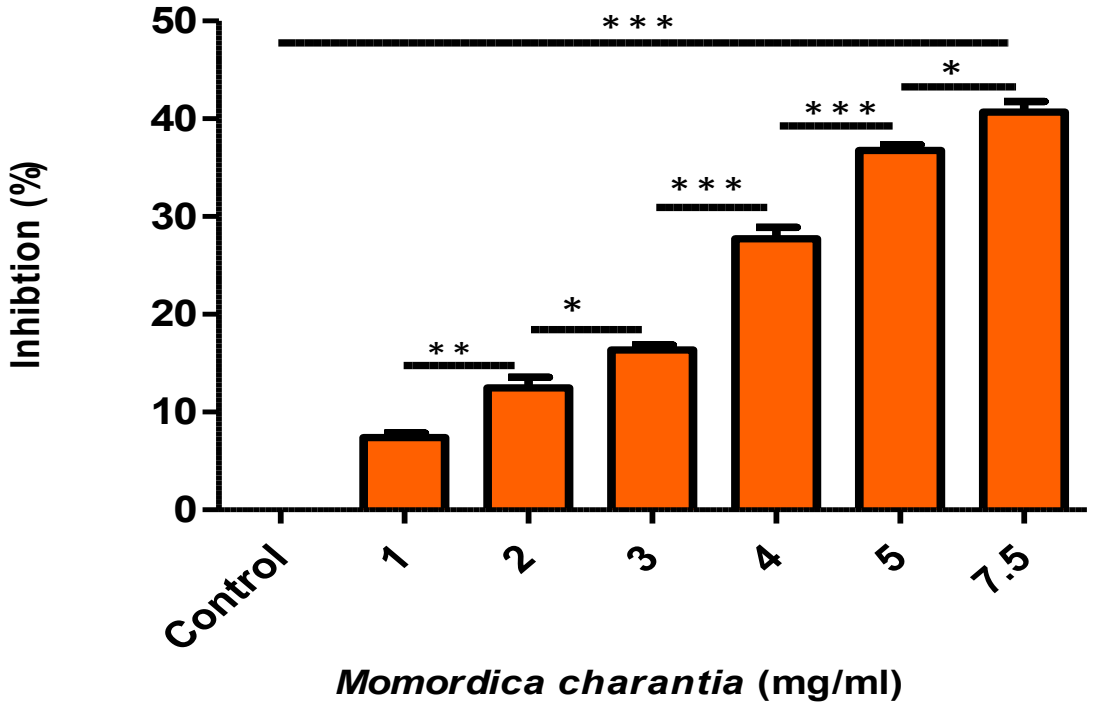


Figure 3.19: Effect of low concentration of HWE of MC on the formation of fluorescent AGE. The graph shows the effect of different concentrations of HWE of MC on fluorescent AGEs formation. Lysozyme (10 mg/ml) was incubated with 50 mM glucose alone or in the presence of different concentrations of HWE of MC in 0.1 M sodium phosphate buffer pH 7.4 at 37 °C for 12 weeks. Each value represents the mean ± SD (n = 3), *$p < 0.05$, **$p < 0.001$ and ***$p < 0.0001$ v.s control (lysozyme + glucose).

3.4.12 Effect of low concentration of CWE of *Momordica charantia* on the formation of fluorescent AGE *in vitro*.

The glycation mixture was incubated with different concentrations of CWE of MC for 12 weeks. The results showed that inhibition of fluorescent cross-linked AGEs formation by CWE of MC was dose-dependent in manner.

Formation of cross-linked AGE *in vitro* was significantly ($p < 0.05$) inhibited by MC extracts as compared to the control sample and this inhibition was dependent on MC extract concentrations as optimum (35%) inhibition was observed in the sample with 7.5 mg/ml of CWE of MC extracts as in Figure 3.20

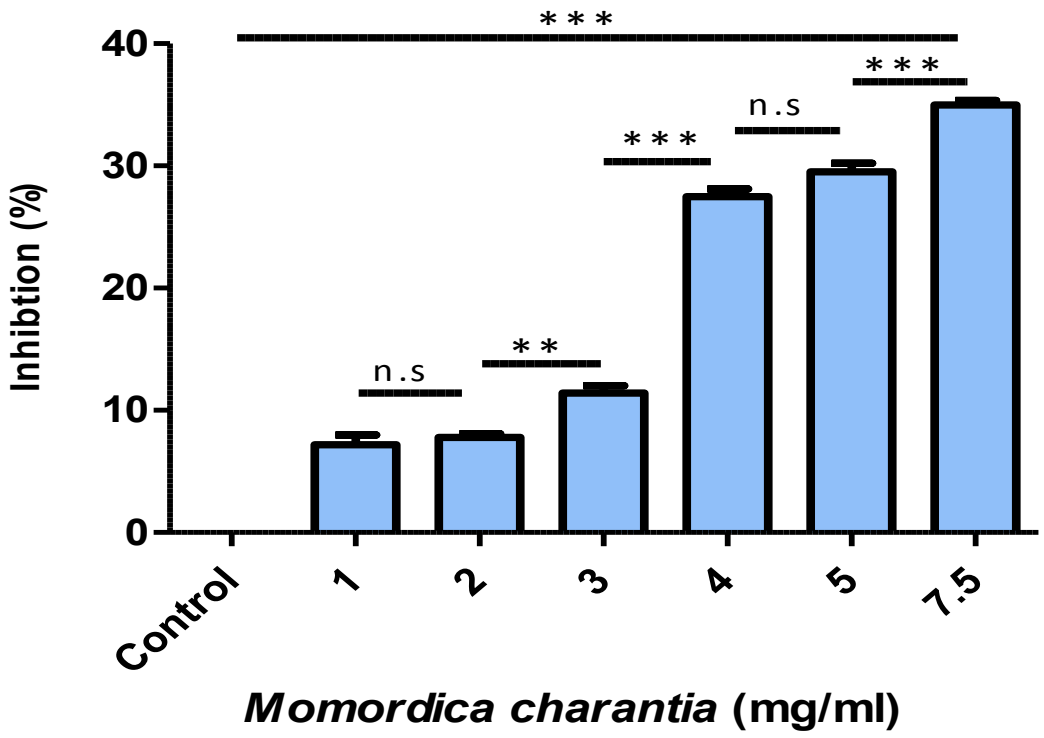


Figure 3.20: Effect of low concentration of CWE of MC on the formation of fluorescent AGE. The graph shows the effect of different concentrations of CWE of MC on fluorescent AGEs formation. Lysozyme (10 mg/ml) was incubated with 50 mM glucose alone or in the presence of different concentrations of CWE of MC in 0.1 M sodium phosphate buffer pH 7.4 at 37 °C for 12 weeks. Each value represents the mean ± SD (n = 3), where n.s = (non-significant), $^{**}p < 0.001$ and $^{***}p < 0.0001$ v.s control (lysozyme + glucose).

3.4.13 Effect of *Momordica charantia* extracts on AGEs levels using ELISA

The glycated product was obtained by incubating BSA (10 mg /ml) with ribose (0.5 M). In contrast with the control containing BSA-AGE, significant inhibition ($p < 0.001$) was illustrated by both MC extracts with the maximum inhibition by the HWE of MC. Furthermore, the inhibitory effects of MC extracts on AGEs level was shown to be in a dose-dependent manner as shown in Figure 3.21. There was a gradual decrease of the level of AGEs after incubation with both MC extracts, where, at the maximum concentration (90 mg/ml), the AGEs production was reduced to 64 % for HWE of MC and 53 % CWE of MC ($p < 0.01$).

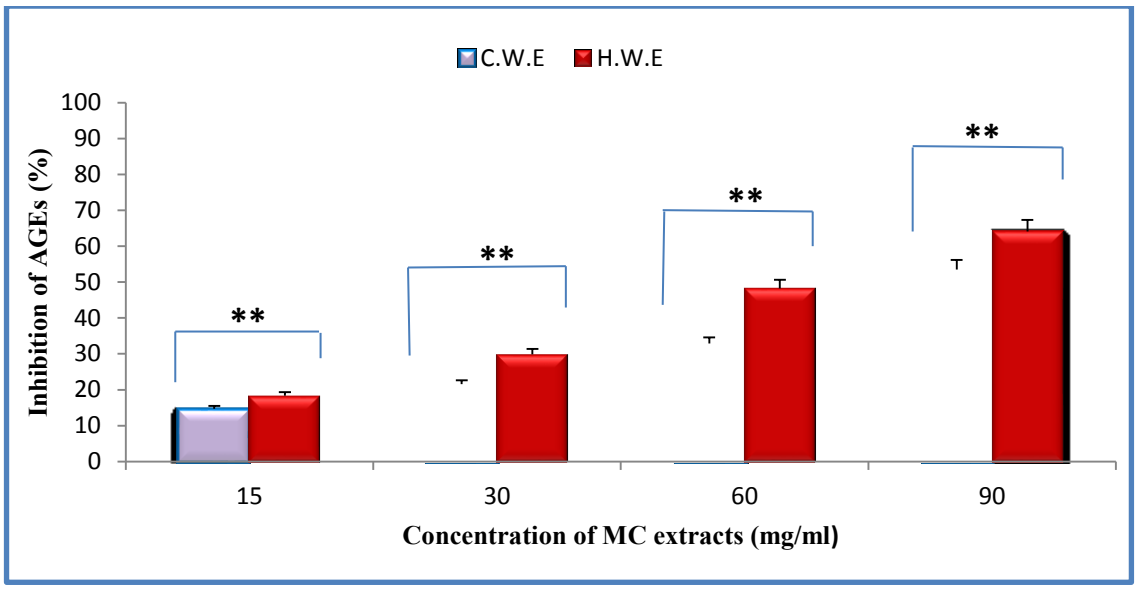


Figure 3.21: Effect of HWE and CWE of MC on AGE formation as measured by an ELISA technique. The graph demonstrates the inhibitory effect of MC extracts on AGEs levels that were determined in the presence of different concentrations of HWE and CWE of MC. Each value represents the mean ± SD (n=3), $^{**}p < 0.01$ v.s control (BSA + AGE).

3.5 Discussion

AGEs can be generated from the amino residues of proteins, lipids and DNA through several different mechanisms, including non-enzymatic glycation by glucose and reactions with metabolic intermediates and reactive dicarbonyl intermediates (methylglyoxal and glyoxal). Consequently, these reactions not only alter the form and structure of the proteins, but also lead to intramolecular and intermolecular cross-link production. The accumulation of cross-linked AGEs in biological compartments such as tissues and plasma plays a vital role in long-term complications associated with diabetes mellitus (Wu *et al*, 2009). The rate of formation and accumulation of tissue AGEs is faster in diabetic tissue (Mashilipa *et al*, 2011). A number of studies have examined the benefits of using plant extracts for inhibiting AGEs production, and may also provide protection to diminish the development of such complications (Leung *et al*, 2009; Tripathi & Chandra, 2009; Verzelloni *et al*, 2011). Diabetes mellitus is accounted as the major chronic disorder worldwide. It has been demonstrated that more than a third of the total of diabetic patients use medicinal plants. MC is one of the most common medicinal plants used as an anti-diabetic remedy (Joseph & Jini, 2013). In the current study, glycation of proteins was examined *in vitro* by incubation of proteins with different reducing sugars (glucose and methylglyoxal) at physiological pH for different time intervals. Furthermore, non-physiological concentrations of reducing sugars were also used to elevate the rate of production of AGEs in a short period. ****Lysozyme is a popular protein for the detection of cross-linked AGEs production, as oligomerisation occurs readily and is easily measurable by SDS-PAGE techniques. A number of researchers have examined the glycation of lysozyme *in vitro* (Kislinger *et al*, 2004). Lysozyme consist 6 lysine and 11 arginine per molecules and modifications of these residues can be achieved by both glucose and methylglyoxal. The results illustrated that elevated cross-linked AGEs formation correlated with glucose concentration and the period of incubation, this is in agreement with previous study (Mashilipa *et al*, 2011). The detection of fluorescence to study the effects of different glucose concentrations and different times of incubation on AGEs production also confirmed the previous findings with SDS-PAGE techniques. The dicarbonyl reducing sugar methylglyoxal was used because it is more reactive than the other reducing sugars, and consists of the carbonyl compounds (aldehyde and ketone). Moreover, the other important features of methylglyoxal are its ability to bind with sulfhydryl, amino, lysine and arginine functional groups in protein molecules (Goldin *et al*, 2006). In this respect, methylglyoxal induced generation of AGEs have been contributed in the progression and development of diabetic microvascular complications such

neuropathy, retinopathy and atherosclerosis (Chang *et al*, 2011) and the concentration of methylglyoxal is elevated during diabetes (Ahmed, 2005). Moreover, methylglyoxal is recognised as a toxic agent, and its toxicity is mainly associated in cell apoptosis. Several studies *in vitro* have demonstrated that methylglyoxal-induced apoptosis in varieties cells for endothelial cells , epithelial cells, human leukaemia cells, Schwaan cells, osteoblasts and rat hippocampal cells (Chan *et al*, 2007; Huang *et al*, 2008; Ota *et al*, 2007). ELISA assay experiments have shown that the incubation of lysozyme with methylglyoxal resulted in elevated generation of AGEs. However, the inhibitory effects of MC extracts were almost similar as determined by SDS-PAGE and fluorescence assays. This activity of MC extracts against AGEs production may be related to the ability to chelate transition metals thus inhibiting autoxidative glycation and glycoxidation reactions, and the antioxidant activity may due to the properties against free radicals activity. It was assumed that MC extracts would compete with protein, and thereby block the modification of lysine residues in the protein.

Sugars that exist in open (acyclic) form, obtain a higher rate of reaction than those of the same sugar in the closed (cyclic) form. Acyclic sugars have a higher rate of producing Schiff base and this rate is directly proportional to the concentration of the acyclic form. In addition to that, glucose is most abundant reducing sugar in the blood and naturally exists in the cyclic form, but when dissolved in the solution changes to acyclic form, this process of changing from cyclic form to acyclic form is named mutoratation (Mashilipa *et al*. 2011). In contrast, methylglyoxal is reported to obtain a higher rate of reaction and is reported to be more reactive than glucose (Gul *et al*, 2009). This reactivity is justified because methylglyoxal exists all in the acyclic form. The reactivity of sugars is dependent on the existence of the-open chain carbonyl form (Ahmed, 2005). This study illustrates for the first time that high and low concentrations of HWE and CWE of MC have significant inhibitory effects on cross-linked and fluorescence AGEs in a dose-dependent manner *in vitro*. Several types of AGEs are produced by oxidation reactions and protein glycation (Ahmed, 2005; Mashilipa *et al*, 2011).

In this study, the potential benefits of utilizing MC extracts as medicinal plants were investigated in lysozyme-glucose system *in vitro*. The formation of cross-linked AGEs was induced by incubation of lysozyme, this is in agreement with previous study (Elosta *et al*, 2012). In addition, low concentrations of the reducing sugar glucose and MC extracts were used in lysozyme-glucose system in vitro. This is to investigate whether the extract inhibit AGE production in relevant diabetic condition *in vitro*. This study illustrates for the first time that

high and low concentrations of HWE and CWE of MC have significant inhibitory effects on cross-linked and fluorescence AGEs in a dose-dependent manner *in vitro*. The results from this study have shown that AGE formation is inhibited by HWE and CWE of MC with the HWE of MC possessing the more potent effect. This result may suggests that MC contains of varieties of amino acid molecules that can reduce the production of AGEs by engulfing carbonyl molecules (Mahomoodally *et al*, 2012; Orlovskaya & Chelombitko, 2007). Moreover, MC consists of a large number of antioxidant compounds, they are therefore able to scavenge the free radicals thereby, reducing the level of AGEs (Leung *et al*, 2009; Shan *et al*, 2012). It has been found that MC extracts have ability to chelate Cu^{2+} ions and protect against LDL oxidation reactions (Hsieh *et al*, 2005). The inhibition of methylglyoxal-induced AGEs by MC extracts may occur through blocking the conversion of dicarbonyl intermediates to AGEs. The results indicate that HWE of MC shows potent antioxidant activity, thus prevent the production of AGEs. The results suggest that the other active compounds under influence of temperature may be present in HWE of MC in addition to those compounds presents in CWE of MC, which provides its antiglycation property, however these compounds have not yet established. In this respect, other methods required to be utilized in order to determine active constituents of MC extracts and study their effects on formation of cross-linked AGEs. In this study, MC extracts inhibited AGE-induced cross-links, as found by its effect on inhibiting glucose- and methylglyoxal-induced lysozyme polymerisation *in vitro*. The results obtained from this study suggest that MC extracts has the ability to break pre-formed AGEs cross-linked *in vitro* and therefore further *in vivo* investigations are needed in order to examine its role in the inhibition of diabetic complications.

Chapter 4. Antioxidant properties of *Momordica charantia* extracts

4.1 Introduction

It has been well established that ROS such as free radical species play a pivotal role in the progression and enhancement of tissue injury and pathological events in living organism ((Tabatabaei-Malazy *et al*, 2013). Therefore, oxidative stress occurs when there is an imbalance between antioxidant compounds and oxidant species and causes damage in all kinds of biomolecules such as protein, lipids, DNA and RNA, thus, the balance between ROS and antioxidants is an important aspect for maintaining biological systems (Sharma & Vig, 2013). Oxidative stress is the most important factor and plays a central role in the progression of diabetic complications, at both microvascular and macrovascular levels (Giacco & Brownlee, 2010). Previous studies have shown that oxidative stress is elevated in insulin-dependent (type1) and non-insulin-dependent (type 2) diabetes (Rahimi *et al*, 2005; Soufi *et al*, 2012). The primary antioxidant enzymes that protect cells from injury mediated by ROS are SOD, CAT and GPx (Chiang *et al*, 2006). Their role as protective enzymes are well established and have been extensively examined *in vivo* and *in vitro* in model systems (Semiz & Sen, 2007). SOD plays a central role in defence system combating oxidative stresses by catalysing ROS to hydrogen peroxide which is subsequently changed to water by CAT (Zhang *et al*, 2011). In this respect, natural antioxidants, particularly those presented in medicinal plant extracts such as phenolics, flavonoids, tannins and anthocyanins, are affordable, safe and are also bioactive (Mohsen & Ammar, 2009). Medicinal plants could be an important sources of natural antioxidant compounds (Ahmad & Ahmed, 2006). The antioxidant properties of these compounds derive from their ability to scavenge free radical species, chelate ion metals and improve antioxidant enzymes activity (Fraga *et al*, 2010). Moreover, the medicinal plants either in the form of supplementary medicine or food products are widely utilized to improve health and inhibit oxidative stress-induced chronic conditions like diabetic mellitus (Kalim *et al*, 2010).

Natural compounds and medicinal plants have become an increasingly attractive option to alleviate diabetic complications and other chronic conditions, because there is growing evidence that these nutritional compounds provide potential adjunctive therapeutic effects on different disorders including diabetes (Amornrit & Santiyanont, 2015). A large number of studies have reported that plant extracts of MC contain different active compounds against oxidative stress (Budrat & Shotipruk, 2009; Mahomoodally *et al*, 2012). Several *in vitro*

antioxidant assays were employed to evaluate the activity of different MC extracts (Semiz & Sen, 2007). Moreover, reports on the *in vivo* antioxidant activity of MC extracts have outlined the elevated efficacy of naturally occurring human antioxidant enzymes such as SOD and CAT (Sathishsekar & Subramanian, 2005). Moreover, previous study has reported that MC extract lower systemic oxidative stress in diabetic rats (Nerurkar *et al*, 2011). In this chapter, the antioxidant property of MC extracts was analysed during this research via a number of *in vitro* antioxidant assays. Determination of phenolic compounds in MC extracts was executed *in vitro* leading to an elaboration of the link between anti-oxidative and antiglycation properties of MC.

4.2 Aim and objectives

The aim of the current study was to investigate and compare the antioxidant activities of HWE and CWE of MC *in vitro*.

The objectives of this study are:

- To investigate and compare the total phenolic content of *Momordica charantia* extracts.
- To examine the ferric ion reducing power of *Momordica charantia* extracts.
- To examine the cupric ion reducing antioxidant capacity of *Momordica charantia* extracts
- To investigate the effect of MC extracts on SOD enzyme activity in BAEC exposed to glucose, methylglyoxal and AGEs
- To examine the effect of MC extracts on CAT enzyme activity in BAEC exposed to glucose, methylglyoxal and AGEs
- To investigate the effects of MC extracts on ROS formation in BAEC exposed to glucose, methylglyoxal and AGEs

4.3 Methods

Total phenolic compounds in MC extracts were measured as defined in section 2.4.10, FRAP antioxidant activity of MC was assessed as in section 2.4.11, CUPRAC was measured as in section 2.4.12, SOD enzyme activity of MC extracts in BAEC were measured as in section 2.4.13, CAT enzyme activity of HWE and CWE of MC in BAEC were assessed as defined in section 2.4.14, ROS enzyme activity of HWE and CWE of MC in BAEC was also measured as in section 2.4.15.

4.4 Results

4.4.1 Total phenolic content of *Momordica charantia* extracts

The amount of phenolic compounds (mg/g) in MC extracts were represented as gallic acid equivalents (GAE). The phenolic amounts in HWE and CWE varied considerably as shown in Table 4.1. The highest amount was found in HWE with a value of 169.7 ± 2.25 mg/g whereas for CWE it was 123.43 ± 1.14 mg/g.

Table 4.1: Total amount of phenolic compounds in *Momordica charantia* extracts. Each value represents the mean ± SD (n = 3) from at least three independent experiments.

Momordica charantia extracts	Total phenolic content (mg GAE/g extract)
HWE	169.7 ± 2.25
CWE	123.43 ± 1.14

4.4.2 Effect of ferric ion reducing power in *Momordica charantia*

The results show that the increasing concentrations of HWE of MC possess high reducing power of 13.68, 16.84, 30.82 and 32.46 with fold inhibitions, respectively. CWE of MC has lower reducing power activity of 10.82, 15.74, 27.28 and 28.63 with fold inhibitions, respectively, at the test concentrations. The lowest reducing activity was found in ascorbic acid of 1.20, 2.58, 7.32 and 11.61 with fold inhibitions, respectively, when tested at the same the concentrations. HWE and CWE of MC possess high reducing power activity compared to the positive control (ascorbic acid). The reducing power of HWE, CWE of MC and ascorbic acid increases with increases in the amount of sample and control concentrations as shown in Figure 4.1

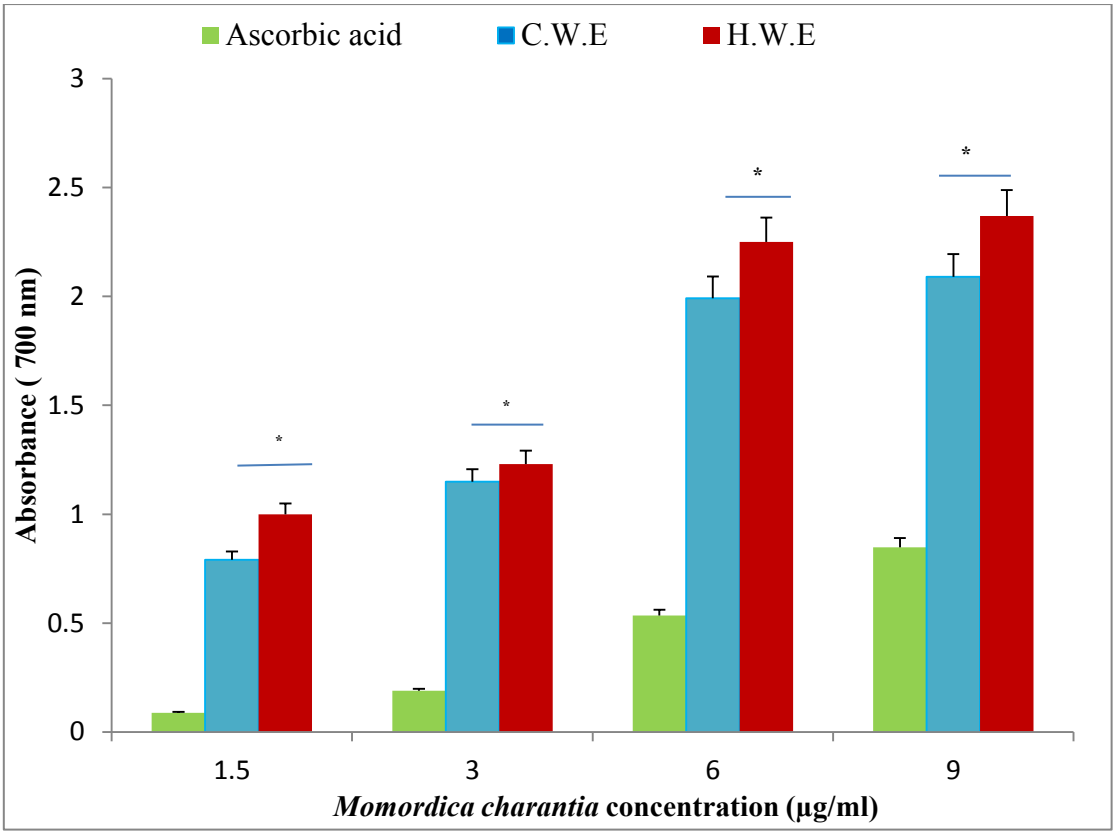


Figure 4.1: Reducing power of HWE and CWE of MC. The effects of different concentrations of HWE and CWE of MC on reducing power. Ascorbic acid was used as a positive control. Results are presented as mean ± SD, n=3. *$p < 0.05$

4.4.3 Effect of cupric ion reducing antioxidant capacity of *Momordica charantia*

The results show that the increasing concentrations of HWE of MC possess high reducing power of 8.24, 8.93, 11.85 and 13.16 with fold inhibitions, respectively. Ascorbic acid has lower reducing power activity of 3.19, 4.86, 10.16 and 10.98 with fold inhibitions, respectively, at the test concentrations. The lowest reducing activety was shown in CWE of MC of 2.14, 2.57, 2.67 and 3.75 with fold inhibitions, respectively, when tested at the same the concentrations. HWE of MC showed the highest reducing power activity compared to ascorbic acid. In contrast, the lowest reducing power activity was found in CWE of MC as demonstrated in Figure 4.2

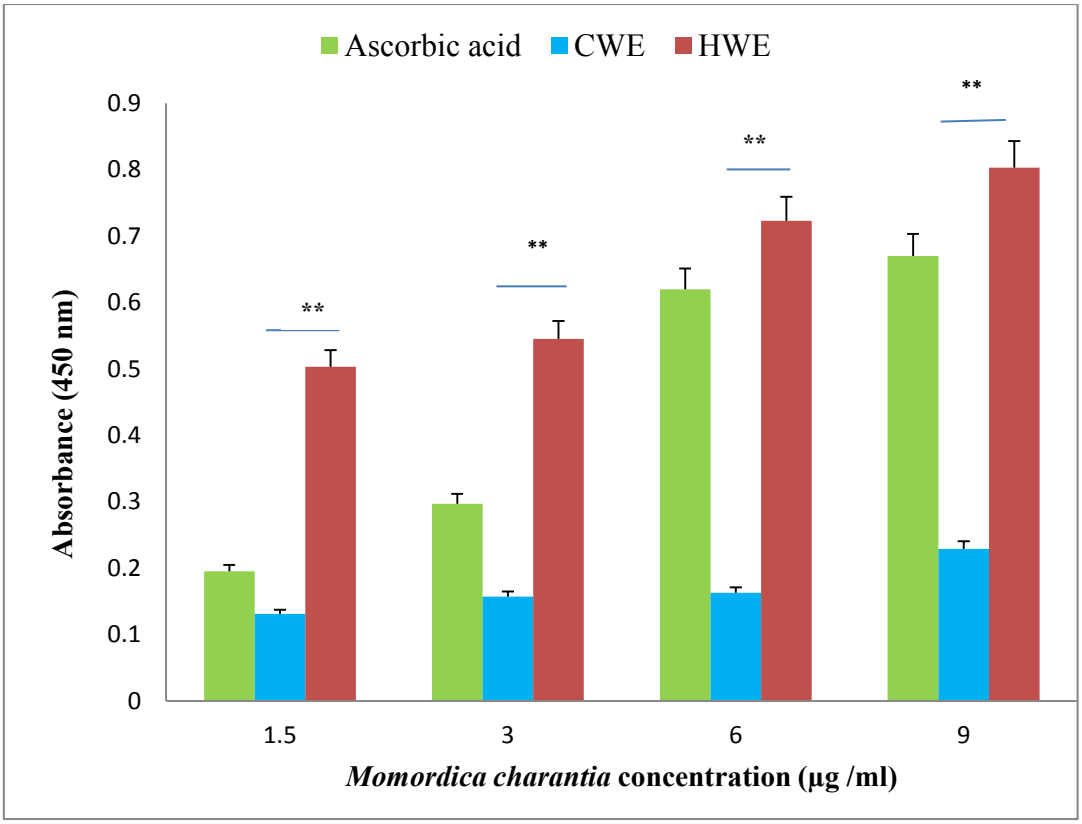


Figure 4.2: Reducing activity of HWE and CWE of MC. The effects of different concentration of HWE and CWE of MC on reducing activity. Ascorbic acid was used as a positive control. Results are presented as mean ± SD, n=3 and $^{**}p < 0.01$.

4.4.4 Effects of *Momordica charantia* on SOD activity in BAEC exposed to glucose

The results show that Trolox induced a significant increase in SOD activity by 1.94 fold ($p = 0.003$), compared to untreated control cells, whereas, CWE of MC induced no significant increase in SOD activity, and HWE of MC induced a significant increase in SOD activity by 1.68 fold ($p = 0.0001$) compared to glucose-treated cells. Moreover, glucose-treated cells induced a non-significant increase in SOD activity compared with untreated cells. HWE of MC possess a more potent SOD activity than the CWE of MC as demonstrated in Figure 4.3

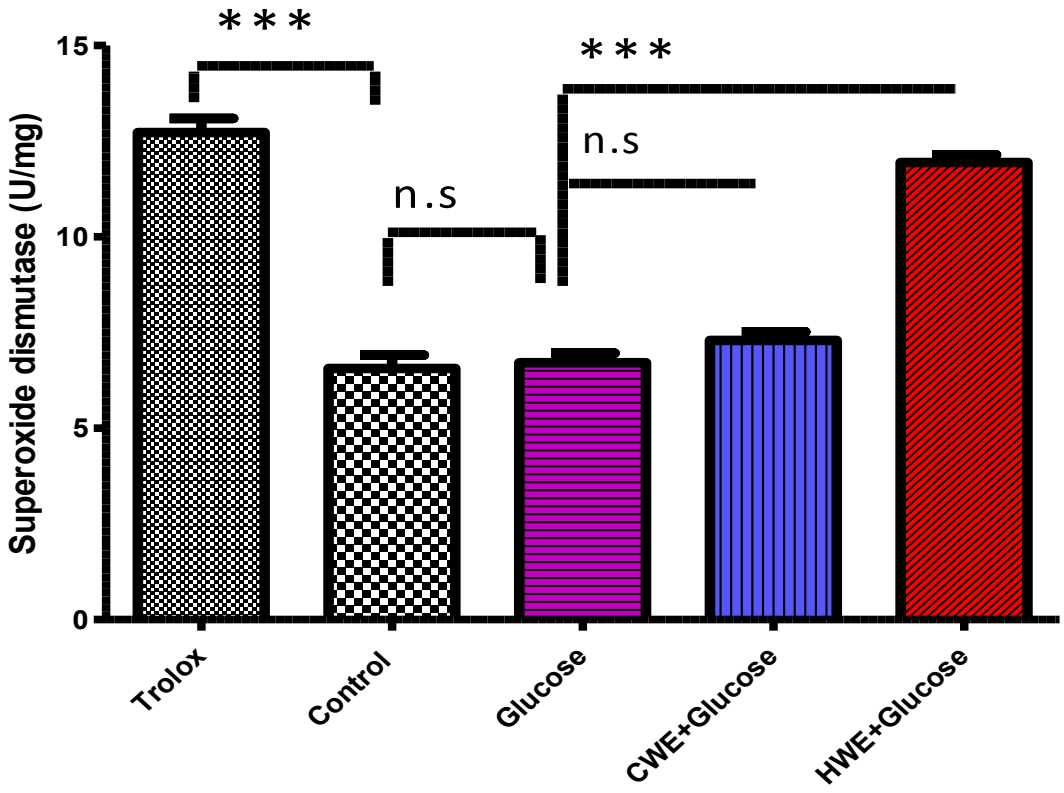


Figure 4.3: Effect of MC extracts on SOD activity in BAEC exposed to glucose. The graph illustrates the effect of 25 mM glucose alone or with 90 mg/ml of MC extracts in BAEC medium cells at 37 ºC for 24 hours. Trolox was used as standard antioxidant. Data are mean ± SD, n= 3. (n.s. = non-significant, ***p< 0.001).

4.4.5 Effects of *Momordica charantia* on SOD activity in BAEC exposed to methylglyoxal

The results show that Trolox induced a significant increase in SOD activity by 1.94 fold (p = 0.0003) compared to untreated control cells, whereas, CWE of MC induced a non-significant increase in SOD activity compared to methylglyoxal-treated cells. On the other hand, the HWE of MC induced a significant increase in SOD activity by 1.53 fold (p = 0.0033) compared to methylglyoxal-treated cells. Moreover, methylglyoxal-treated cells induced a non-significant increase in SOD activity compared with untreated cells. HWE of MC possess a more potent SOD activity than the CWE of MC as shown in Figure 4.4

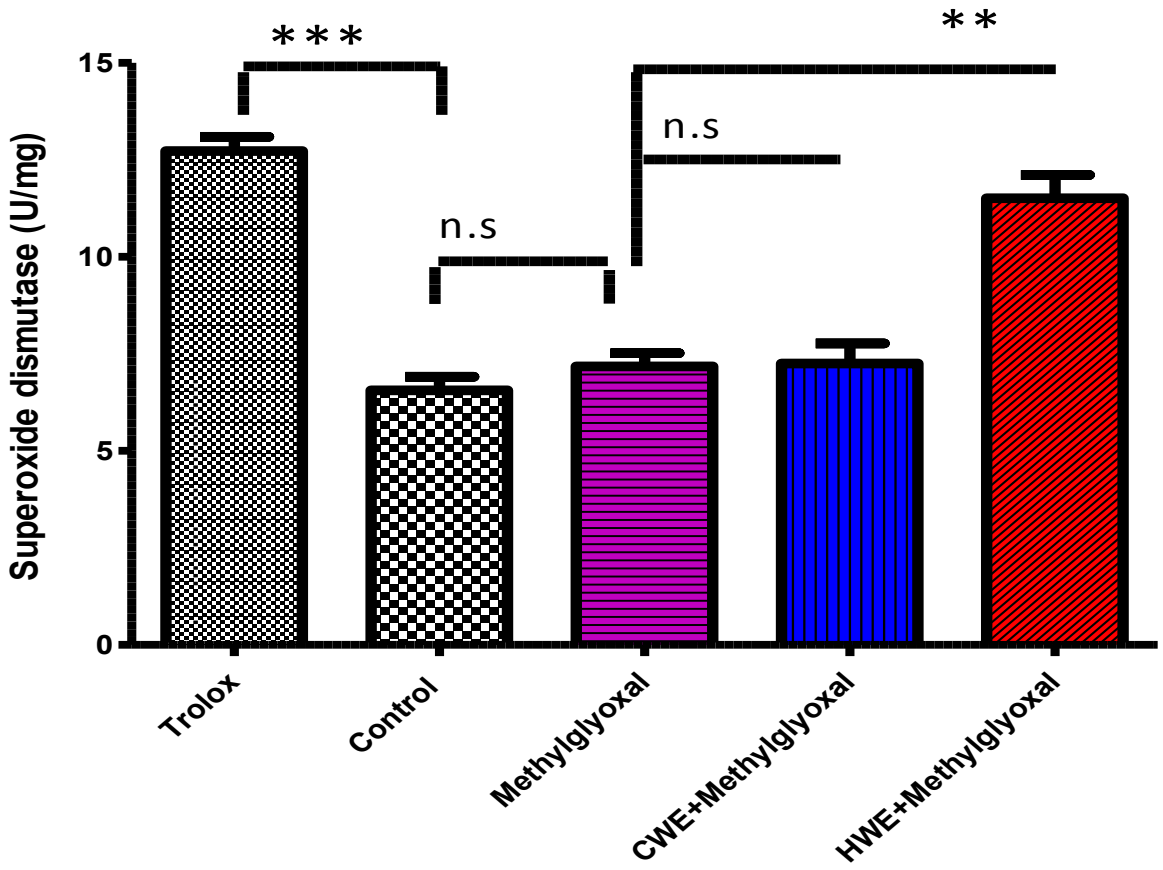


Figure 4.4: Effect of MC extracts on SOD activity in BAEC exposed to methylglyoxal. The graph illustrates the effect of 5 mM M methyglyoxal alone or with 90 mg/ml of MC extracts in BAEC medium cells at 37 ºC for 24 hours. Trolox was used as standard antioxidant. Data are mean ± SD, n= 3 (n.s. = non-significant, ***p*< 0.01, ****p*> 0.001).

4.4.6 Effects of *Momordica charantia* on SOD activity in BAEC exposed to AGEs

Figure 4.5 showed that Trolox induced a significant increase in SOD activity by 1.94 fold (p = 0.0003), compared to untreated control cells, whereas, CWE of MC induced a significant increase in SOD activity by 1.32 fold (p = 0.0003) compared to AGEs-treated cells. Similarly, the HWE of MC induced a significant increase in SOD activity by 1.94 fold (p = 0.0001) compared to AGEs-treated cells. Moreover, AGEs- treated cells had no significant effect in SOD activity compared with control cells. HWE of MC possess a more potent SOD activity than the CWE of MC.

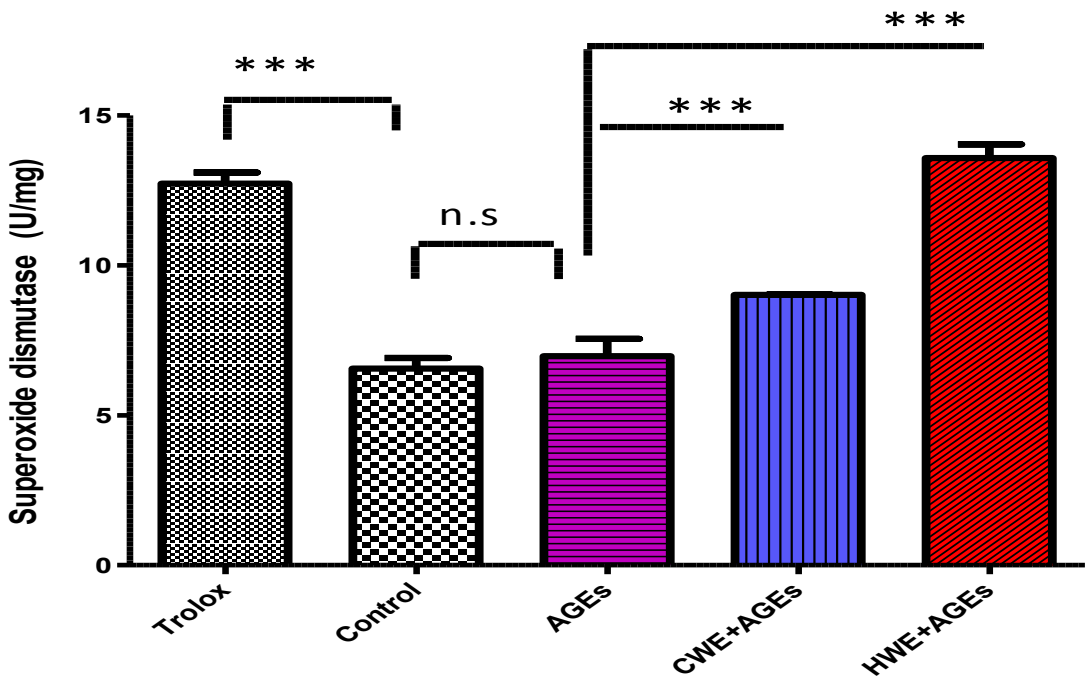


Figure 4.5 Effect of MC extracts on SOD activity in BAEC exposed to AGEs. The graph illustrates the effect of 10 µg/ml AGE alone or with 90 mg/ml of MC extracts in BAEC medium cells at 37 ºC for 24 hours. Trolox was used as standard antioxidant. Data are mean ± SD, n= 3 (n.s. = non-significant, ***$p < 0.001$).

4.4.7 Effects of *Momordica charantia* on CAT activity in BAEC exposed to glucose

The results show that Trolox induced a significant increase in CAT activity by 2.94 fold ($p = 0.0001$), compared to untreated control cells. However, HWE and CWE of MC induced a significant increase in CAT activity by 2.15 fold ($p = 0.0001$), and by 2.73 fold ($p = 0.0001$) respectively compared to glucose-treated cells. However, glucose-treated cells had no effect on CAT activity compared with untreated cells as shown in Figure 4.6

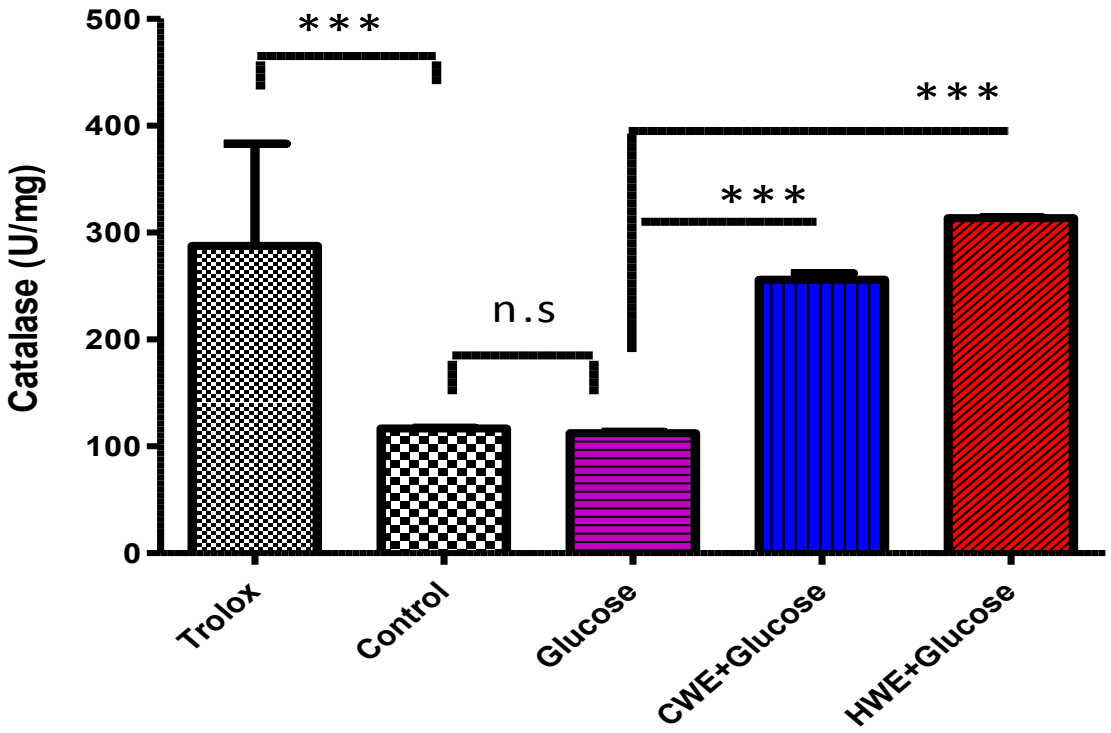


Figure 4.6: Effect of MC extracts on CAT activity in BAEC exposed to glucose. The graph demonstrates the effect of 25 mM glucose alone or with 90 mg/ml of MC extracts in BAEC medium cells at 37 ºC for 24 hours. Trolox was used as standard antioxidant. Data are mean ± SD, n= 3. (n.s. = non- significant, ***$p < 0.001$).

4.4.8 Effects of *Momordica charantia* on CAT activity in BAEC exposed to methylglyoxal

The results show that Trolox induced a significant increase in CAT activity by 2.94 fold ($p = 0.0001$), compared to untreated control cells, whereas, CWE and HWE of MC induced a significant increase in CAT activity by 1.65 fold ($p = 0.0001$), 2.23 fold ($p = 0.0001$) compared to methylglyoxal-treated cells, respectively. Moreover, methylglyoxal-treated cells had no effect on CAT activity compared with untreated cells. HWE of MC possesses a more potent CAT activity than the CWE of MC as shown in Figure 4.7

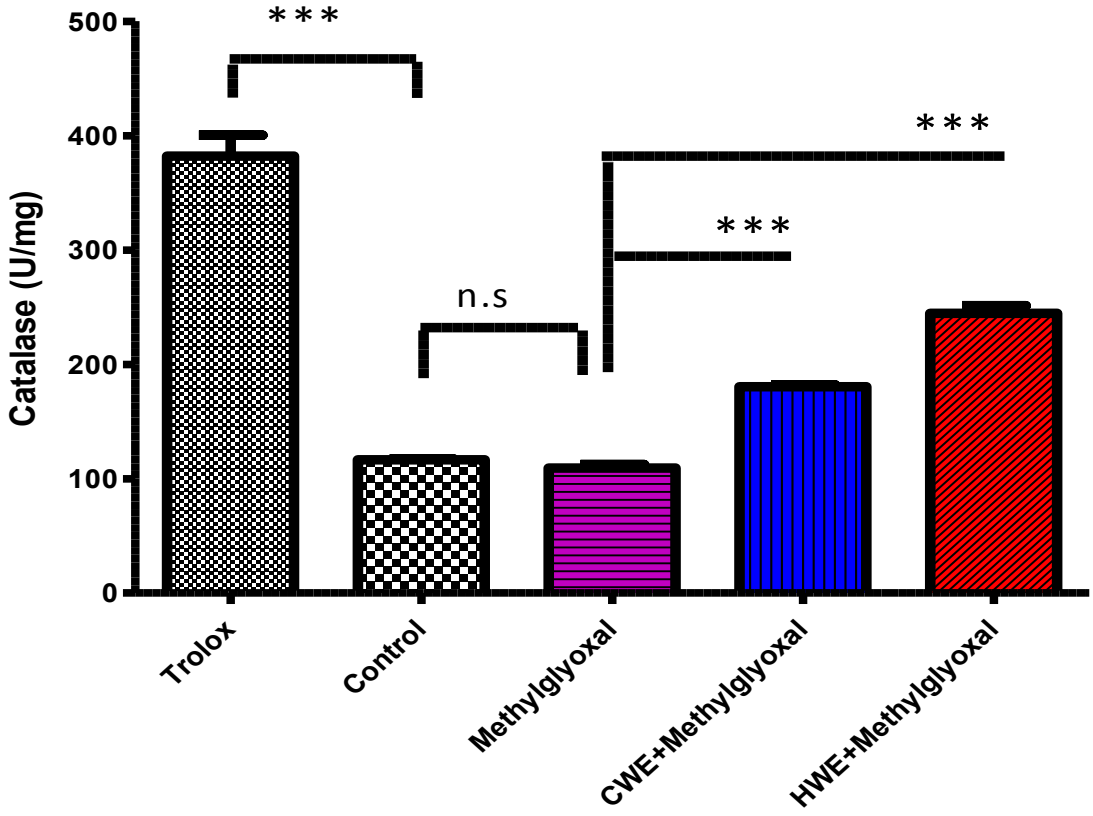


Figure 4.7: Effect of MC extracts on CAT activity in BAEC exposed to methylglyoxal. The graph illustrates the effect of 5 mM methylglyoxal alone or with 90 mg/ml of MC extracts in BAEC medium cells at 37 ºC for 24 hours. Trolox was used as standard antioxidant. Data are mean ± SD, n= 3. (n.s. = non- significant, ***$p < 0.001$).

4.4.9 Effects of *Momordica charantia* on CAT activity in BAEC exposed to AGEs

The results shown that Trolox induced a significant increase in CAT activity by 2.94 fold ($p = 0.0001$), compared to untreated control cells, whereas, CWE of MC induced a significant increase in CAT activity by 2.02 fold ($p = 0.0001$), and HWE of MC induced a significant increase in CAT activity by ($p = 0.0001$) compared to AGEs-treated cells. Moreover, AGEs-treated cells also induced a significant decrease in CAT activity by 1.44 fold ($p = 0.0002$) compared with untreated cells. HWE of MC possess a more potent CAT activity than the CWE of MC as shown in Figure 4.8

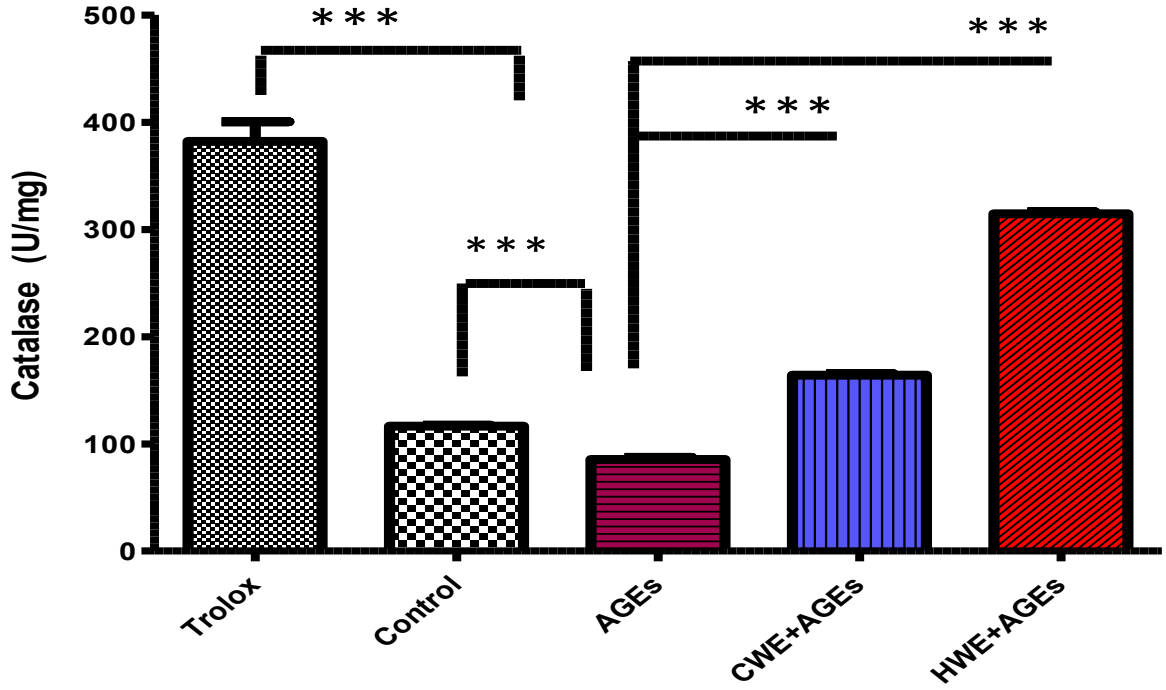


Figure 4.8: Effect of MC extracts on CAT activity in BAEC exposed to AGEs. The graph shows the effect of 10 µg/ml AGE alone or with 90 mg/ml of MC extracts in BAEC medium cells at 37 ºC for 24 hours. Trolox was used as standard antioxidant. Data are mean ± SD, n= 3, ***$p < 0.001$

4.4.10 Effects of *Momordica charantia* on glucose–induced oxidative stress in BAEC

The results show that Trolox did not increase ROS levels, compared to untreated control cells, whereas CWE of MC had no effect on ROS compared to glucose-treated cells. In contrast, the HWE of MC induced a significant decreased in ROS levels by 0.78 fold ($p = 0.0034$) compared to glucose-treated cells. Moreover, glucose-treated cells also induced a significant increase in ROS levels by 0.89 fold ($p = 0.0075$) compared to untreated cells as shown in Figure 4.9

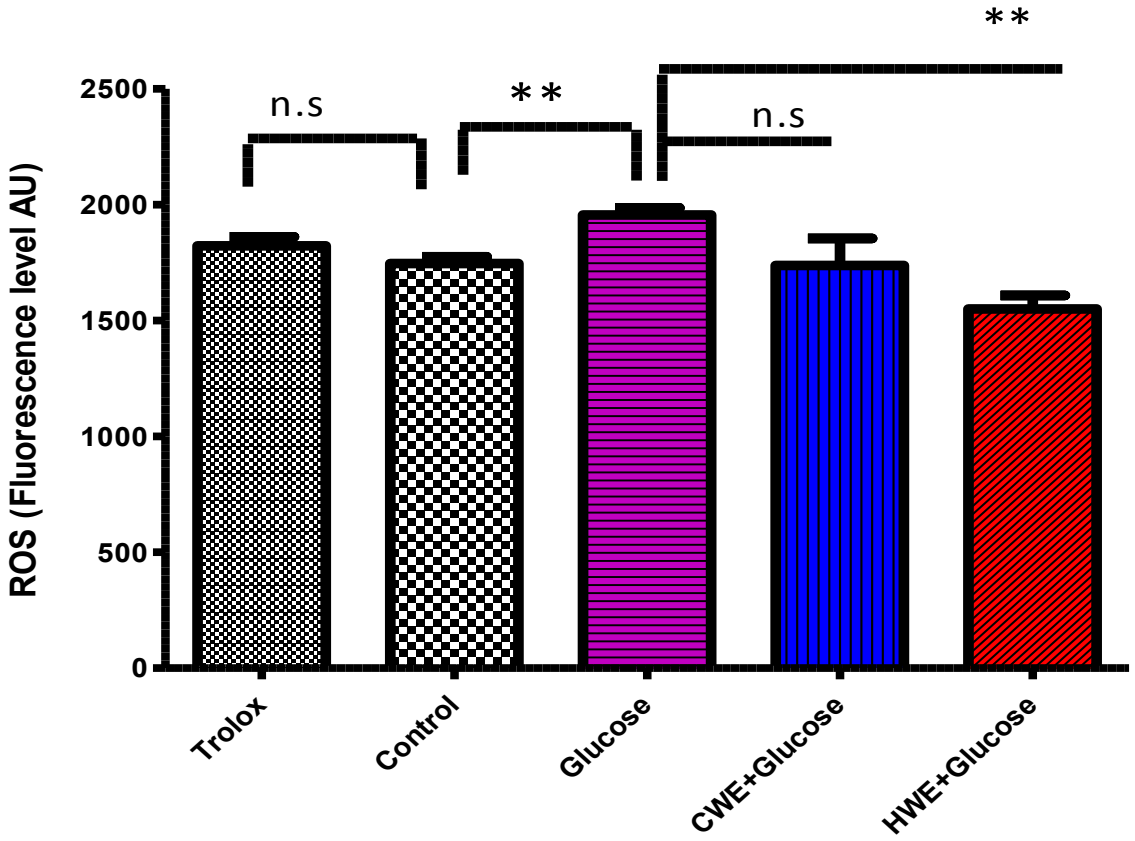


Figure 4.9 Effect of MC extracts on glucose-induced oxidative stress in BAEC. The graph shows the effect of 25 mM glucose alone or with 90 mg/ml of MC extracts in BAEC medium cells at 37 ºC for 24 hours. Trolox was used as standard antioxidant. Data are mean ± SD, n= 3, (n.s. = non-significant, $^{**}p < 0.001$).

4.4.11 Effects of *Momordica charantia* on methylglyoxal–induced oxidative stress in BAEC

The results show that Trolox did not increase ROS levels compared to untreated control cells, whereas, CWE and HWE of MC induced a significant decrease in ROS levels by 1.20 fold `($p = 0.0002$), 1.08 fold ($p = 0.0004$), respectively compared to methylglyoxal-treated cells. Moreover, methylglyoxal-treated cells also induced a significant increase in ROS levels 1.10 fold ($p = 0.0003$) compared to untreated cells as demonstrated in Figure 4.10

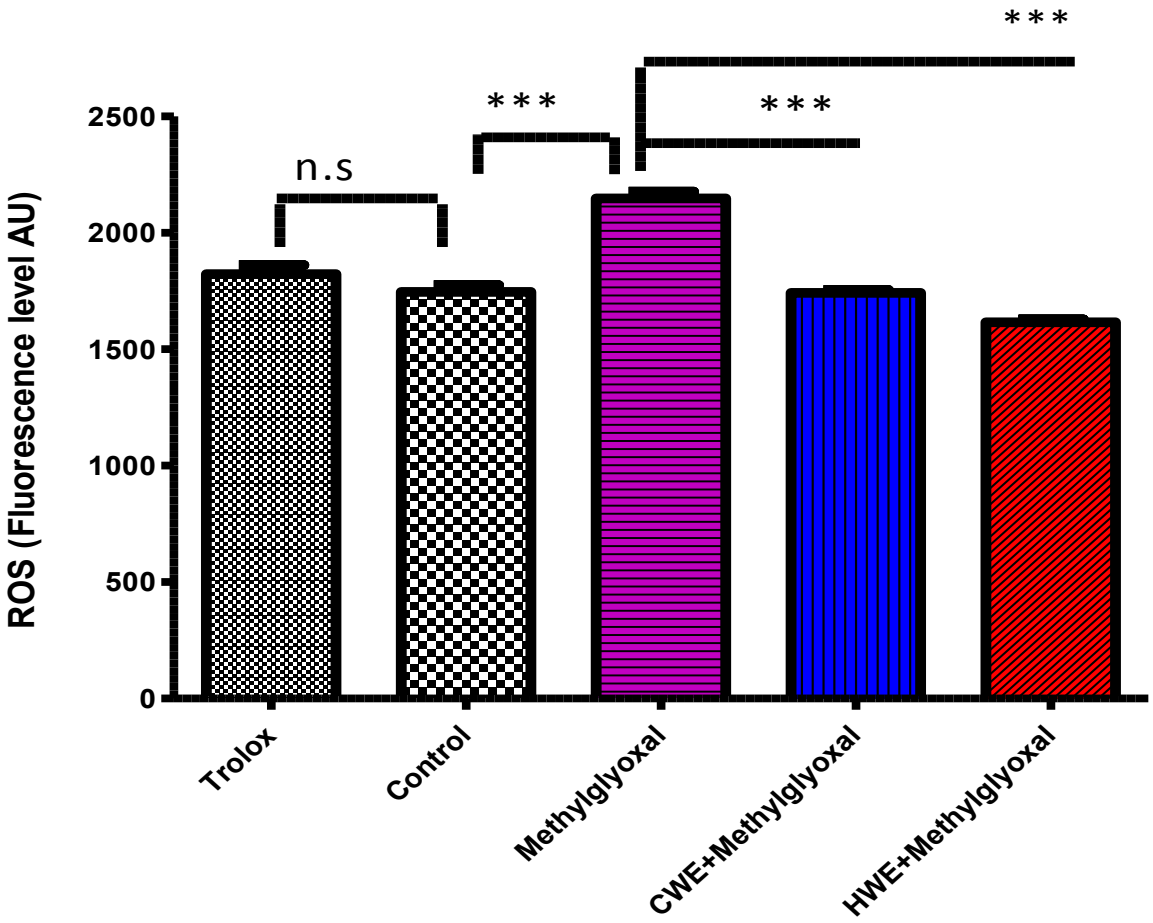


Figure 4.10 Effect of MC extracts on methylglyoxal-induced oxidative stress in BAEC. The graph shows the effect of 5 mM methylglyoxal alone or with 90 mg/ml of MC extracts in BAEC medium cells at 37 ºC for 24 hours. Trolox was used as standard antioxidant. Data are mean ± SD, n=3, n.s = non-significant, ***$p< 0.001$.

4.4.12 Effects of *Momordica charantia* on AGE−induced oxidative stress in BAEC

Figure 4.11 showed that Trolox did not increase ROS levels compared to untreated control cells, whereas CWE and HWE of MC induced a significant decrease in the oxidative stress level by 0.95 fold (p = 0.0001), 0.98 fold (p = 0.0001) compared to AGEs-treated cells. Moreover, AGEs-treated cells also induced a significant increase in ROS levels 1.38 fold (p = 0.0001) compared to untreated cells.

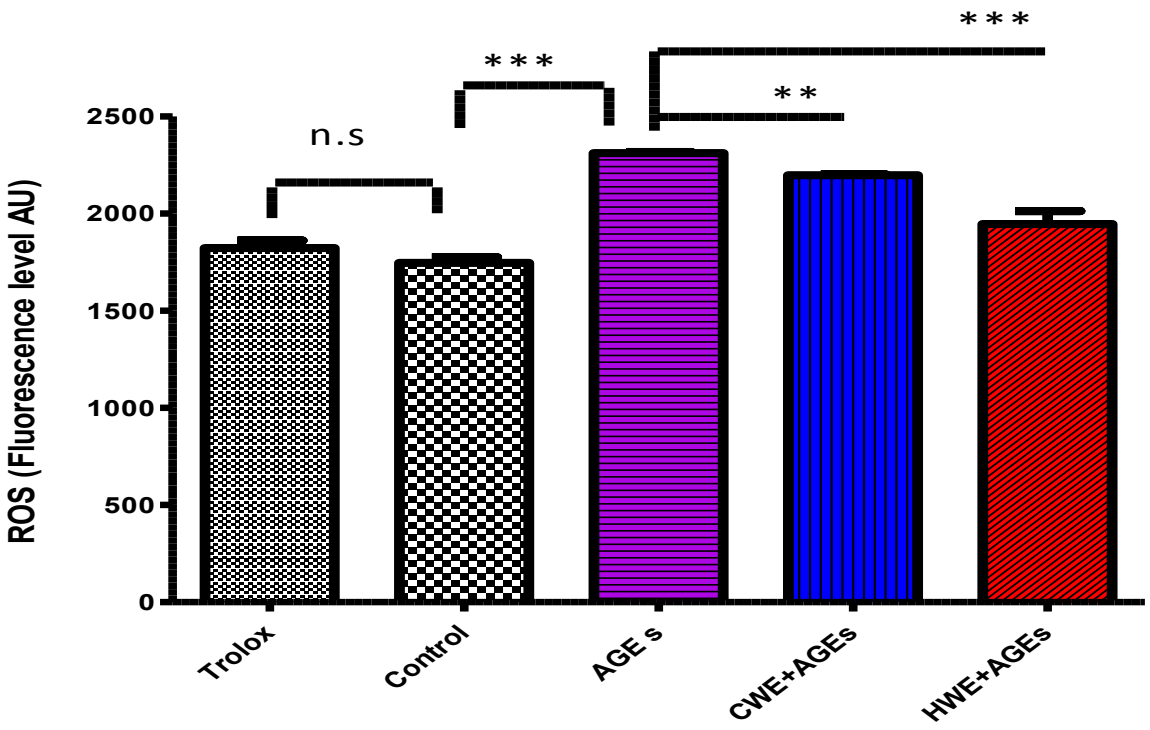


Figure 4.11 Effect of MC extracts on AGEs-induced oxidative stress in BAEC. The graph shows the effect of 10 µg/ml AGE alone or with 90mg/ml of MC extracts in BAEC medium cells at 37 ºC for 24 hours. Trolox was used as standard antioxidant. Data are mean ± SD, n= 3, n.s. = non-significant, $^{**}p< 0.01$ and $^{***}p< 0.001$.

4.5 Discussion

In past two decades, it has become obvious that nearly all age-related diseases such as diabetic mellitus are associated with reactive oxygen species (ROS) such as superoxide anion radicals, hydroxyl radicals and hydrogen peroxide (Zahin *et al*, 2013). Oxidative stress occurs from either elevated free radical generation, a reduction in endogenous antioxidant compounds, or both (Chen *et al*, 2011). Previous studies have indicated that the phenolic molecules which are found in plant materials have potent antioxidant properties (Dhar *et al*, 2012). Phenolic compounds are essential plant components with antioxidant abilities that contribute directly to their redox activities, which demonstrate their importance in scavenging free radical molecules (Wang & Ballington, 2007).

At present, the most frequently used assay for determining antioxidant properties is FRAP and CUPRAC. In FRAP assay, an important effect of all inhibitors is to donate an electron was examined, where an increase in blue colour of the reaction indicates a higher ferric reducing power (Zheng *et al*, 2008). The results obtained from this study indicate that HWE of MC reduces a ferricyanide complex to ferrous ions more effectively than the CWE of MC. This results matches with the similar study (Krishnaiah *et al*, 2011). Similarly, the CUPRAC reducing power assay demonstrated that HWE of MC reduces Cu^{2+} to Cu^{+} more strongly than the CWE of MC. The CUPRAC method that has been used in this study has shown responses in a dose-dependent manner to several antioxidant compounds such as vitamin C, flavonoids and phenolics (Apak *et al*, 2004). The CUPRAC findings correlated well with the results of FRAP, both being electron-transfer-based antioxidant capacity assays. It has been established that the reducing power it might be linked to the hydroxyl molecules in the aromatic rings of polyphenolic compounds, which were proven to apply antioxidant activities by donating hydrogen atom (Xing *et al*, 2005; Kubola & Siriamornpum, 2008). However, the reducing capacity of a compound may serve as an important indicator of its potential antioxidant activity.

Momordica charantia extracts were found to have higher total phenolic contents in HWE than the CWE. These differences might be due to the extraction of MC in hot water, this findings similar to the results was reported previously (Kenny *et al*, 2013) who reported that MC extract with small amount of phenolic compound represent low antioxidant activity. The HWE of MC was found to contain the highest total phenolic compound levels and to be a potent, active extract in all *in vitro* antioxidant assays tested. The strong antioxidant properties of the plant extract are correlated with the high level of phenolic molecules (Hwang *et al*, 2013).

Momordica charantia extracts were studied for their antioxidant effects by investigating antioxidant enzymes as well as its protective effects on ROS damage in BAECs exposure to AGEs-induced oxidative stress were evaluated. AGEs are well known complex molecules cause endothelial cell dysfunction through the generation of reactive free radical species (Tupe *et al*, 2014). Antioxidant prevents the formation of ROS and play an important role in to inactivate them. Antioxidant activity of medicinal plants may be through inhibitory actions on formation of ROS or by direct scavenging of free radical species. Several studies further indicate that the antioxidant enzymes and antioxidant molecules can prevent free radical generation by chelating ion metals, decreasing concentrations of ROS and scavenging free radicals (Rolo *et al*, 2006). SOD and CAT are amongst the major antioxidant enzymes combating oxidative stress through scavenging free radical species that have shown to be down regulated in a number of pathophysiological conditions and diseases like diabetic mellitus (Li *et al*, 2013).

In this study, we tested whether MC extracts could scavenger ROS and preserve the activities of antioxidant enzymes (SOD and CAT). The results of the present study show that the activities of SOD and CAT were higher and ROS was lower in BAECs treated with MC extracts. Furthermore, HWE of MC has better scavenger ROS and potent inducer SOD and CAT when compared with CWE of MC. These results suggest that MC extracts may attenuate oxidative stress by reducing ROS and increasing antioxidant enzyme activities *in vitro*. This finding was confirmed by a previous study which showed that *in vitro* and *in vivo* treatment with MC extracts enhances antioxidant enzymes such as SOD and CAT activities and lowers the concentration of free radical species (Semiz & Sen, 2007; Gong *et al*, 2015). It is more likely that a related synergistic mechanism may act against oxidative stress and carbonyl-promoted cytotoxicity in *in vitro* models (Fenercioglu *et al*, 2010). BAECs were challenged by different oxidants including AGE, methylglyoxal and glucose. The results showed that HWE of MC potent than CWE of MC as compared to the control cells in all these assays. Hence, the activation of these antioxidant enzymes by the administration of MC extracts clearly demonstrates that MC extracts possess free radical scavenging property, which could provide beneficial effect against structural and functional alterations of cell components caused by the presence of free radical species such as superoxide and hydroxide radicals.

These findings clearly show that the antioxidant activity of MC extracts in BAEC exposed to different oxidants-induced oxidative stress. In this study, we have shown for first time that HWE of MC contains high amount of phenolic compounds than the CWE of MC, which is a

promising source of antioxidant compounds that may be responsible for its antioxidant activity. In this context, further studies are required to isolate the active principles, as well as to define the role of several interacting active compounds in influencing therapeutic potential and efficacy *in vivo*. In addition, the present study supports the antioxidant potency of the plant extracts as proved by elevated level of these antioxidant enzymes in extract treated BAECs.

Chapter 5. Inhibitory effect of *Momordica charantia* extracts on oxidative stress signalling pathways in BAEC.

5.1 Introduction

Oxidative stress occurs as a result of an increased production of ROS, and it is associated with a variety of chronic diseases including diabetes mellitus. Increased amounts of ROS are associated with endothelial cell death and apoptosis and. It is well established that oxidative stress increases the expression of certain genes in varieties of cells such as endothelial cells and smooth muscle cells (Chaturvedi *et al*, 2010). ROSs are produced from a variety of sources such as the mitochondrial transport system, xanthine oxidase, cytochrome p450, NADPH oxidase (NOX), uncoupled NO synthase (NOS), and the NOX family are the main sources of ROS (oxidant signalling) in endothelial cells (Frey *et al*, 2009). The NOXs family consists of seven members (NOX1-5 and DUOX1 and 2) which differ in location and mechanism of activation. NOX converts molecular oxygen ($O_{2)}$ to the superoxide anion ($O_2^{\bullet-}$), SOD then converts these products to hydrogen peroxide (H_2O_2), or to more reactive species such the hydroxyl radical ($OH^{\bullet-}$). Afterwards, they are converted to water (H_2O) by CAT and GPx (Pinnell, 2003).

A previous study has reported that direct exposure of endothelial cells to hyperglycemic concentration of reducing sugar such as glucose elevates the generation of ROS, thus activation of NADPH oxidase enzyme is directly associated in this process. AGEs exert their cellular effects by binding to particular receptors named RAGE. RAGE-mediated induction of cellular oxidant stress triggers a cascade of intercellular signals involving $p21_{ras}$ (Lander *et al*, 1997). Interacting AGEs with RAGE generates cellular signals that activate NFkB, which leads to the transcription of diverse pro-oxidative stress genes (Stefanie *et al*, 2012). Moreover, ROS can be generated from activation of the growth-promoting enzyme Janus kinase 2 (JAK2) and its latent signal transducers and activators of transcription (STAT). Antioxidant compounds including enzymes and non-enzymes act as a free radical scavenging system and play a key role in first-line defence against ROS. In this regards, the most important action of a cell defence system is to counter oxidative stress is the induction of new gene expression in response to specific oxidative compounds. Some of the genes induced can be antioxidant enzymes such as SOD and CAT or pro-oxidative stress genes such p21(Simon *et al*, 1998). SOD and CAT are among antioxidant enzymes generate inside the human body to counter the

effects of ROS. Moreover, antioxidant can be obtain from external sources like medicinal plants (Snee *et al*, 2011). MC extracts contains natural antioxidants which can provide considerable activity against oxidative stress (Semiz & Sen, 2007).

In this study we investigated the hypothesis that MC extracts have inhibitory effects on the activation of NADPH oxidase (NOX4, NOX5), p-JAK2, total JAK2, RAGE and P21 by AGE-RAGE interaction. This interaction might attribute, at least in part, to formation of ROS and creating a series signalling pathways, which leading to altered gene expression in endothelial cells (Wautier *et al*, 2001). Excessive generation of ROS causes oxidative stress that contribute to protein oxidation, and subsequent dysfunction (Ulrich & Cerami, 2001). Hence, identifying the inhibitory effects of MC extracts on AGE-induced oxidative stress in BAECs *in vitro* is an essential step in investigating the vascular protective effect of MC extracts in diabetic vascular disease.

5.2 Aim and objectives

The aim of this work was to examine the effects of MC extracts on the oxidative stress signalling pathways in BAECs treated with AGEs *in vitro*.

The objectives were:

- To investigate the effect of different concentrations of MC extracts and AGEs on BAEC proliferation *in vitro*.

- To determine the cytotoxic effects of AGEs, methylglyoxal, glucose alone or in combination with MC extracts on BAECs *in vitro*.

- To investigate whether MC extracts can enhance antioxidant enzymes and inhibit oxidative stress expression genes in endothelial cells exposed to AGEs.

- To examine the inhibitory effects of MC extracts on pro-oxidative stress proteins in endothelial cells exposed to AGEs.

5.2 Methods

BAECs were used to determine the effect of MC extracts in the presence or absence of BSA-AGEs on cell proliferation (2.4.20). Cell viability was examined as explained in section (2.4.21). The effects of MC extract on the oxidative stress gene expressions were investigated by qRT-PCR as mentioned in section (2.4.27). Furthermore, the effects of MC extract on AGE-induced oxidative stress signalling pathways were investigated by western blotting as described in section (2.4.22)

5.3 Results

5.3.1 Effects of *Momordica charantia* extracts, glucose, methylglyoxal and BSA-AGEs on BAEC proliferation

Figures 5.1 -5.4 demonstrate the effects of different concentrations of MC extracts on BAEC proliferation after treatment for 24 hours. To check the cell response, FGF-2 was used as a positive control; and 25 ng/ml of FGF-2 induced a significant increase in the cell number by 2.14-fold (p = 0.0002), compared to control cells. The addition of increasing concentrations (15, 30, 60, and 90 μg/ml) of MC extracts significantly improved BAEC proliferation in a dose-dependent manner, with the greatest stimulation at 90 μg/ml (Figures 5.1 and 5.2). The 15, 30, 60, and 90 μg/ml of HWE of MC elevated (p< 0.0001) significantly BAEC proliferation by 2.11 fold, 2.20 fold, 2.45 fold and 2.56-fold respectively, compared to untreated control cells (Figure 5.1). At the same concentrations of CWE also significantly (p< 0.0001) increased BAEC proliferation by 2.04 fold 2.16 fold 2.41 fold and 2.48 fold respectively, compared to control cells as shown in Figure 5.2

Figure 5.3 shows exposure of BAECs to 100, 200, 600 μg/ml of HWE of MC for 24 hours resulted in a significant increase of 1.73-fold (p = 0.0002), 1.64-fold (p = 0.0006), 0.43- fold (p = 0.0013), 1.20- fold (p = 0.04) in BAEC proliferation compared with the control. However, 800 and 1000 μg/ml HWE of MC significantly inhibited BAEC proliferation by 0.71-fold (p< 0.02), 0.48 (p< 0.02) compared with the control.

Figure 5.4 shows exposure of BAECs to 100, 200, 400 μg/ml CWE of MC for 24 hours resulted in a significant increase of 1.65-fold (p = 0.0004), 1.54-fold (p= 0.0006), 0.43- fold (p = 0.0013) in BAEC proliferation compared with the control. 600 and 800 μg/ml CWE of MC resulted in non-significant change in BAEC proliferation compared to the control Whereas, 1000 μg/ml CWE of MC significantly inhibited BAEC proliferation by 0.47-fold (p = 0.0016) compared with the control cells.

Figure 5.5 shows at a concentration of 10.1 mM, glucose had no effect on BAEC proliferation compared with the control cells. Whereas, at 30.1, 55.1 and 105.1 mM, glucose significantly (p< 0.0001) inhibited BAEC proliferation compared to the control (5.1 mM). The inhibitory effects on BAEC proliferation by glucose was dose-dependent. Similarly, methylglyoxal at concentrations of 1, 2, 4 and 5 mM, demonstrated significant (p< 0.001) decreasing effects in BAEC proliferation compared to the control (Figure 5.6). This inhibition occurred in a dose-

dependent manner. Moreover, BSA-AGEs at concentrations of 10, 50, 75 and 100 µg/ml showed significant (p< 0.001) inhibition of BAEC proliferation compared to the control cells. This inhibition occurred in a concentration–dependent manner as illustrated in Figure 5.7

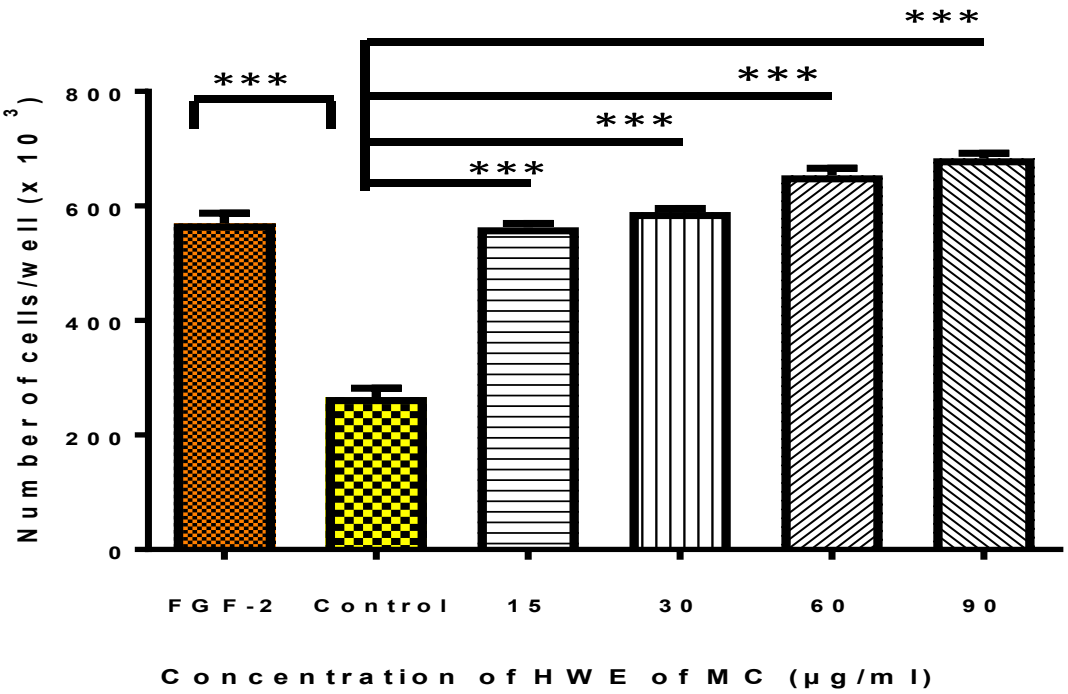


Figure 5.1: Effect of different concentrations (15 to 90 µg/ml) of HWE of MC on BAEC proliferation. Bovine aortic endothelial cells (2 x 10^5 cells/ml) were seeded in 48-well plates and incubated with different concentrations of HWE of MC for 24 hours. Fibroblast growth factor 2 (25 ng/ml) was used as a positive control. Control is cells cultured under the same condition without any treatment. Results are presented as mean ± SD (n=3). *** p < 0.0001.

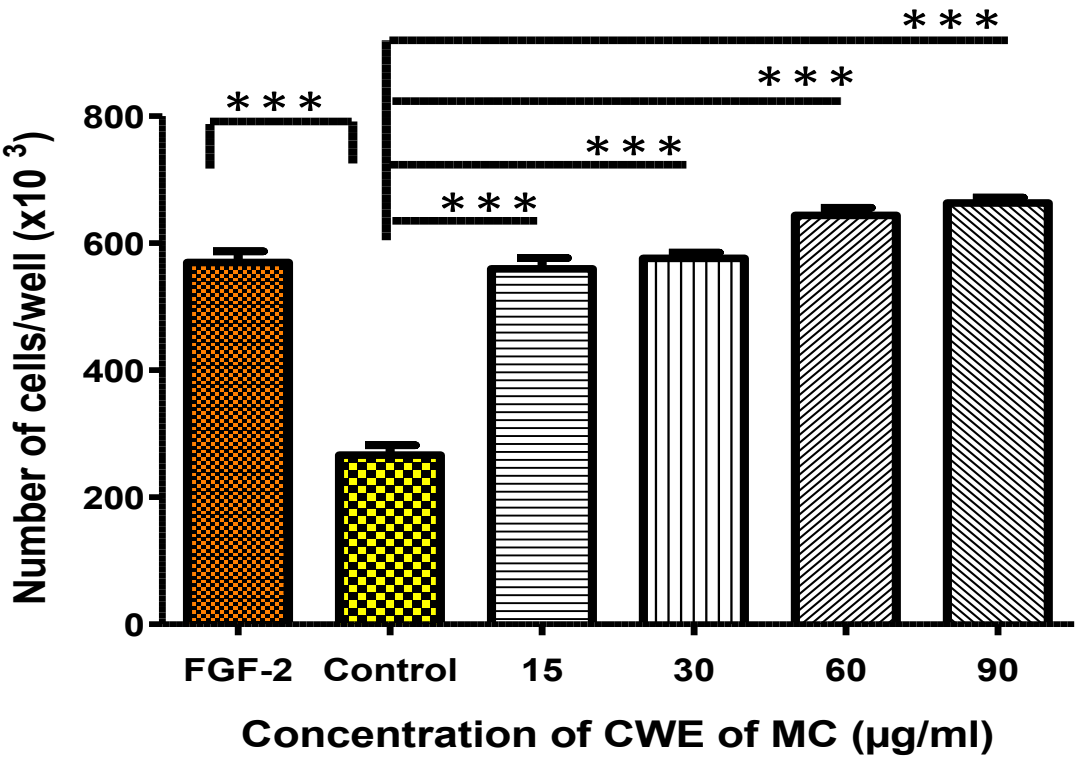


Figure 5.2: Effect of different concentrations (15 to 90 µg/ml) of CWE of MC on BAEC proliferation. Bovine aortic endothelial cells (2 x 10^5 cells/ml) were seeded in 48-well plates and incubated with different concentrations of CWE of MC for 24 hours. Fibroblast growth factor 2 (25 ng/ml) was used as a positive control. Control is cells cultured under the same condition without any treatment. Results are presented as mean ± SD (n=3). ***$p < 0.0001$.

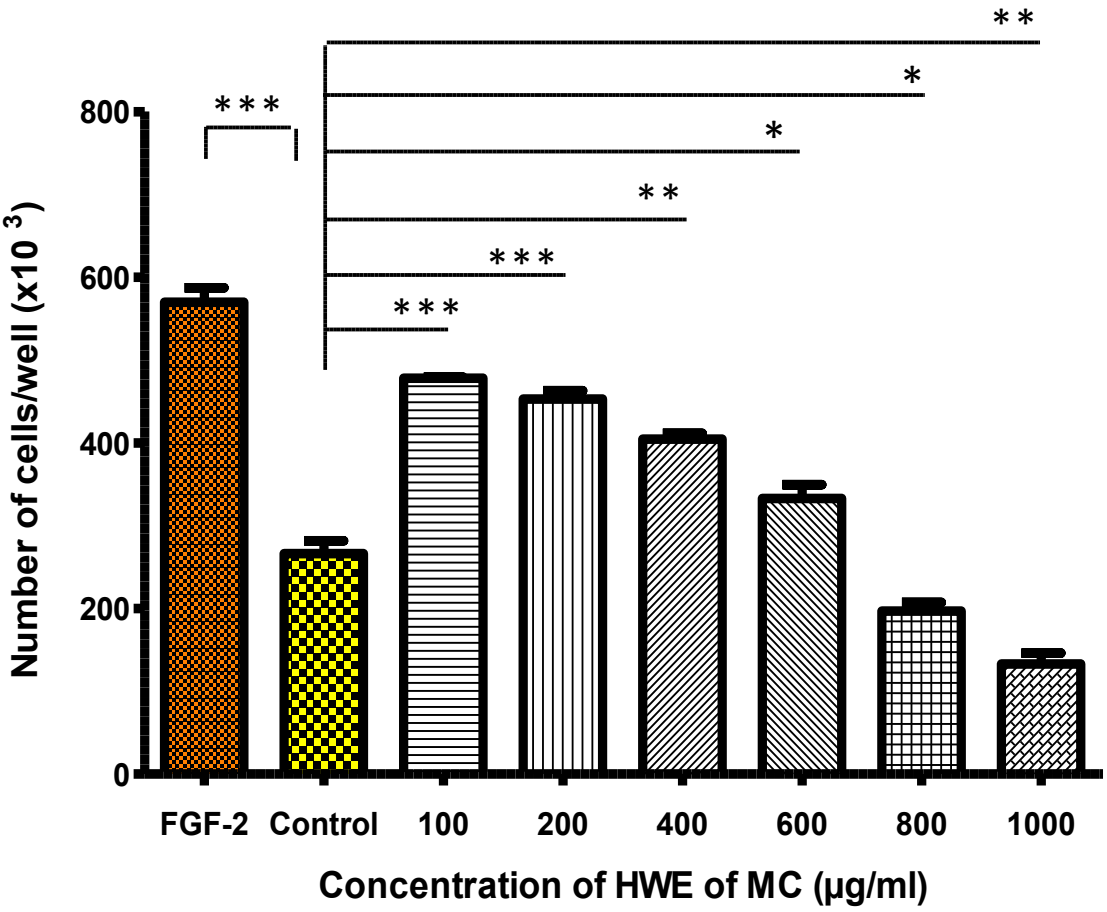


Figure 5.3: Effect of higher concentrations (100 to 1000 µg/ml) of HWE of MC on BAEC proliferation. Bovine aortic endothelial cells ($2x10^5$ cells/well) were seeded in 48-well plates and incubated with different concentrations of HWE of MC for 24 hours. Fibroblast growth factor 2 (FGF-2) (25 ng/ml) was used as a positive control. Control is cells cultured under the same condition without any treatment. Results are represented as mean ± SD (n=3). Where n.s. = non-significant, * $p < 0.05$ ** $p < 0.001$ and ***$p < 0.0001$.

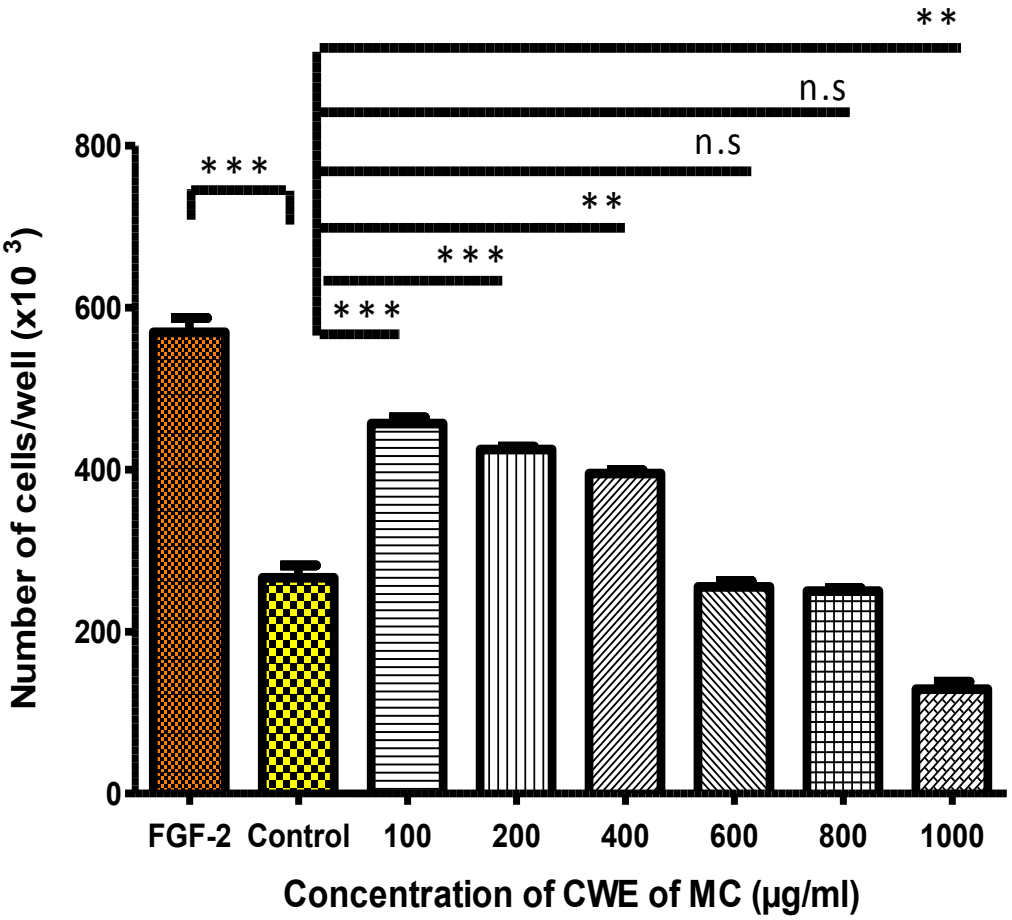


Figure 5.4: Effect of higher concentrations (100 to 1000 µg/ml) of CWE of MC on BAEC proliferation. Bovine aortic endothelial cells ($2x10^5$cells/well) were seeded in 48-well plates and incubated with different concentrations of CWE of MC for 24 hours. Fibroblast growth factor-2 (25 ng/ml) was used as a positive control. Control is cells cultured under the same condition without any treatment. Results are represented as mean ± SD (n=3). Where n.s. = non-significant, ** $p < 0.001$ and ***$p < 0.0001$.

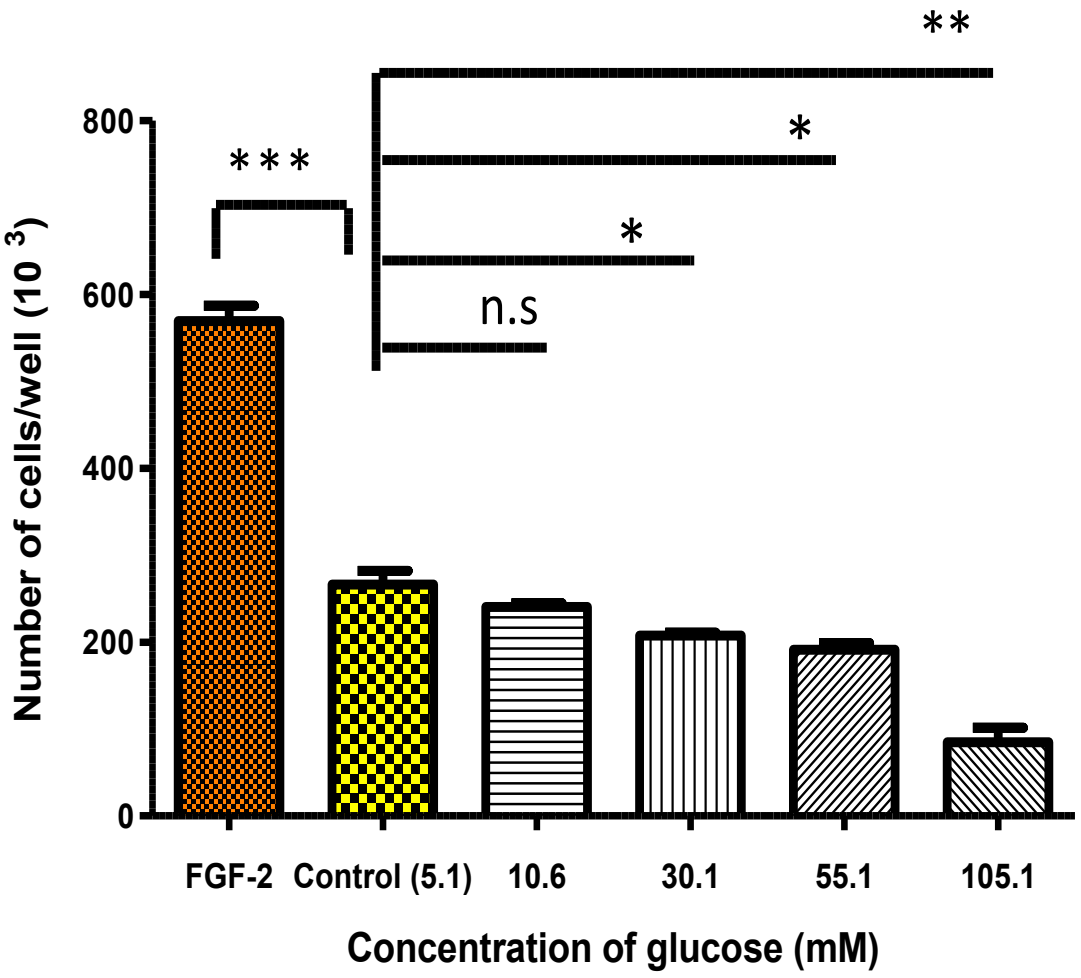


Figure 5.5: Effect of different concentrations of glucose on BAEC proliferation. Bovine aortic endothelial cells (2 x 10^5 cells/ml) were seeded in 48-well plates and incubated with different concentrations of glucose for 24 hours. Fibroblast growth factor-2 (25 ng/ml) was used as a positive control. Control is cells cultured under the same condition without any treatment. Results are presented as mean ± SD (n=3). n.s = non-significant, *$p < 0.05$ and ** $p < 0.001$ and *** $p < 0.0001$.

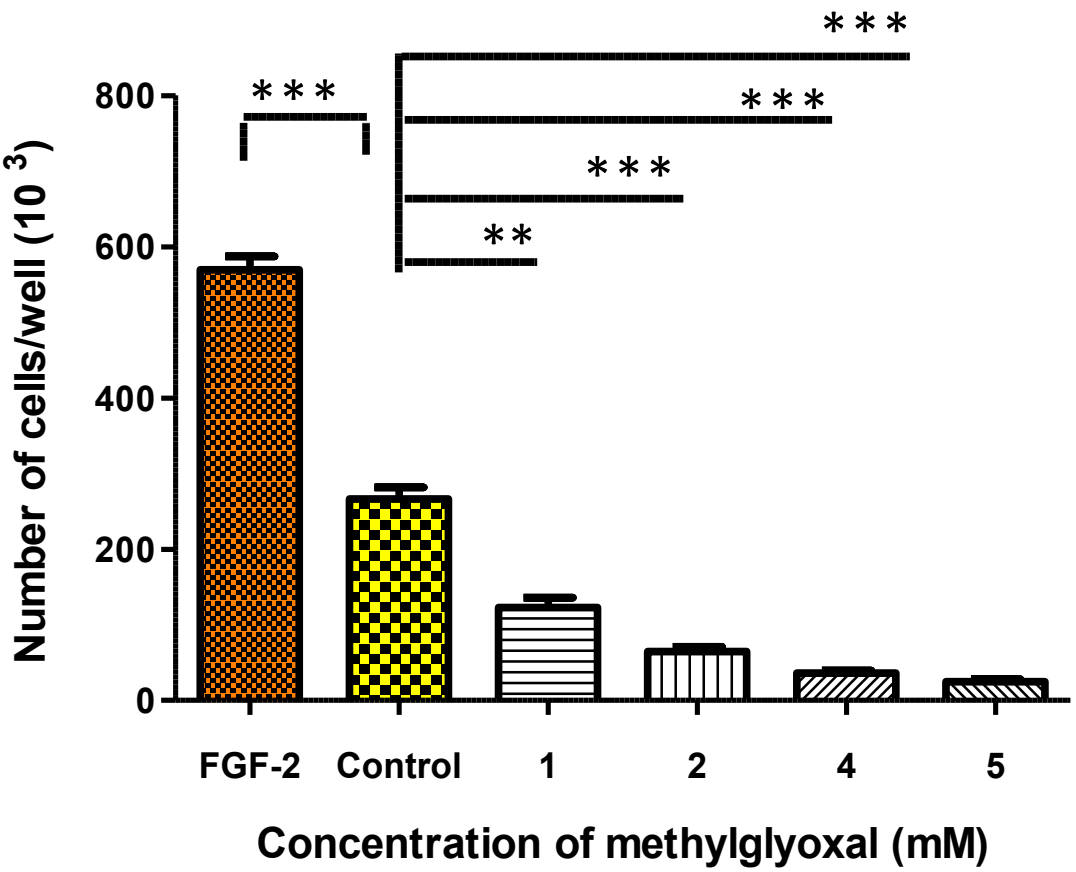


Figure 5.6: Effect of different concentrations of methylglyoxal on BAEC proliferation. Bovine aortic endothelial cells (2 x 10^5 cells/ml) were seeded in 48-well plates and incubated with different concentrations of methylglyoxal for 24 hours. Fibroblast growth factor-2 (25 ng/ml) was used as a positive control. Control is cells cultured under the same condition without any treatment. Results are presented as mean ± SD (n=3). ** $p < 0.001$, *** $p < 0.0001$.

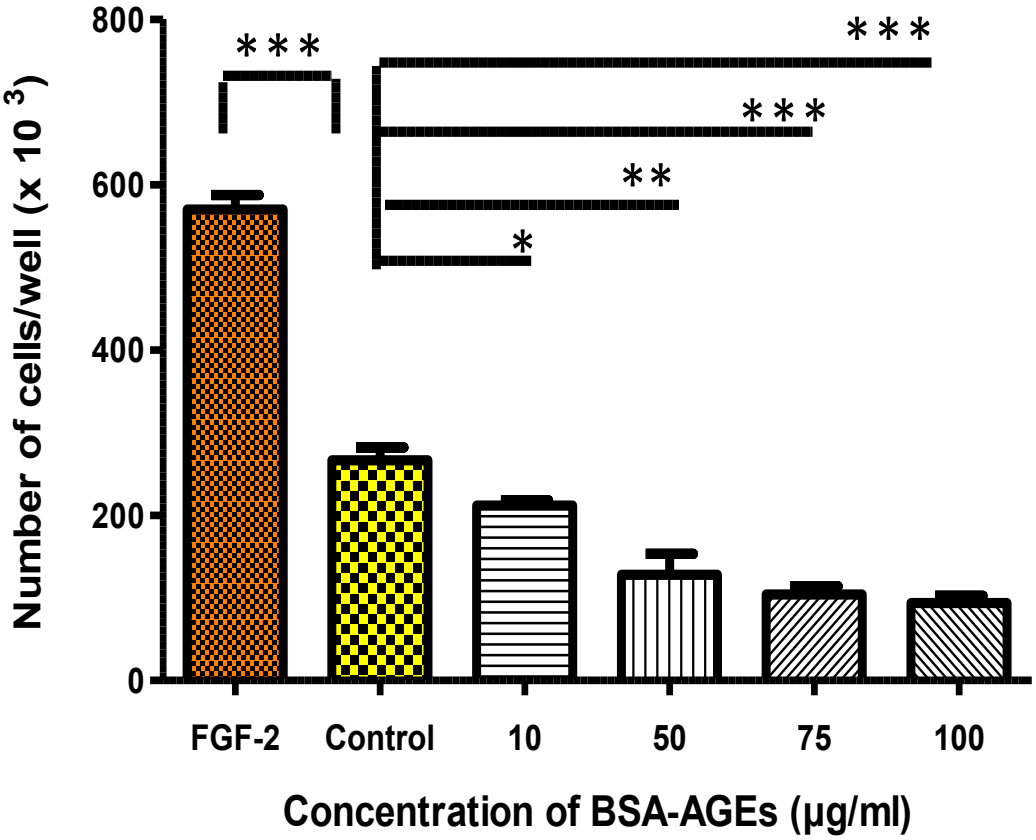


Figure 5.7: Effect of different concentrations of BSA-AGEs on BAEC proliferation. Bovine aortic endothelial cells (2 x 10^5 cells/ml) were seeded in 48-well plates and incubated with different concentrations of BSA-AGEs for 24 hours. Fibroblast growth factor-2 (25 ng/ml) was used as a positive control. Control is cells cultured under the same condition without any treatment. Results are presented as mean ± SD (n=3). * $p < 0.05$, ** $p < 0.001$ and *** $p < 0.0001$.

5.3.2 Effects of *Momordica charantia* extracts on glucose, methylglyoxal and AGEs-induced inhibition of endothelial cell proliferation

Figure 5.8 shows that Glucose (30.1 mM) inhibited cell proliferation by 0.61 fold (p = 0.0139) when compared to untreated control cells (5.1mM). The addition of CWE of MC, reduced glucose's inhibitory effect on the proliferation of BAEC by 1.11 fold (p = 0.013) and HWE did so by 1.32-fold (p = 0.0052). Similarly, MC extract reduced methylglyoxal's (1 mM) inhibitory effect on the proliferation of BAEC by 1.24-fold (p = 0.0001) with the addition of CWE, and by 1.54-fold (p = 0.0004) with the addition of HWE (Figure 5.9). At 10 µg/ml, BSA-glucose-AGEs inhibited cell proliferation by 0.48-fold (p = 0.0038) when compared to the control cells. The addition of MC extract reduced BSA-AGEs inhibitory effect on the proliferation of BAEC by 1.69-fold (p = 0.0001) with CWE and by 1.86-fold (p = 0.0004) with the addition of HWE as shown in Figure 5.10

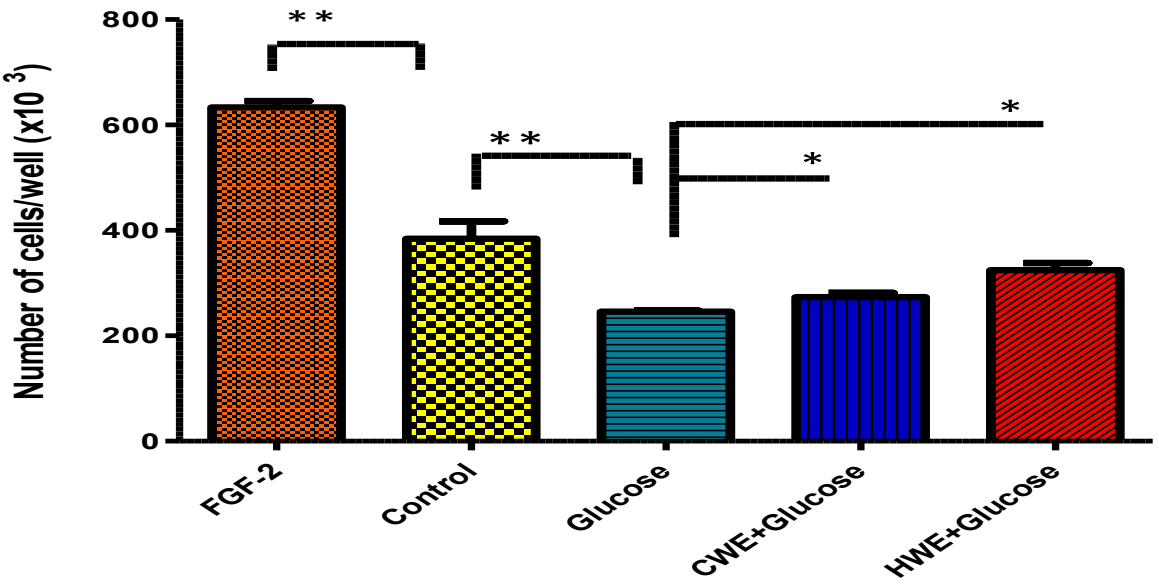


Figure 5.8: Effect of MC extracts on glucose-induced inhibition of endothelial cell proliferation. Bovine aortic endothelial (2 x 10^5 cells/ml) were cultured in 48-well plates and incubated with glucose 30.1 mM alone or in presences of 90 µg/ml of HWE or CWE of MC respectively for 24 hours. FGF-2 (25 ng/ml) was used as a positive control. Control is cells cultured under the same condition without any treatment. Results are presented as mean ± SD (n=3). * $p < 0.05$ and ** $p < 0.001$.

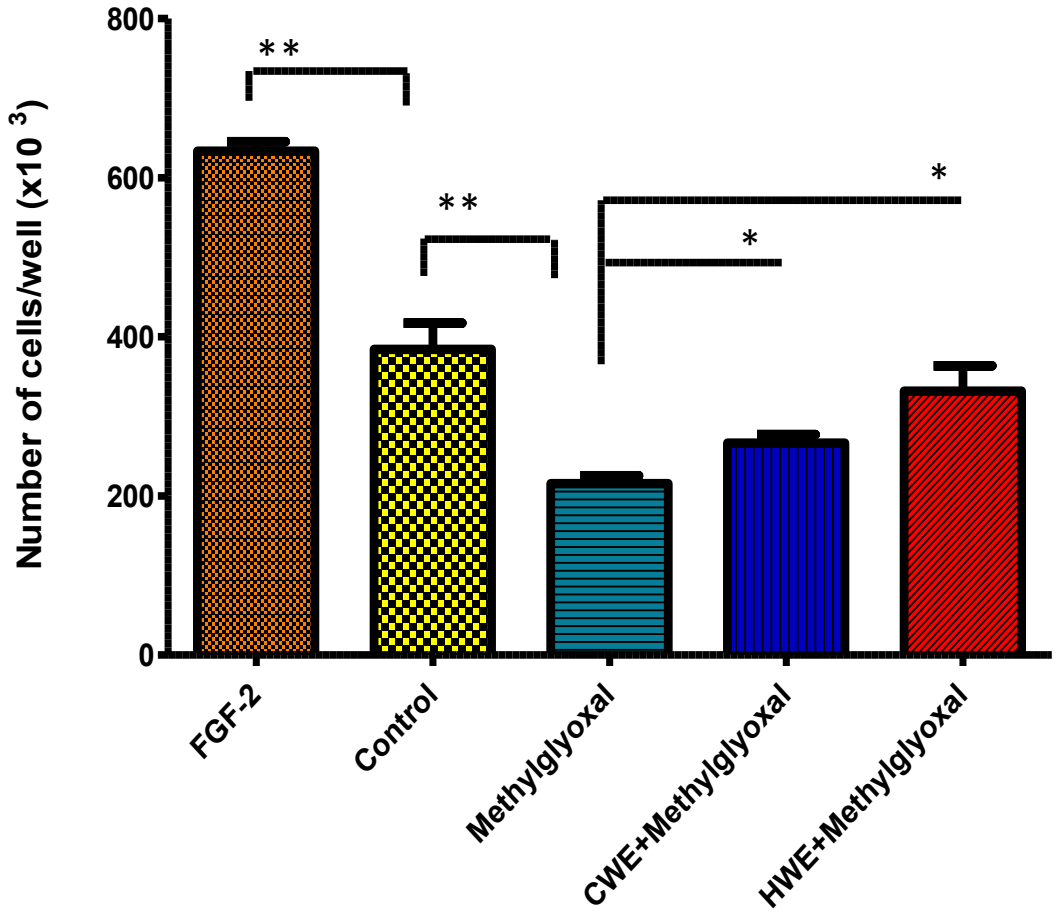


Figure 5.9: Effect of MC extracts on methylglyoxal-induced inhibition of endothelial cell proliferation. Bovine aortic endothelial cells (2 x 10^5 cells/ml) were cultured in 48-well plates and incubated with methylglyoxal 1 mM alone or in presences of 90 µg/ml of HWE or CWE of MC respectively for 24 hours. FGF-2 (25 ng/ml) was used as a positive control. Control is cells cultured under the same condition without any treatment. Results are presented as mean ± SD (n=3). * $p < 0.05$ and ** $p < 0.001$.

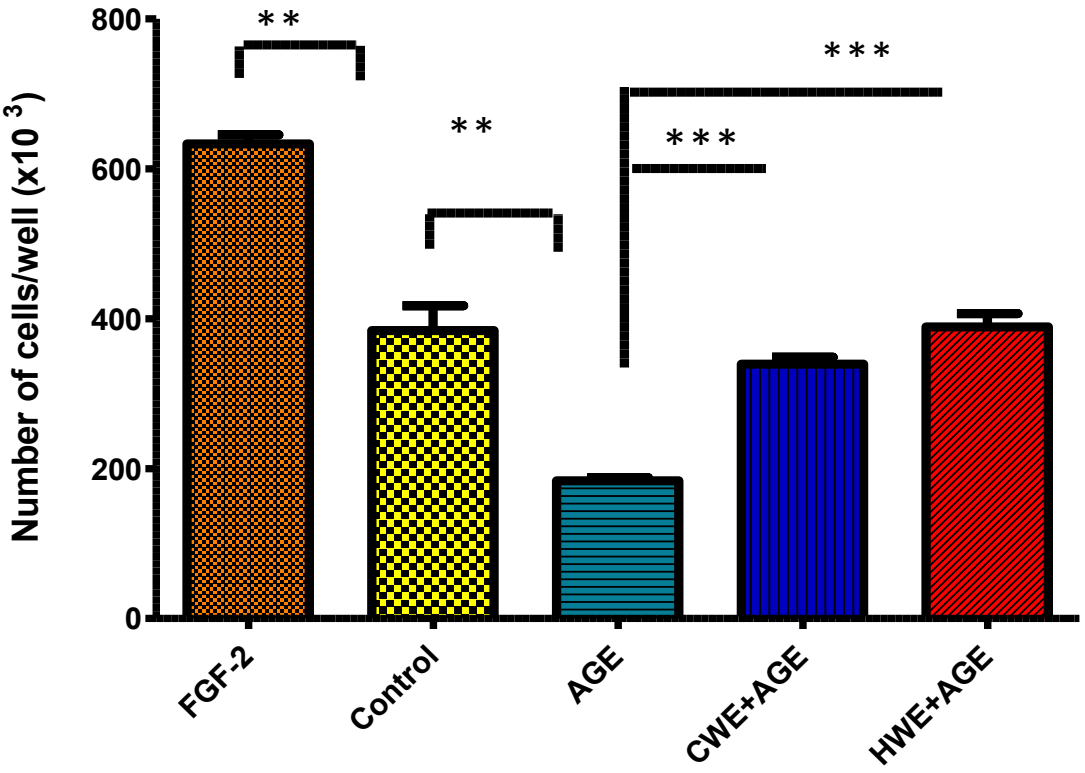


Figure 5.10: Effect of MC extracts on BSA-glucose-AGEs-induced inhibition of endothelial cell proliferation. Bovine aortic endothelial cells (2 x 10^5 cells/ml) were cultured in 48-well plates and incubated with 10 µg/ml BSA-glucose-AGEs alone or in presences of 90 µg/ml of HWE or CWE of MC respectively for 24 hours. FGF-2 (25 ng/ml) was used as a positive control. Control is cells cultured under the same condition without any treatment. Results are presented as mean ± SD (n=3). ** $p < 0.01$ and *** $p < 0.001$.

5.3.3 Effects of *Momordica charantia* extracts, glucose, methylglyoxal, BSA-AGEs on BAEC cell viability

Figures 5.11 and 5.12 show that HWE and CWE of MC at concentrations of 15 to 90 µg/ml had no cytotoxic effects on the viability of BAEC, compared to the control. Glucose at 10.1 mM had no cytotoxic effects on BAEC cell, whereas at 30.1, 55.1 and 105.1 mM, it significantly reduced BAEC viability by 7 %. 26 %, and 28 % , respectively, compared with untreated control cells as demonstrated in Figure 5.13. Figure 5.14 shows the BAEC viabilities were significantly reduced by 8 %, 17 %, 22.3 % and 23 % , following the treatment of 1, 2, 4 and 5 mM methylglyoxal for 24 hours respectively, compared to the control. At the concentrations of 10, 50, 75 and 100 ug/ml, BSA-AGEs slightly reduced BAEC viabilities by 6 %, 7.5 %, 10 % and 21 % , respectively, compared with untreated control cells. This inhibition was dose-dependent as shown in Figure 5.15

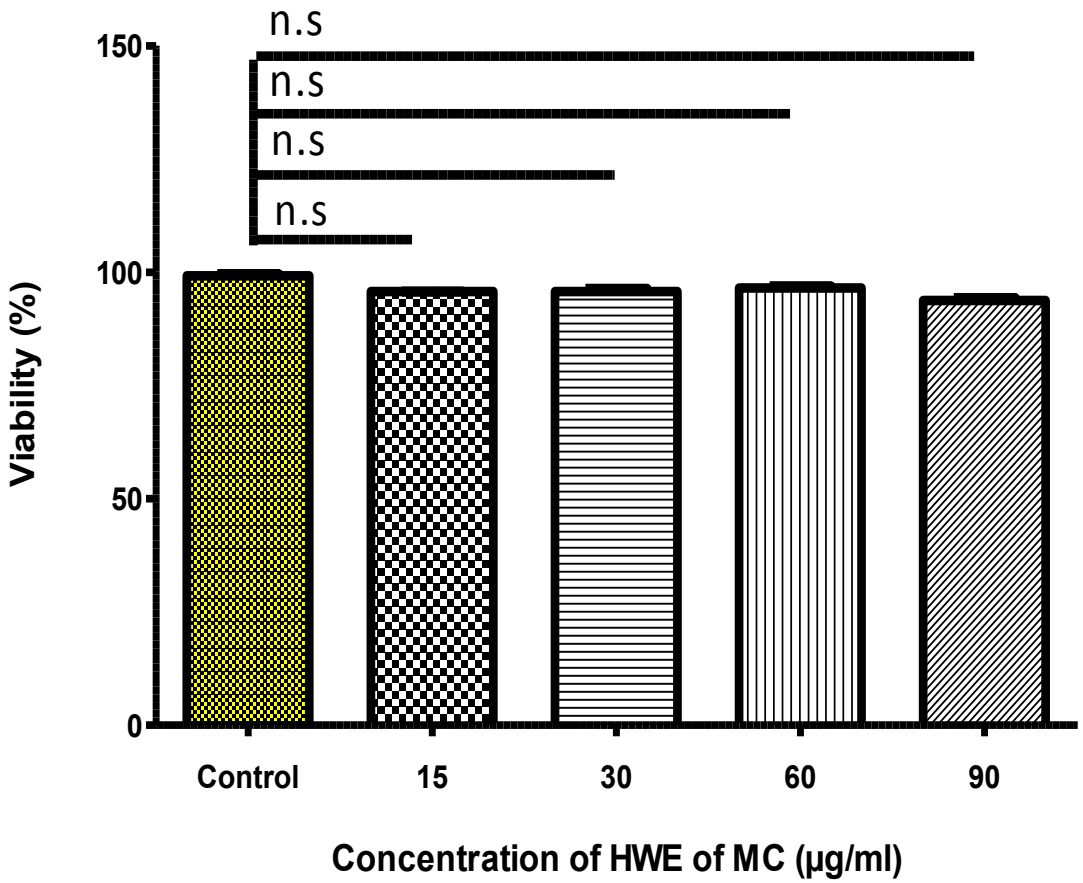


Figure 5.11: Effect of HWE of MC on BAEC viability. Bovine aortic endothelial cells (2 x 10^5 cells/ml) were seeded in 48-well plates and incubated with different concentrations of HWE of MC extracts for 24 hours. Results are expressed as mean ± SD (n = 3) and n.s = non-significant.

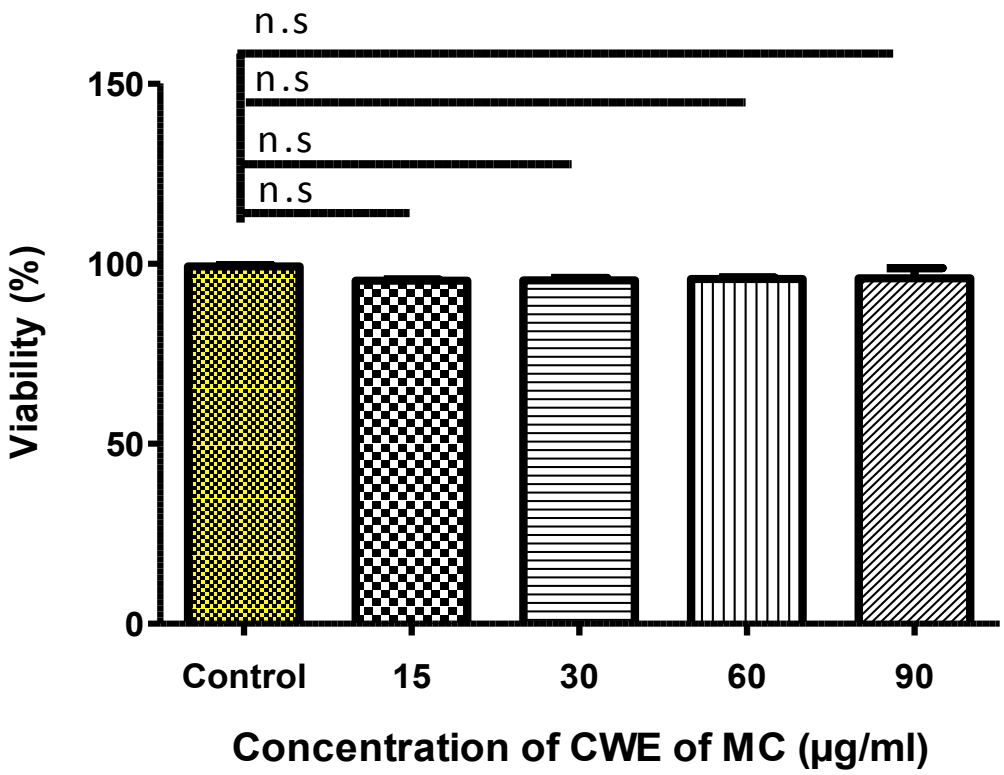


Figure 5.12: Effect of CWE of MC extracts on BAEC viability. Bovine aortic endothelial cells (2 x 10^5 cell/well) were seeded in 48-well plates and incubated with different concentrations of CWE of MC extracts after 24 hours. Results are expressed as mean ± SD (n = 3) and n.s = non-significant.

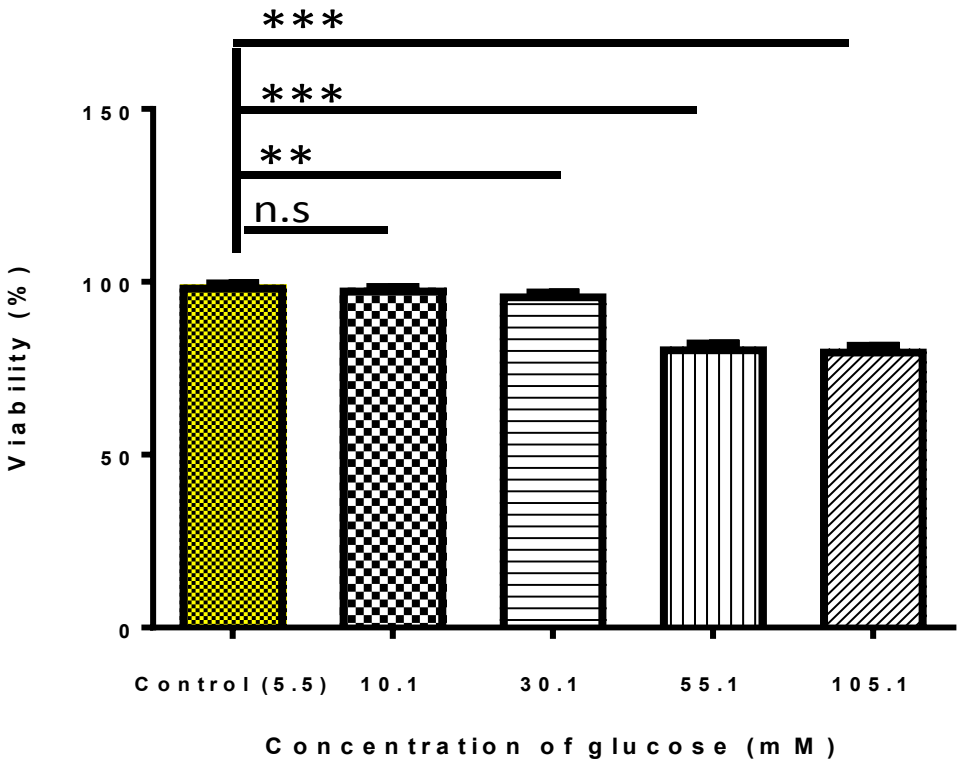


Figure 5.13: Effect of glucose on BAEC viability. Bovine aortic endothelial cells (2 x 10^5 cells/ml) were seeded in 48-well plates and incubated with different concentrations of glucose after 24 hours. Results are expressed as mean ± SD (n = 3). ** $p < 0.01$, *** $p < 0.001$ and n.s = non-significant.

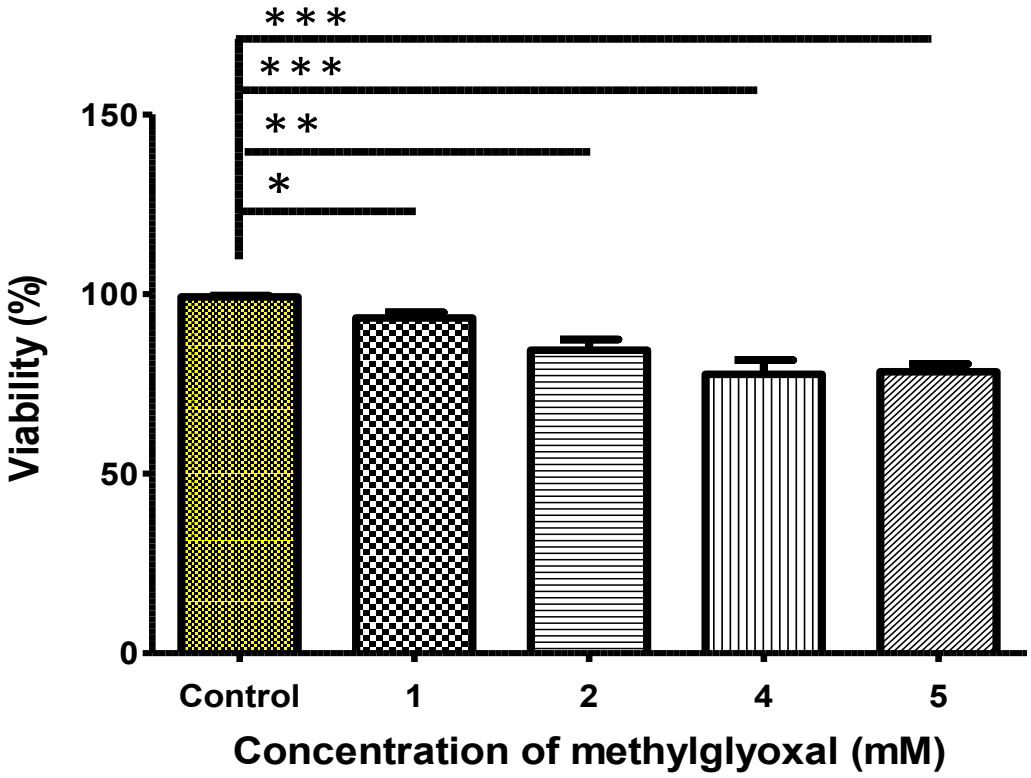


Figure 5.14: Effect of methylglyoxal on BAEC viability. Bovine aortic endothelial cells (2 x 10^5 cells/ml) were seeded in 48-well plates and incubated with different concentrations of methylglyoxal for 24 hours. Results are expressed as mean ± SD (n = 3). * $p < 0.05$ **, $p < 0.01$ and *** $p < 0.001$.

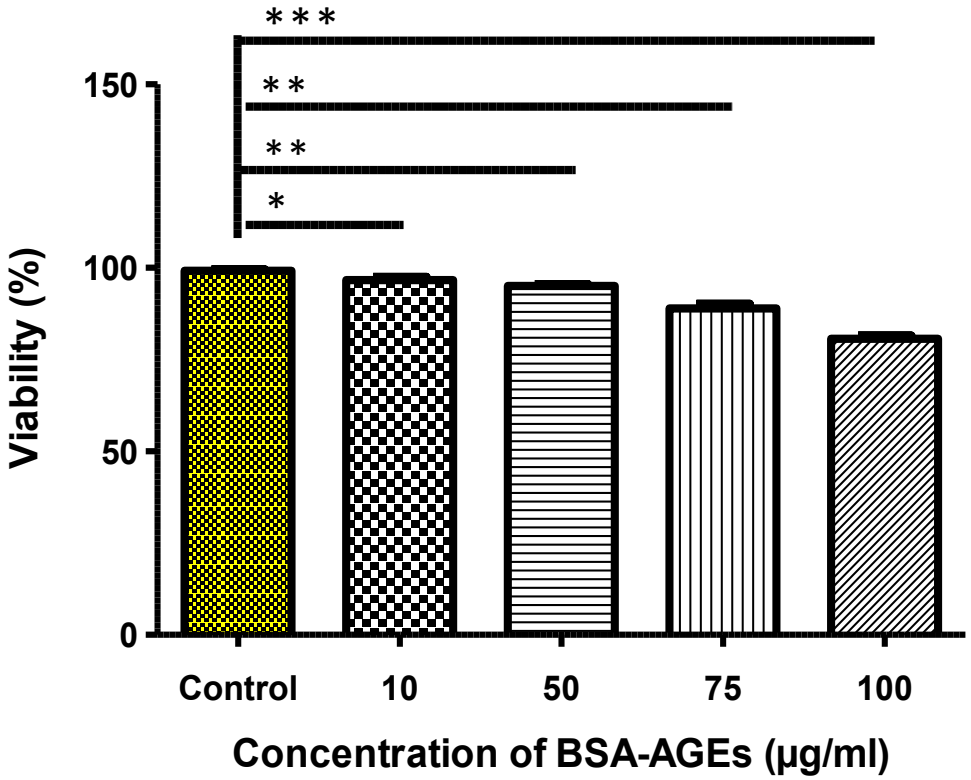


Figure 5.15: Effect of BSA-AGEs on BAEC viability. Bovine aortic endothelial cells (2 x 10^5 cells/ml) were seeded in 48-well plates and incubated with different concentrations of BSA-AGEs for 24 hours. Results are expressed as mean ± SD (n = 3).* $p < 0.05$ ** $p < 0.01$ and *** $p < 0.001$.

5.3.4 Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) analysis of selected oxidative stress gene expression in BAECs

Figure 5.16 illustrates the relative mRNA expression of *CAT*, *SOD2*, *HOX 1, p47 phox, NOX5*, *NOX4, p22phox,* and *GPx* genes in BAECs in response to HWE or CWE of MC plus AGEs treatment compared to the cells treated with AGEs alone for 24 hours by qRT-PCR. The results show that expression of *CAT* and *SOD2* in AGEs-induced oxidative stress in BAECs increases significantly by3.36-fold ($p< 0.03$), 3.31-fold ($p< 0.04$) respectively, following 24 hours treatment with 10 μg/ml of AGEs and 90 μg/ml of HWE of MC. Similar effects of CWE plus AGEs on the expressions of *CAT* and *SOD2* were observed in BAECs with upregulations by 2.06-fold ($p< 0.005$), and 2.81-fold ($p< 0.0043$) respectively, following 24 hours treatment. In contrast, the expressions of *HOX1*, *NOX5*, *NOX4*, $p22^{phox}$ and *GPx* in AGEs-induced oxidative stress in BAEC decreases by by 0.20-fold ($p< 0.047$), 0.23-fold ($p< 0.036$), 0.26-fold ($p< 0.044$), 0.27-fold ($p< 0.049$) and 0.29-fold ($p< 0.037$) respectively, following 24 hours treatment with HWE and AGEs. Similarly, *HOX1*, *NOX5*, *NOX4*, $p22^{phox}$ and *GPx* in AGEs-induced oxidative stress in BAEC decreases by 0.14-fold ($p< 0.037$), 0.18-fold ($p< 0.02$), 0.23-fold ($p< 0.043$, 0.20-fold ($p< 0.023$) and 0.25-fold ($p< 0.012$) respectively, following 24 hours treatment with CWE and AGEs. Only the expression of $p47^{phox}$ did not change ($p>0.05$) following the treatment of HWE+AGEs or CWE+AGEs compared to treatment with AGEs alone.

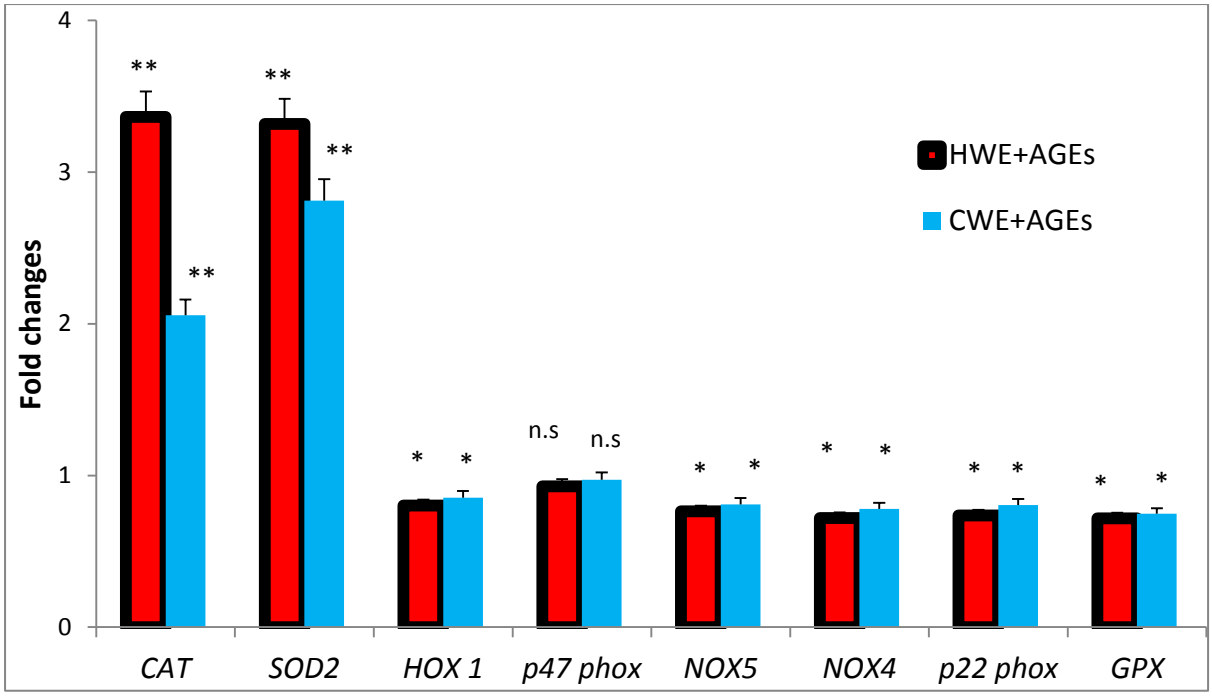


Figure 5.16 Effects of HWE or CWE of MC on mRNA expression of *CAT, SOD2, p47 phox, NOX5, NOX4, p22 phox and GPx* in BAEC. The relative gene expression in cells treated with HWE or CWE of MC plus AGEs were compared to the cells treated with AGEs alone (used as control, value=1) and normalised to the housekeeping gene *ACTB* expression. Columns represent mean ± SD (n=3). * $p < 0.05$, ** $p < 0.001$.

5.3.5 Effects of *Momordica charantia* extracts on AGE-induced oxidative stress signalling pathways in BAEC.

To optimise the incubation time corresponding to the maximal inhibitory effects of MC extracts on the oxidative stress cell signalling induced by BSA-glucose-AGEs, the expression of RAGE and p-JAK2 in BAECs treated with 10 µg/ml AGEs alone or AGEs with 90 µg/ml MC extracts were investigated at 10 minutes, 1, 6 and 24 hours of incubation times. After 10 minutes incubation, the cells treated with AGEs alone significantly increased the level of phosphorylated JAK-2 (p-JAK2) and RAGE compared to the basal level of p-JAK2 and RAGE expressed in untreated control cells. The expressions of p-JAK2 and RAGE were reduced in the cells treated with HWC of MC and AGEs compared to their expressions in cells treated with AGEs alone ($p< 0.001$). The highest inhibitory effect was shown after 24-hours treatment as demonstrated in Figures 5.17 and 5.18

The effects of different concentrations of HWE or CWE of MC on the protein expressions of molecules regulating oxidative stress signalling such as NOX4, NOX5, total JAK-2, p-JAK2,

p21 and RAGE in BAECs after 24 hours treatment were investigated and demonstrated in Figures 5.19 to 5.30. Figure 5.19 shows that AGEs significantly induced the expression of NOX4 by 1.61 fold ($p< 0.0001$), compared to the basal level of NOX4 in control cells. However, the inhibitory effects of 15, 30, 60 μg/ml HWE on the expression of NOX4 induced by AGEs were not significant after 24 hours incubation ($p > 0.05$), compared to cells treated with AGEs alone. The addition of 90 μg/ml of HWE of MC significantly ($p< 0.0001$) reduced NOX4 expression by 0.37 fold, compared to cells treated with AGEs alone. The CWE shows greater inhibitory effects on NOX4 expression than HWE. The addition of 15, 30, 60, or 90 μg/ml of CWE of MC significantly ($p< 0.001$) reduced NOX4 expression by 5.7-fold, 6-fold, 8.1-fold, 8.2-fold respectively, compared to cells treated with AGEs alone as shown in Figure 5.20

AGEs significantly ($p< 0.0001$) induced NOX5 expression by 1.59-fold, compared to the basal level of NOX5 in untreated control cells. The addition of 15, 30, 60 and 90 μg/ml of HWE of MC as shown in Figure 5.21 significantly ($p< 0.001$) decreased NOX5 by 0.33, 0.39, 0.44 and 0.57 fold, at same concentration CWE as demonstrated in Figure 5.22 significantly ($p<0.001$) reduced NOX5 by 0.36, 0.48, 0.49 and 0.59 fold, compared to cells treated with AGEs alone.

Moreover, AGEs significantly ($p< 0.001$) induced p-JAK2 expression by 2.53-fold, compared to the basal level of p-JAK2 in untreated control cells. The addition of 15, 30, 60 and 90 μg/ml of HWE of MC as demonstrated in Figure 5.23 significantly ($p< 0.001$) reduced p-JAK2 overexpression by 0.35-fold, 0.40-fold, 0.45-fold and 0.72-fold, compared to cells treated with AGEs alone. However, the inhibitory effects of 15μg/ml of CWE on the expression of p-JAK2 induced by AGEs was not significant after 24 hours incubation ($p >0.05$), compared to cells treated with AGEs alone. The addition of 30, 60 and 90 μg/ml of CWE of MC as demonstrated in Figure 5.24 significantly ($p< 0.01$) reduced p-JAK2 expression by 0.33-fold, 0.38-fold, and 0.48-fold, compared to cells treated with AGEs alone.

Figures 5.25 and 5.26 show AGEs significantly ($p< 0.001$) induced total JAK2 overexpression by 1.68- fold, compared to the basal level of total JAK2 in untreated control cells. The inhibitory effects of 15, 30 and 60 μg/ml of HWE of MC on expression of total JAK2 induced by AGEs were not significant ($p> 0.05$), compared to cells treated with AGEs alone. Whereas, the addition of 90 μg/ml of HWE of MC significantly ($p< 0.001$) reduced total JAK2 expression by 0.65-fold, compared to cells treated with AGEs alone. The inhibitory effects of 15μg/ml of CWE on the expression of total JAK2 induced by AGEs was not significant ($p> 0.05$) compared

to cells treated with AGEs alone. In contrast, the addition of 30µg/ml of CWE of MC significantly ($p< 0.05$) reduced total JAK2 overexpression by 0.49-fold, compared to cells treated with AGEs alone. Furthermore, the addition of 60 and 90 µg/ml of CWE of MC significantly ($p< 0.001$) reduced total JAK2 overexpression by 0.69-fold and 0.77-fold respectively, compared to cells treated with AGEs alone.

AGEs significantly ($p< 0.001$) induced p21 overexpression by 1.52 fold, compared to the basal level of p21 in untreated control cells. The addition of 15, 30, 60 and 90 µg/ml of HWE of MC significantly ($p< 0.0001$) reduced p21 overexpression by 0.25, 0.36, 0.40 and 0.65 fold respectively, compared to cells treated with AGEs alone as shown in Figure 5.27. The inhibitory effects of 15 µg/ml of CWE of MC on expression of p21induced by AGEs was not significant ($p> 0.05$), compared to cells treated with AGEs alone. Whereas, the addition of 30, 60 and 90 µg/ml of CWE of MC significantly reduced p21expression by 0.07-fold ($p< 0.05$), 0.11-fold ($p< 0.001$) and 0.57-fold ($p< 0.0001$) respectively, compared to cells treated with AGEs alone as shown in Figure 5.28

Figure 5.29 shows AGEs significantly ($p< 0.001$) induced RAGE overexpression by 1.55-fold increase, compared to the basal level of RAGE in untreated control cells. The addition of 15, 30 60 and 90 µg/ml of HWE of MC significantly reduced p21 overexpression by 0.42-fold ($p< 0.01$), 0.52-fold ($p< 0.001$), 0.56-fold ($p< 0.001$) and 0.58-fold ($p< 0.001$) respectively, compared to cells treated with AGEs alone. The inhibitory effects of 15 µg/ml of CWE of MC on expression of RAGE induced by AGEs was not significant ($p> 0.05$), compared to cells treated with AGEs alone. In the contrary, the addition of 30, 60 and 90 µg/ml of CWE of MC significantly ($p< 0.001$) reduced RAGE overexpression by 0.21-fold ($p< 0.01$), 0.25-fold ($p< 0.001$) and 0.37-fold ($p< 0.001$) respectively, compared to cells treated with AGEs alone as shown in Figure 5.30

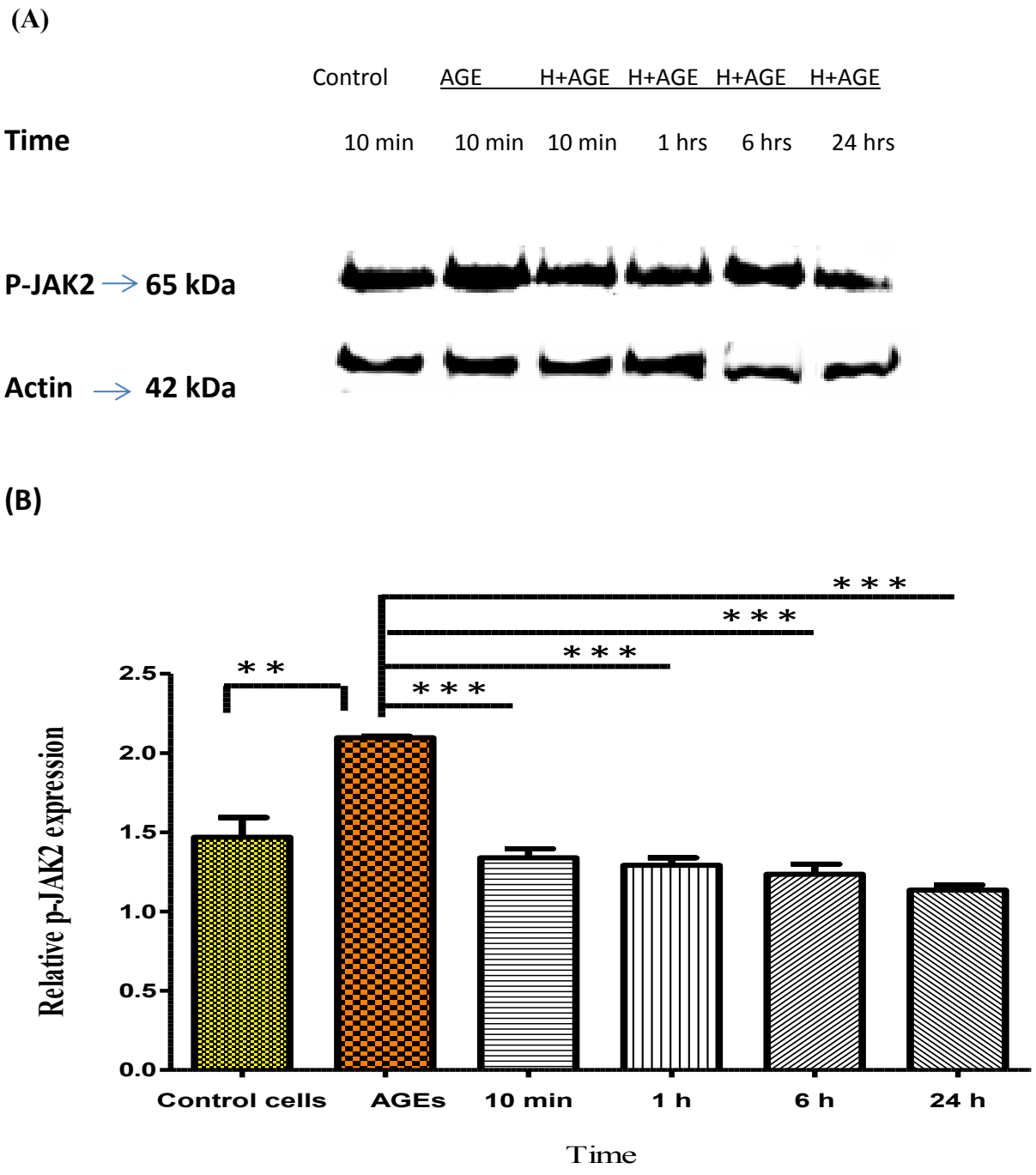


Figure 5.17: Time-course of p-JAK2 protein expression in BAECs treated with AGEs and MC extracts. (A) A representative Western blot analysis showing the effect of 90 µg/ml HWE of MC on p-JAK2 expression in BAECs after incubation with 10µg/ml AGEs for 10 minutes, 1, 6, or 24 hours, compared with cells treated with AGEs alone or untreated control cells. **(B)** Bar graph showing the relative expression of p.JAK2, which were normalized to actin expression. Actin was employed as loading control. Results are presented as mean ± SD (n = 3). ** $p < 0.001$ and *** $p < 0.0001$

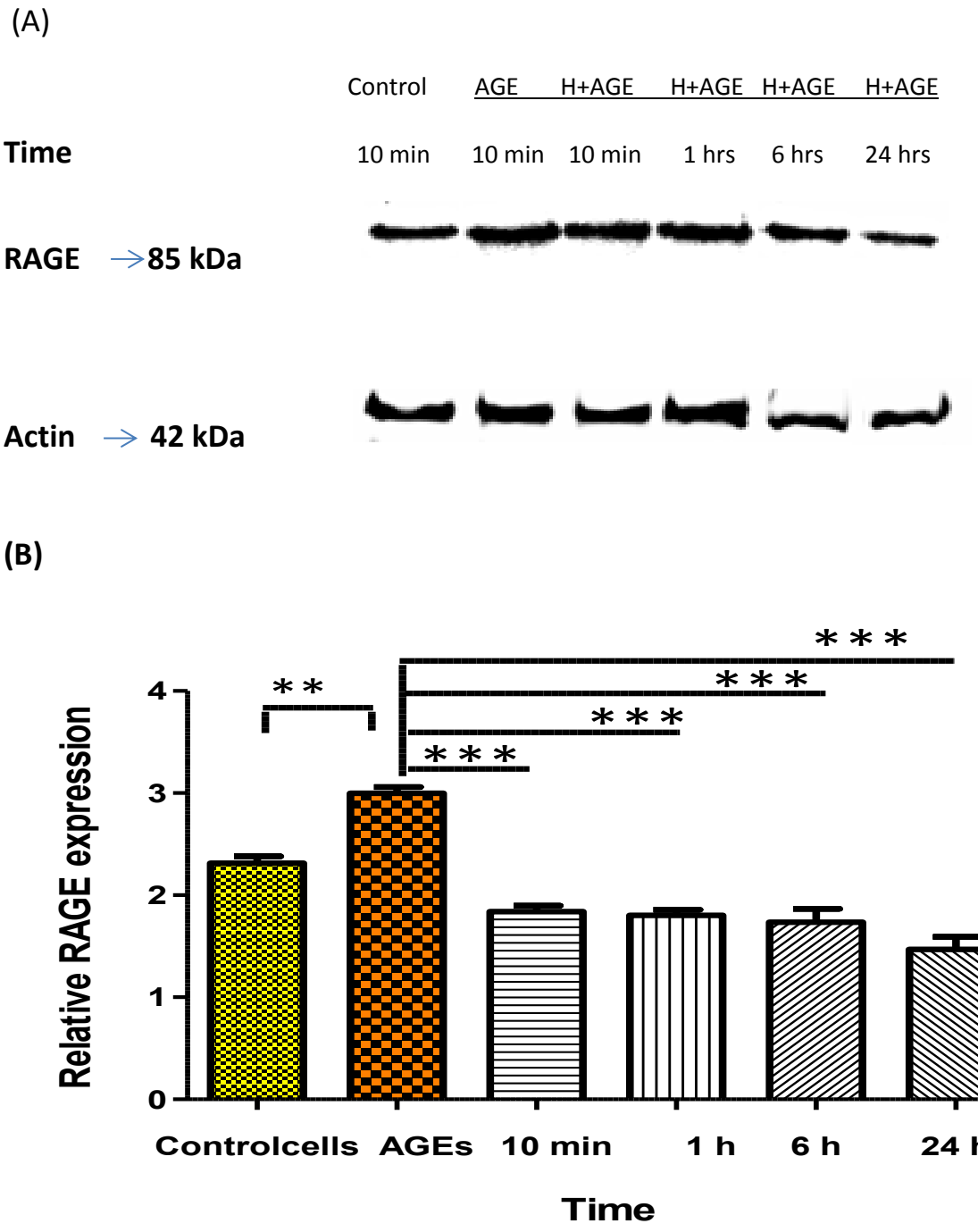
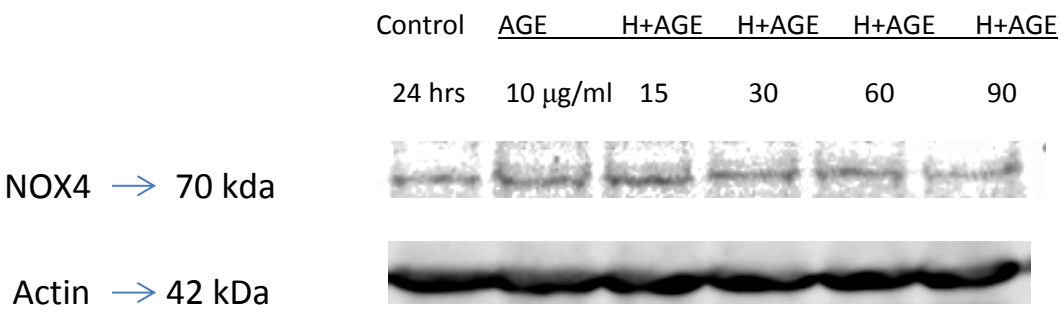


Figure 5.18: Time-course of RAGE protein expression in BAECs treated with AGEs and MC extracts. (A) A representative Western blot analysis showing the effect of 90 µg/ml HWE of MC on RAGE expression in BAECs after incubation with 10 µg/ml AGEs for 10 minutes, 1, 6, or 24 hours, compared with cells treated with AGEs alone or untreated control cells. **(B)** Bar graph showing the relative expression of RAGE, which were normalized to actin expression. Actin was employed as loading control. Results are presented as mean ± SD (n = 3). ** $p < 0.001$ and *** $p < 0.0001$

(A)



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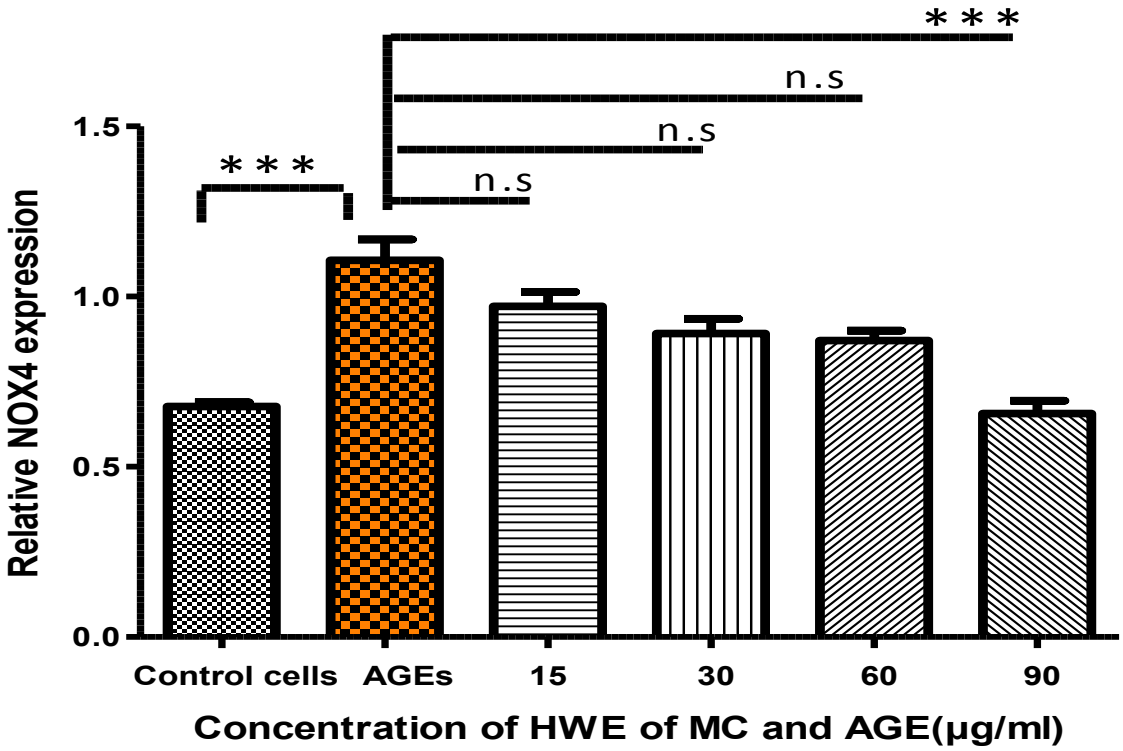
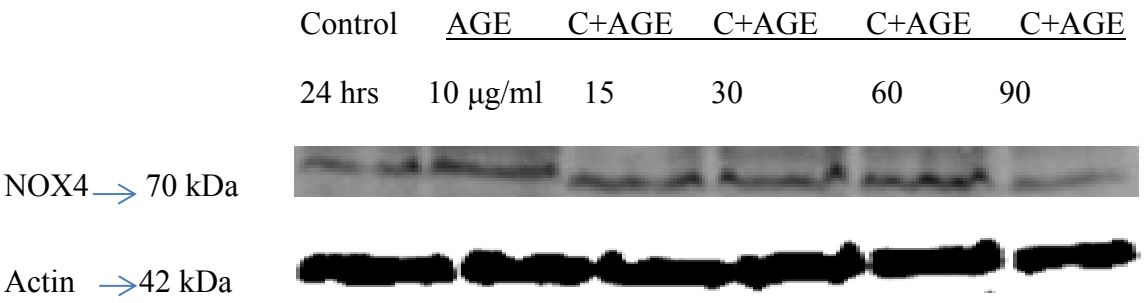


Figure 5.19: Effect of different concentrations of HWE of MC on NOX4 expression in BAECs . (A) A representative western blot showing the expression of NOX4 in BAECs after treatment with different concentrations of HWE of MC plus AGEs (10 µg/ml), or with AGEs alone. **(B)** The bar shows the relative expression of NOX4 in control cells, or in cells treated with AGEs (10 µg/ml) alone or AGEs plus HWE at different concentrations for 24 hours. To check the equal loading of proteins, actin was employed as loading control. Data are mean ± SD, n=3. All values were compared to AGEs (value=1) and normalised to ß-actin. ***$p <$ 0.0001 and n.s. = non-significant.

(A)



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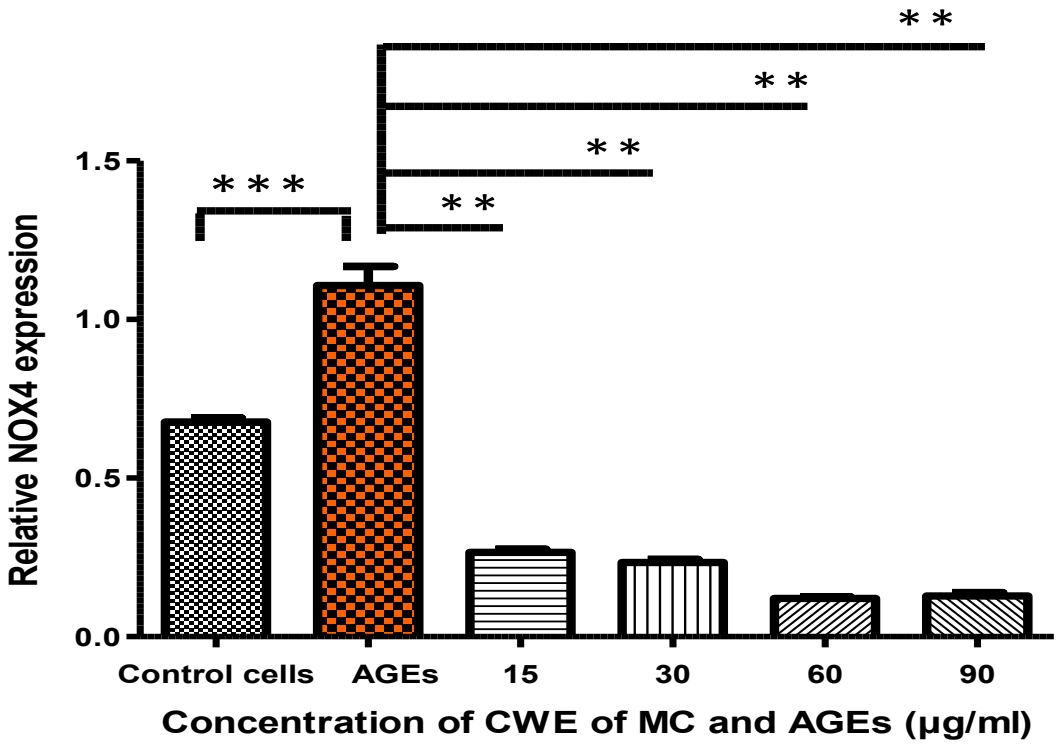


Figure 5.20: Effect of different concentrations of CWE of MC on NOX4 expression in BAECs. (A) A representative western blot showing the expression of NOX4 in BAECs after treatment with different concentrations of CWE of MC plus AGEs (10 µg/ml), or with AGEs alone. **(B)** The bar shows the relative expression of NOX4 in control cells, or in cells treated with AGEs (10 µg/ml) alone or AGEs plus CWE at different concentrations for 24 hours. To check the equal loading of proteins, actin was employed as loading control. Data are mean ± SD, n=3. All values were compared to AGEs (value=1) and normalised to ß-actin. $^{**}p < 0.001$ and $^{***}p < 0.0001$.

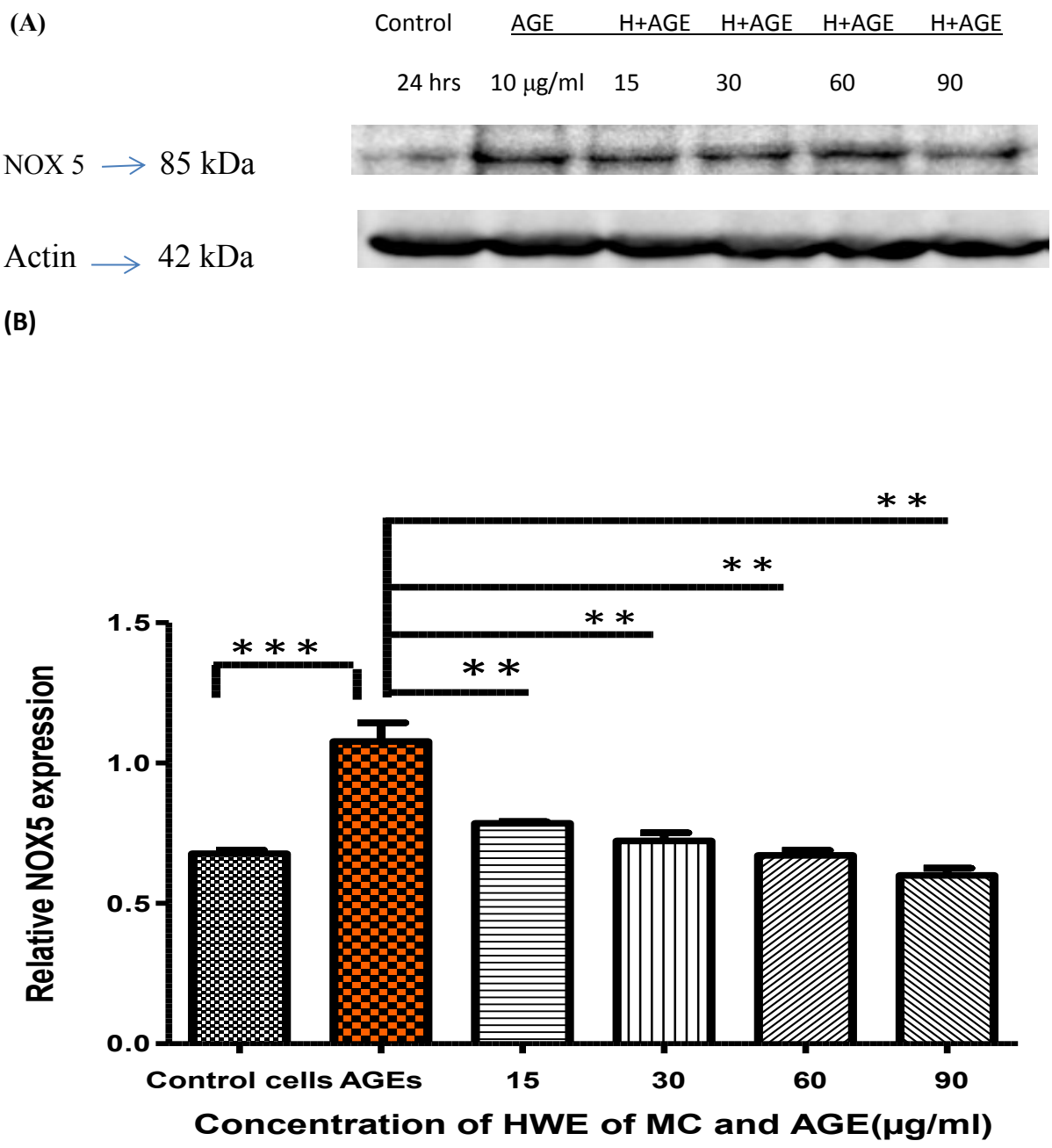


Figure 5.21: Effect of different concentrations of HWE of MC on NOX5 expression in BAECs. (A) A representative western blot showing the expression of NOX5 in BAECs after treatment with different concentrations of HWE of MC plus AGEs (10 µg/ml), or with AGEs alone. **(B)** The bar graph shows the relative expression of NOX5 in control cells, or in cells treated with AGEs (10 µg/ml) alone or AGEs plus HWE at different concentrations for 24 hours. To check the equal loading of proteins, actin was employed as loading control. Data are mean ± SD, n=3. All values were compared to AGEs (value=1) and normalised to ß-actin. ** $p < 0.001$ and *** $p < 0.0001$

(A)

(B)

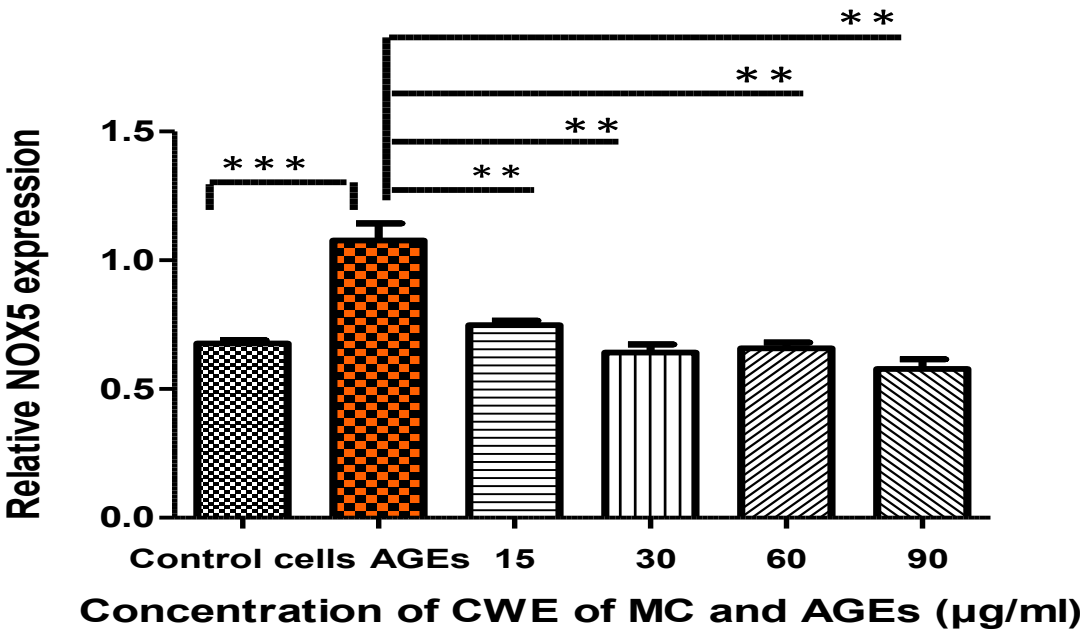
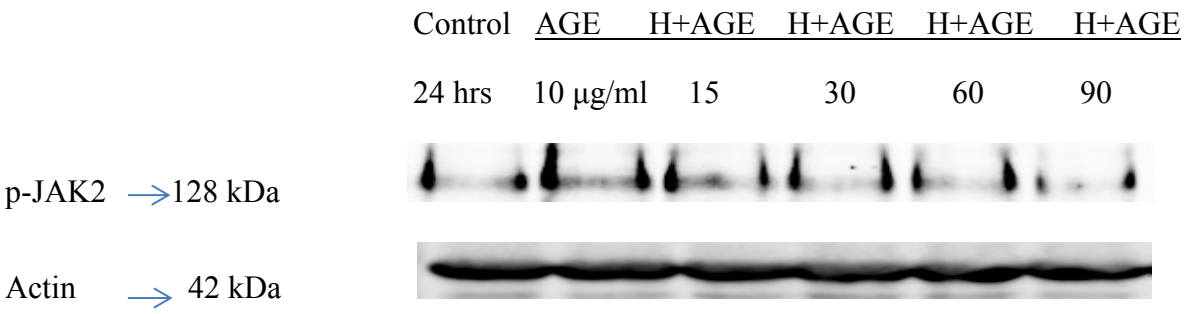


Figure 5.22: Effect of different concentrations of CWE of MC on NOX5 expression in BAECs . (A) A representative western blot showing the expression of NOX5 in BAECs after treatment with different concentrations of CWE of MC plus AGEs (10 µg/ml), or with AGEs alone. **(B)** The bar shows the relative expression of NOX5 in control cells, or in cells treated with AGEs (10 µg/ml) alone or AGEs plus CWE at different concentrations for 24 hours. To check the equal loading of proteins, actin was employed as loading control. Data are mean ± SD, n=3. All values were compared to AGEs (value=1) and normalised to ß-actin. $^{**}p < 0.001$ and $^{***}p < 0.0001$

(A)



(B)

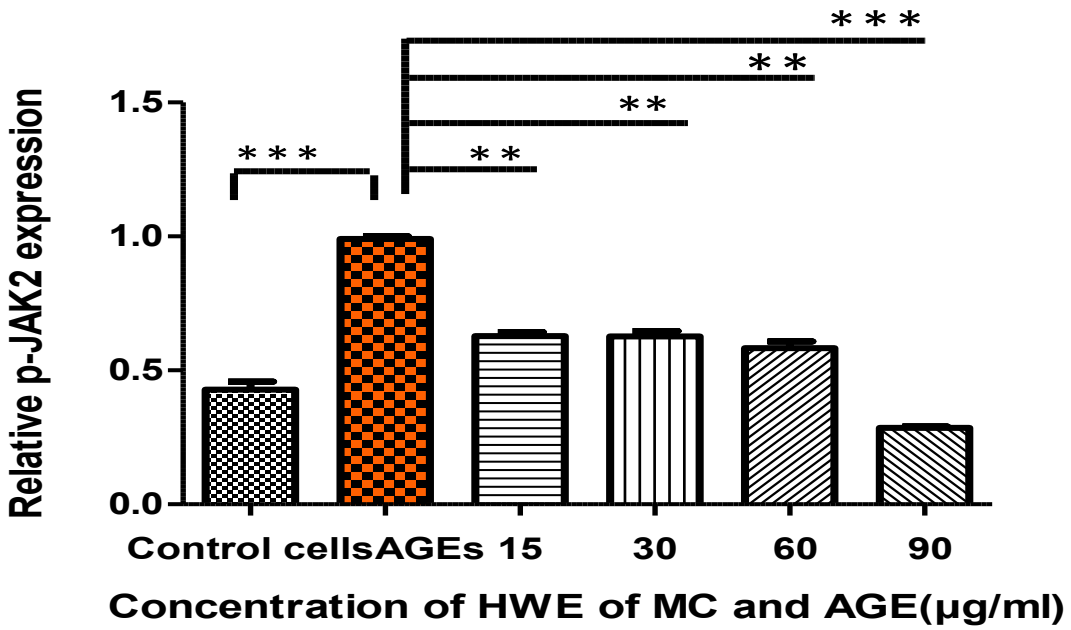


Figure 5.23: Effect of different concentrations of HWE of MC on p.JAK2 expression in BAECs. (A) A representative western blot showing the expression of p.JAK2 in BAECs after treatment with different concentrations of HWE of MC plus AGEs (10 µg/ml), or with AGEs alone. **(B)** The bar shows the relative expression of p.JAK2 in control cells, or in cells treated with AGEs (10 µg/ml) alone or AGEs plus HWE at different concentrations for 24 hours. To check the equal loading of proteins, actin was employed as loading control. Data are mean ± SD, n=3. All values were compared to AGEs (value=1) and normalised to ß-actin. $^{**}p < 0.001$ and $^{***}p < 0.0001$.

(A)

(B)

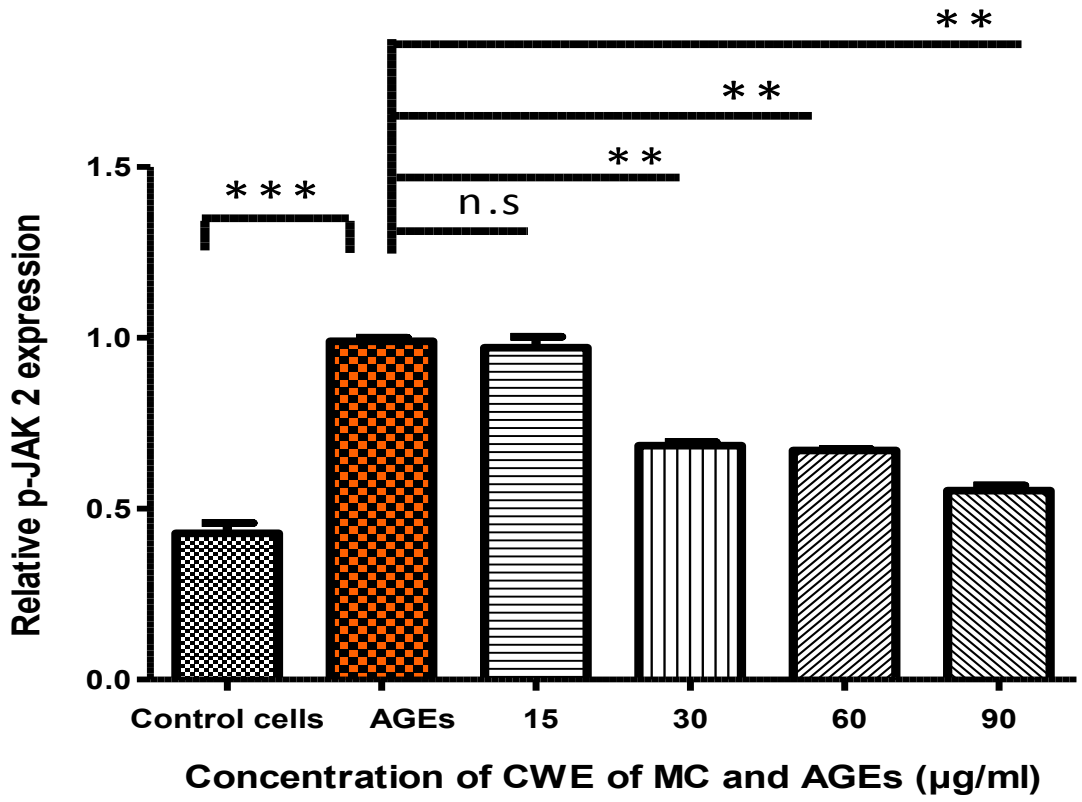
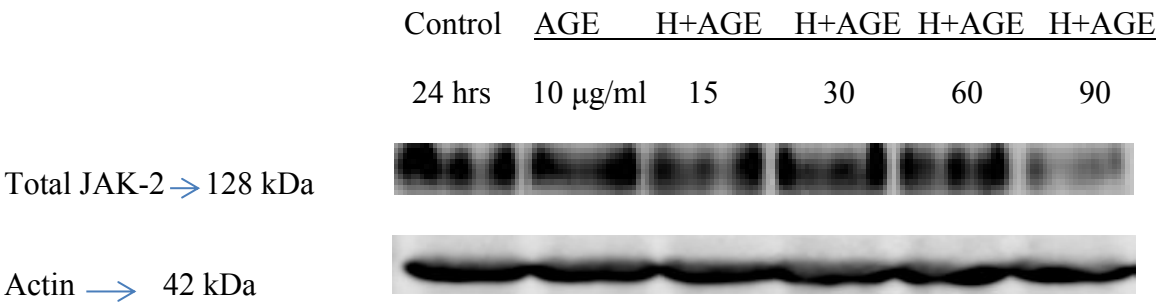


Figure 5.24: Effect of different concentrations of CWE of MC on p.JAK2 expression in BAECs. (A) A representative western blot showing the expression of p.JAK2 in BAECs after treatment with different concentrations of CWE of MC plus AGEs (10µg/ml), or with AGEs alone. **(B)** The bar shows the relative expression of p.JAK2 in control cells, or in cells treated with AGEs (10µg/ml) alone or AGEs plus CWE at different concentrations for 24 hours. To check the equal loading of proteins, actin was employed as loading control. Data are mean ± SD, n=3. All values were compared to AGEs (value=1) and normalised to ß-actin. $^{**}p < 0.001$, $^{***}p < 0.0001$, and n.s. = non-significant.

(A)



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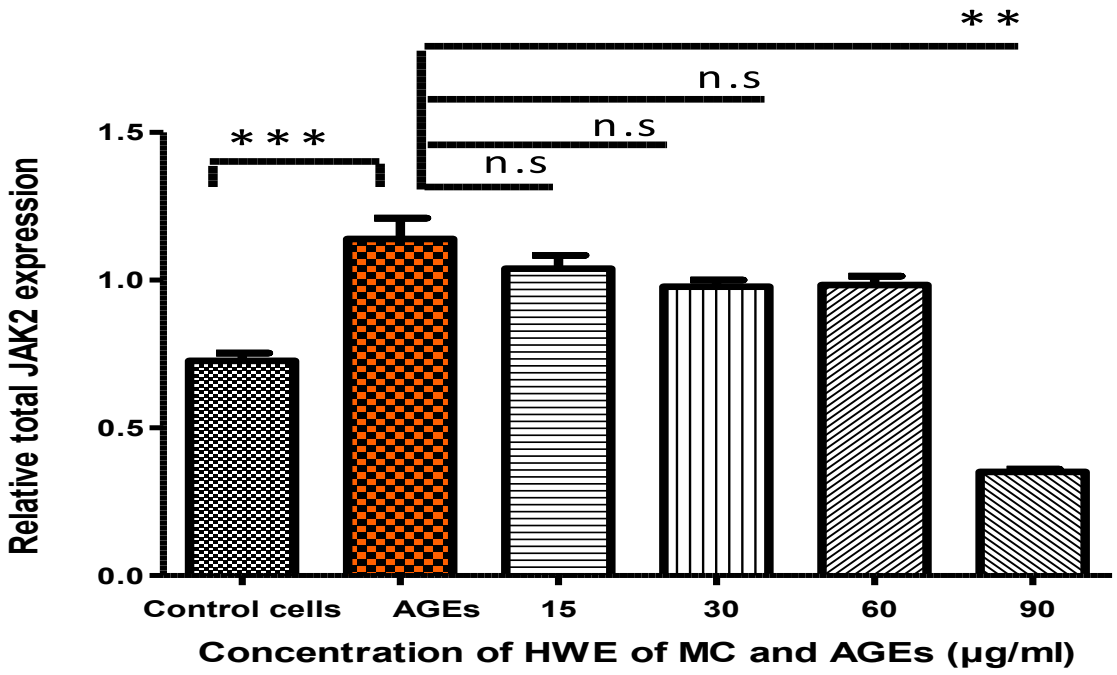
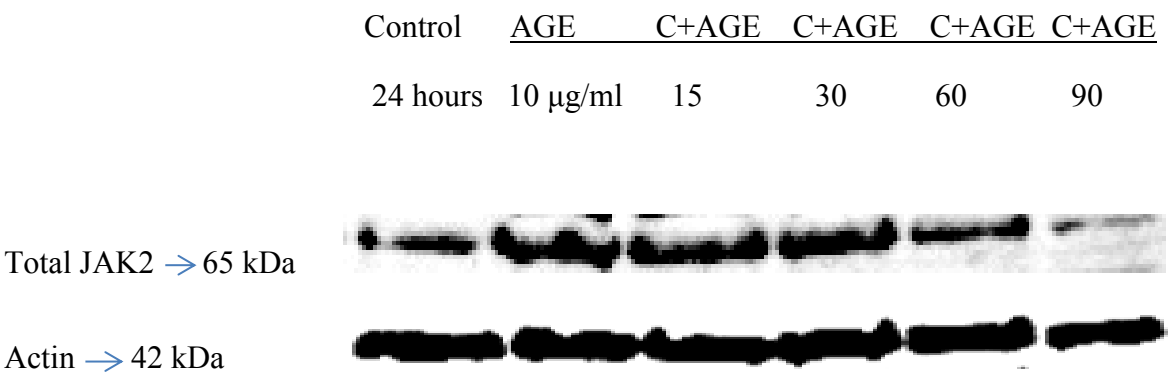


Figure 5.25: Effect of different concentrations of HWE of MC on total JAK2 expression in BAECs. (A) A representative western blot showing the expression of total JAK2 in BAECs after treatment with different concentrations of HWE of MC plus AGEs (10 µg/ml), or with AGEs alone. **(B)** The bar shows the relative expression of total JAK2 in control cells, or in cells treated with AGEs (10 µg/ml) alone or AGEs plus HWE at different concentrations for 24 hours. To check the equal loading of proteins, actin was employed as loading control. Data are mean ± SD, n=3. All values were compared to AGEs (value=1) and normalised to ß-actin. $^{**}p < 0.001$, $^{***}p < 0.0001$ and n.s. = non-significant.

(A)



(B)

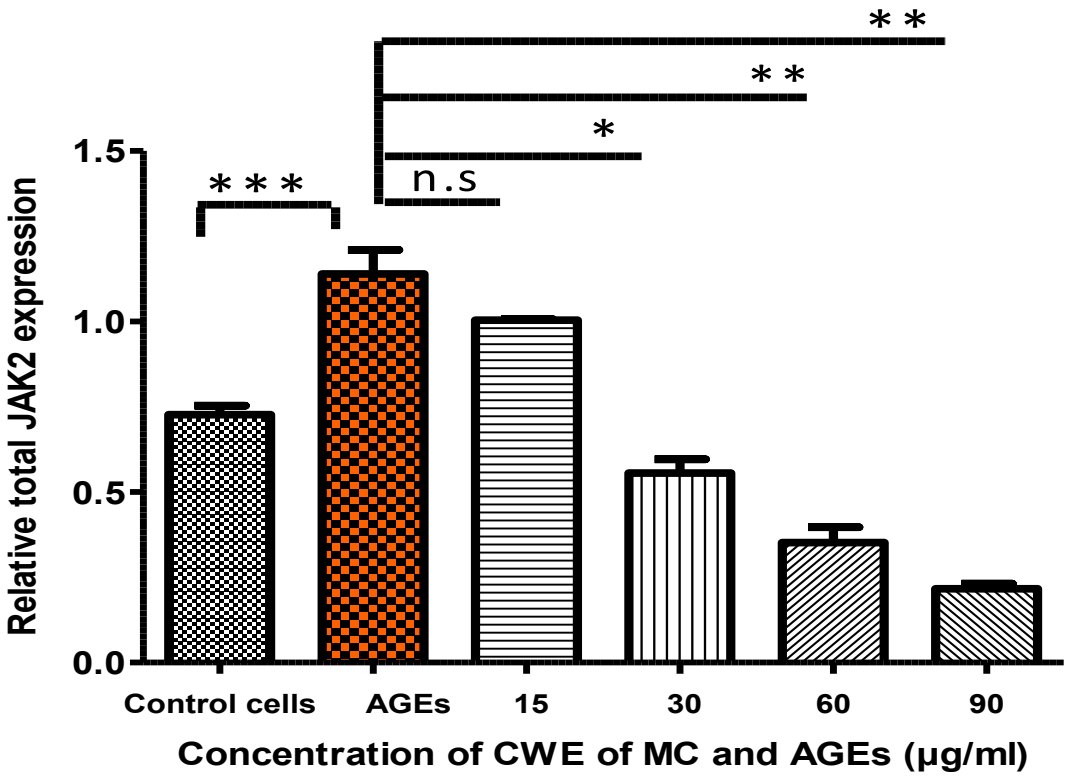


Figure 5.26: Effect of different concentrations of CWE of MC on total JAK2 expression in BAECs. (A) A representative western blot showing the expression of total JAK2 in BAECs after treatment with different concentrations of CWE of MC plus AGEs (10 µg/ml), or with AGEs alone. **(B)** The bar shows the relative expression of total JAK2 in control cells, or in cells treated with AGEs (10 µg/ml) alone or AGEs plus CWE at different concentrations for 24 hours. To check the equal loading of proteins, actin was employed as loading control. Data are mean ± SD, n=3. All values were compared to AGEs (value=1) and normalised to ß-actin. *$p < 0.05$, *$p < 0.001$, **$p < 0.001$ and n.s. = non-significant.

(A)

(B)

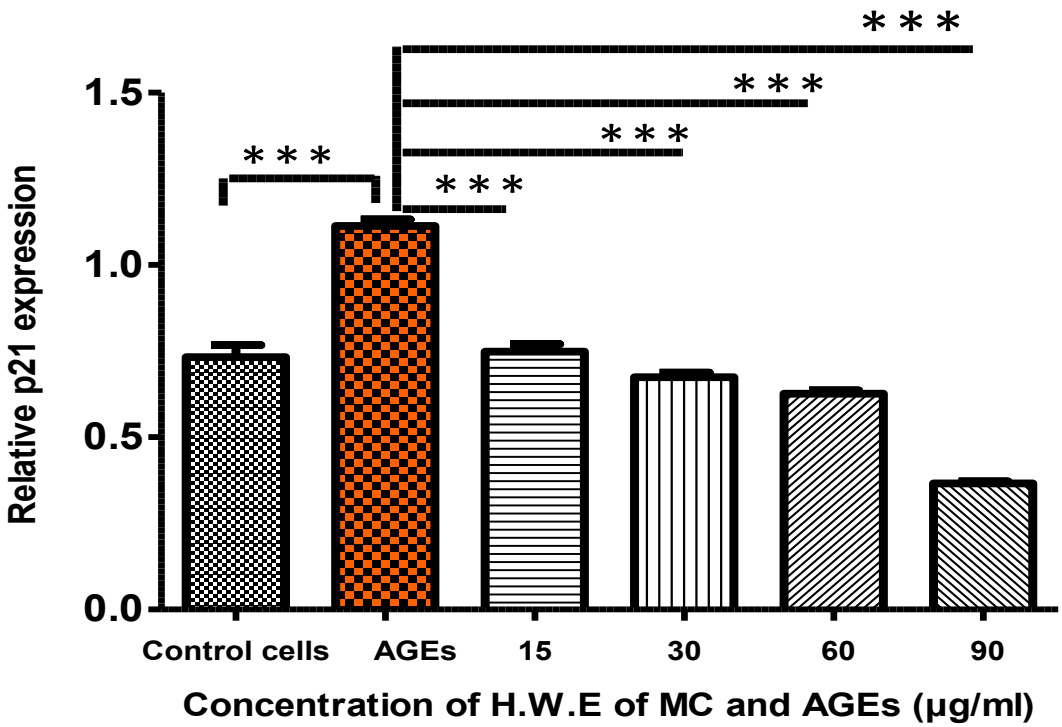
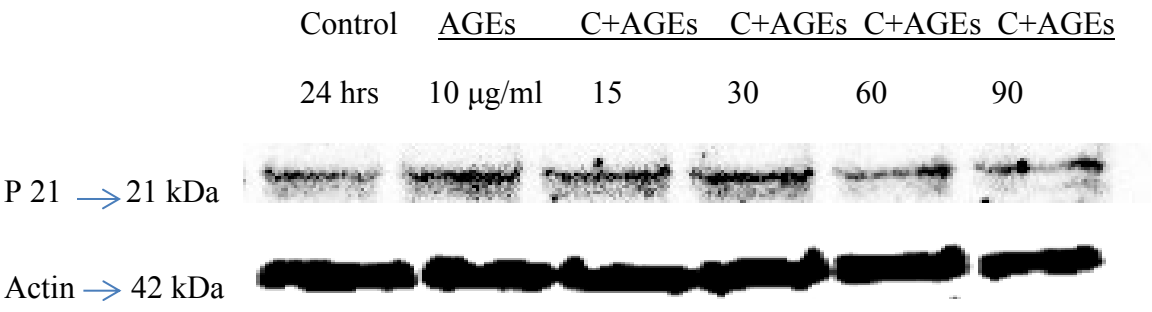


Figure 5.27: Effect of different concentrations of HWE of MC on p21 expression in BAECs. (A) A representative western blot showing the expression of p21 in BAECs after treatment with different concentrations of HWE of MC plus AGEs (10 µg/ml), or with AGEs alone. **(B)** The bar shows the relative expression of p21 in control cells, or in cells treated with AGEs (10 µg/ml) alone or AGEs plus MC extracts at different concentrations for 24 hours. To check the equal loading of proteins, actin was employed as loading control. Data are mean ± SD, n=3. All values were compared to AGEs (value=1) and normalised to ß-actin. ***$p <$ 0.0001.

(A)



(B)

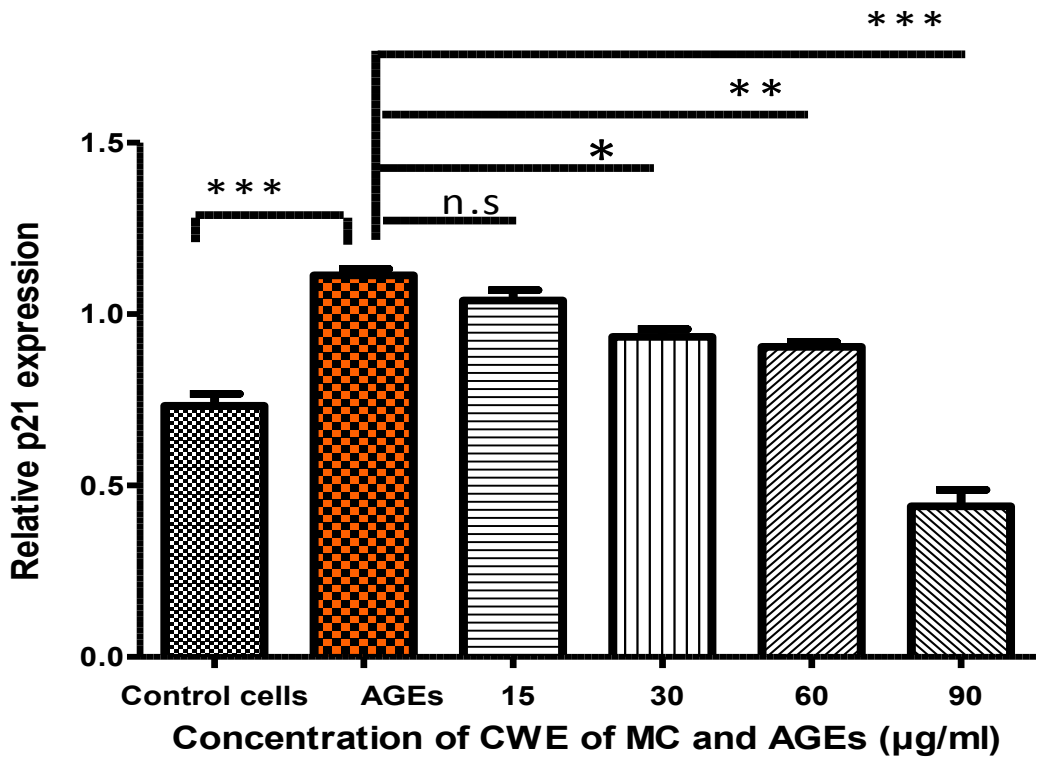


Figure 5.28: Effect of different concentrations of MC extract on p21 expression in 24 hours. (A) A representative Western blot demonstrating the expression of p21induced by 10 µg/ml AGEs alone or in the presence of MC extracts at different concentrations. **(B)** The effects of AGEs 10 µg/ml alone or with MC extracts at different concentrations in 24 hours on p21 expression. To check the equal loading of proteins, actin was employed as loading control. Data are mean ± SD, n=3. All values were compared to AGEs (value=1) and normalised to ß-actin. *$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$ and n.s = non-significant.

(A)

(B)

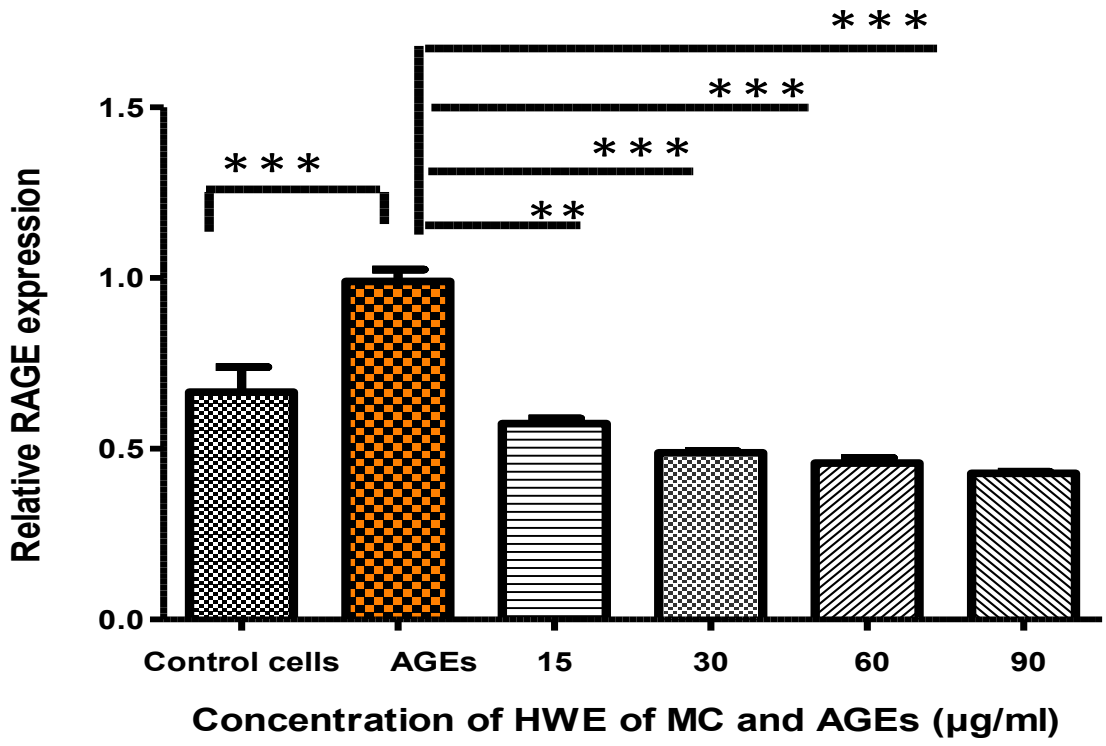
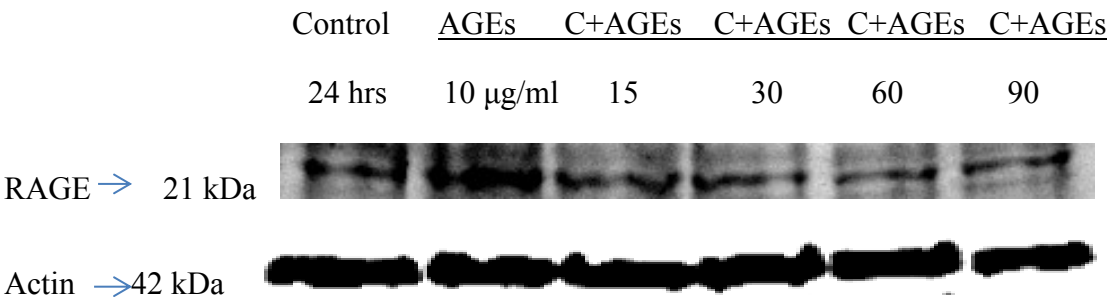


Figure 5.29: Effect of different concentrations of HWE of MC on RAGE in BAECs. (A) A representative western blot showing the expression of RAGE in BAECs after treatment with different concentrations of HWE of MC plus AGEs (10 µg/ml), or with AGEs alone. **(B)** The bar shows the relative expression of RAGE in control cells, or in cells treated with AGEs (10 µg/ml) alone or AGEs plus HWE at different concentrations for 24 hours. To check the equal loading of proteins, actin was employed as loading control. Data are mean ± SD, n=3. All values were compared to AGEs (value=1) and normalised to ß-actin. $^{**}p < 0.001$ and $^{**}p < 0.0001$.

(A)



(B)

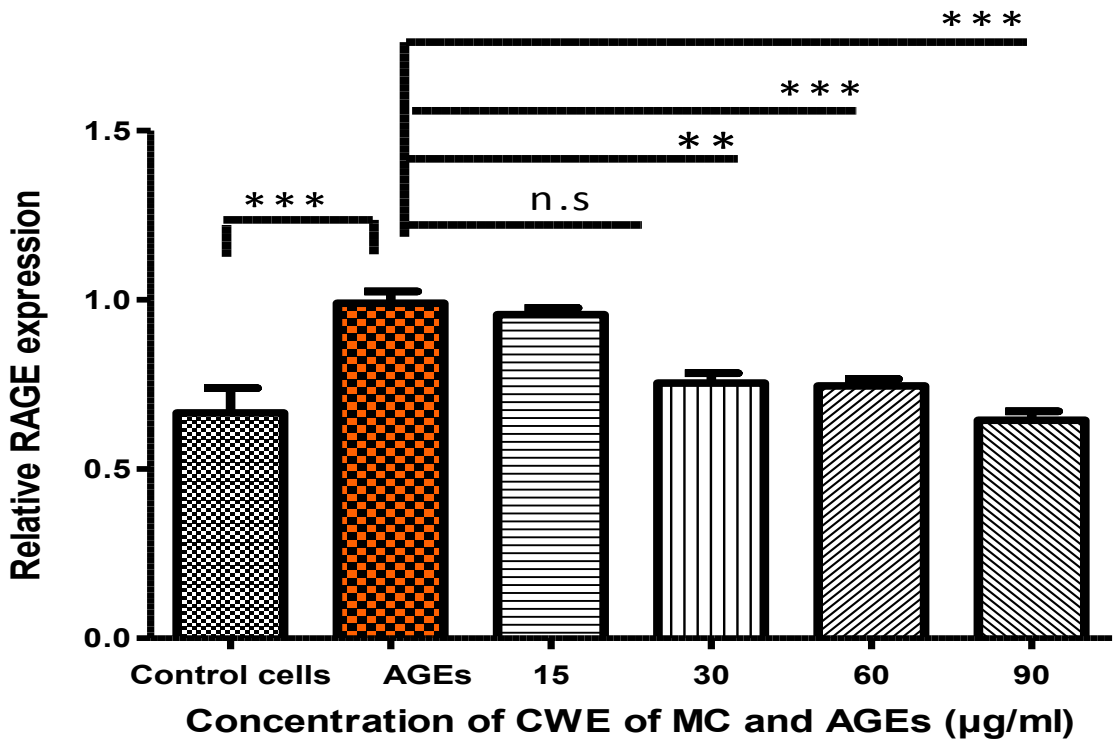


Figure 5.30: Effect of different concentrations of CWE of MC on RAGE expression in BAECs. (A) A representative western blot showing the expression of RAGE in BAECs after treatment with different concentrations of CWE of MC plus AGEs (10 µg/ml), or with AGEs alone. **(B)** The bar shows the relative expression of RAGE in control cells, or in cells treated with AGEs (10 µg/ml) alone or AGEs plus CWE at different concentrations for 24 hours. To check the equal loading of proteins, actin was employed as loading control. Data are mean ± SD, n=3. All values were compared to AGEs (value=1) and normalised to ß-actin. $^{**}p < 0.001$, $^{***}p < 0.0001$ and n.s. = non-significant.

5.4 Discussion

Long-term exposure to hyperglycaemia cause formation of AGEs which plays a central role in the pathogenesis of diabetic complications such as retinopathy, nephropathy, neuropathy and delayed wound healing (Ahmed, 2005). Furthermore, it has been reported that the hyperglycemia decreases endothelial cell proliferation, migration and growth blood vessels formation *in vitro*, hence results in to damage angiogenesis (Stitt & Curtis, 2005). However, estimation of AGEs is great important for clinicians and researchers concerned with the management and prevention of diabetic complications and the pathological role of AGEs has encouraged the research for inhibitors of AGEs.

This study reported that both HWE and CWE of MC increased cell proliferation in BAEC expouserd to glucose, methylglyoxal and AGEs-induced oxidative stress. Figures 5.1 and 5.2 show that MC extracts ranging from 15 to 90 µg/ml increased endothelial cell proliferation in a dose-dependent manner. The stimulation effects on BAEC cell proliferation by 90 µg/ml MC extracts were even stronger than by 25 ng/ml FGF-2. FGF-2 is a potent mitogen that induces cell proliferation, migration, blood vessel growth, and plays a pivotal role in tissue reformation and stimulate wound healing (Mashilipa *et al*, 2011). FGF2 at a concentration of 25 ng/ml showed significant elevate BAEC proliferation (Hussain *et al*, 2009). The results obtained from this study indicate that like FGF2, 15 to 90 µg/ml MC extract might have angiogenic property. Proliferation study proposed that HWE of MC consist more-insulin-like proteins or other stimulating molecules than CWE of MC. It has been reported that MC extracts contain varieties of chemical compounds, among them insulin-like molecules, which act as pro-angiogenic growth factors by the activation of tyrosine kinase via binding to insulin receptors (Kim & Kim, 2011). Previous research has reported that topical insulin effectively improves skin wound-healing in diabetic rats (Lima *et al*, 2012). Cytotoxicity studies as demonstrated that MC extracts had no effects on BAEC viability at low concentrations of 15, 30, 60 and 90 µg/ml, but high concentrations of 100 to 1000 µg/ml of HWE and CWE of MC decreased cell viability indicating the characteristic pattern of BAEC cell death as shown in Figure 5.3 and 5.4, respectively. Moreover, MC extracts (2-100 µg/ml) was not affected cell viability in ß-cells (Kim & Kim, 2011).

It is well established that in DM, hyperglycaemia usually ranges from 6 to 30 mM glucose and in severe condition the levels may reach 55 mM glucose (Facchiano *et al*, 2006). To examine the effect of high glucose concentration *in vivo*, BAECs were cultured in medium containing 10.6, 30.6, 55.6 and 105.6 mM glucose to mimic different physiological conditions. The results have shown that high concentrations of glucose inhibit BAEC proliferation as shown in Figure 5.5, which is in accordance with previous studies reported that high glucose concentration inhibited cell proliferation of BAEC *in vitro* (Jiaojun *et al*, 2004). The results suggest that glucose may had mitogenic effect on BAECs cultured at 30.6 mM glucose compared to control; this effect may be related to the elevation of nutrients in the growth medium before starting to cause cellular toxicity. At the higher concentration of glucose 30.6. 55.6 and 105.6 mM cell growth reduced in dose-dependent manner compared to control. This reduction in cell growth may not as a results of cellular toxicity due to hyper-osmolarity, but related to high glucose concentration in the growth medium (Mashilipa *et al*, 2011: Zhang *et al*, 2006). Moreover, previous studies have reported that high glucose concentrations can enhance cell cytotoxicity of retinal endothelial cells (Anitha *et al*, 2008), and decreases the cell viability of BAECs which can be involved in macrovascular complications accompanying diabetes mellitus (Duffy *et al*, 2006). The results have shown that high concentrations of glucose at (30.6 to 105.6 mM) significantly reduced cell viability as shown in Figure 5.13. The exact mechanism of how high glucose induced reduced cell growth and viability not yet fully understood. It may be speculated that exposure of endothelial cells to high concentrations of glucose can activate oxidative stress transcription factor NFkB, believing that hyperglycemia could activate gene expression through activation of transcription factor of NFkB (Spanier *et al*, 2009)**.**

Figures 5.6 and 5.14 demonstrate that 1, 2, 4 and 5mM methylglyoxal inhibited BAEC proliferation and reduced viability. These effects occurred in dose-dependent manner compared to control. These results suggest that methylglyoxal induced oxidative stress by inactivation of antioxidant enzymes such as SOD, CAT and GPx by their glycation. Moreover, AGE-RAGE interaction results in strong oxidant formation such as superoxide anion, hence this leads to endothelial dysfunction. These results are in accordance with a previous study, which also found that 5 mM methylglyoxal inhibited the cell growth and induced cell cytotoxicity of BAEC *in vitro* (Tatsunami *et al*, 2009).

A large number of *in vivo* and *in vitro* studies have indicated that production and excessive accumulation of AGEs results in chronic hyperglycaemia and lead to diabetic complications

(Goh & Cooper, 2008). Subsequently, it has been suggested that a pivotal role in angiogenesis is played by the glycation of proteins through AGE synthesis. In the present study, AGEs have been utilized at a concentration of 10 μg/ml which was selected according to a previous study that reported 10 μg/ml AGEs may represent the lowest level of plasma AGEs detected in patients suffering from diabetes (Mashilipa *et al*, 2011). This study showed that 10 to 100 μg/ml AGEs inhibited BAEC proliferation *in vitro* as shown in Figure 5.7 and had a cytotoxic effect on BAECs as illustrated in Figure 5.15. The results suggested that AGEs may leads cell toxicity in human endothelial, epithelial and neuronal cells through elevation of oxidative stress have been shown in dose-dependent manner (Amornrit & Santiyanont, 2015), it is nevertheless possible exposure BAECs to AGEs may result to alteration in the cytoskeleton and cell permeability as mentioned by Esposito et al, (1989). These findings are consistent with previous studies, which also found that 10 μg/ml BSA-AGEs inhibit the growth of mesangial cells, and cause cytotoxicity in neuronal cells (Woltjer *et al*, 2003). Altogether, these effects are suggested to be induced by interaction of AGE-RAGE which are presented in different cell lines (Mruthinti *et al*, 2006).

The elevated formation of free radicals, and together with a decrease in the antioxidant potential can cause oxidative stress, which leads to injury of the cells components like proteins, lipids, DNAs and RNAs (Naziroğlu & Butterworth, 2005). Previous research illustrated that compounds with antioxidant property have therapeutic potential in the protection against AGEs-induced cellular toxicity (Zhang *et al*, 2006). The antioxidant properties of MC extracts were demonstrated in Chapter 4. Both HWE and CWE reduced the cell toxicity effects of glucose methylglyoxal and AGEs on BAEC cell proliferation (Figure 5.8, 5.9 and 5.10) respectively. Whether low concentrations of MC extracts can protect against AGEs-reduced BAEC viability need further study. These results suggested that this protection effect of MC extracts may be related to the combination of several bioactive compounds and proteins as mentioned previously. Recent investigations demonstrated that these compounds had a protective property on endothelial dysfunction and reduced the pathological progress of diabetic complications (Kumar R, 2009: Kubola & Siriamornpun, 2008). Nevertheless, more studies will be required to investigate radical scavenging activity in HWE and CWE of MC extracts to confirm that the inhibitory effects shown in the current results are from antioxidant properties resulting in cell protection by decreasing the toxicity of glucose, methylglyoxal and AGEs-induced oxidative stress in the cells.

AGEs deposit in the inner part of the blood vessel wall, which are could damage cell structure and function. AGES generated inside the body or obtained from ingested food could bind to RAGE, hence this leads to activating a cascade of intercellular signalling pathways, which are associated with diabetic complications. AGE/RAGE interaction results in intercellular ROS formation signalling pathways via NADPH oxidase, p38 MAP Kinase, NF-kB, $P21^{Ras}$ and JAK/STAT pathways (Lee *et al*, 2010). These pathways result in cellular responses including the changes in cell proliferation and cytotoxicity in endothelial cells (Odjakova *et al*, 2012).

Momordica charantia extracts downregulated the expressions of the pro-oxidative stress genes *NOX4*, *NOX5*, *HOX*1, $p47^{phox}$, $p22^{phox}$ and *GPx,* and upregulated the expressions of antioxidant enzyme genes *SOD2* and *CAT* in AGEs-treated BAECs (Figures 5.16). These data suggest that the protective effects of MC extracts against oxidative damage are likely due to the upregulation of endogenous cellular antioxidant enzymes such SOD and CAT rather than the direct ROS scavenging property of MC extracts. Moreover, upregulation of antioxidant enzyme and oxidative stress genes expressions by HWE of MC are greater than by CWE of MC. This may be related to higher amount of phenolic compounds in HWE of MC, because of that, the measurement of this compound was carried out in this study as shown in Table 4.1. However, further study requires to be done to identify the compounds that are responsible for the inducing antioxidant enzyme genes and inhibiting pro-oxidative stress genes.

Increased oxidative stress play a central role in the pathogenesis of several chronic conditions such as vascular diabetic complications (Förstermann, 2008). It has been well established that several enzymes such NADPH oxidases, xanthine oxidase, uncoupled endothelial NO synthase are involved in the excessive production and accumulation of ROS, but NADPH oxidase are more likely to be the major source of ROS in the wall of blood vessels especially in those patients suffered from vascular disorders like diabetic patients (Guzik *et al*, 2006).

In this study, 10 µg/ml AGEs significantly stimulated NOX4, NOX5, p-JAK2, total JAK2, p21 and RAGE expressions in BAECs as demonstrated in Figures 5.22-5.30. However, MC extract at the concentration of 15, 30, 60 and 90 µg/ml significantly attenuated AGE-induced oxidative stress signals. In addition, the inhibitory activity of MC extracts on AGE-induced oxidative stress was shown in a dose dependent manner (Figures 5.29 to 5.30). These findings suggest that MC extracts may intervene AGE-RAGE interaction to attenuate AGEs-induced oxidative damage.

In this study, MC extracts reduce the expression of NOX4, which is the main NOX family member in endothelial cells. Moreover, MC extracts-mediated attenuation of AGEs-induced oxidative stress in BAECs may correlate with downregulation of p-JAK and total JAK2. It has been reported p-JAK2 and total JAK were expressed at high levels in patients with diabetic complications. High glucose level cause formation of ROS via JAK-2 overexpression in mesangial cells (Berthier *et al*, 2009). Therefore, MC extracts have potential to be utilized in controlling ROS formation in diabetic patients by inhibiting JAK2 expression. These results obtained from this study indicated the contribution of a specific pathways, activation of NADPH oxidase (NOX4, NOX5), p-JAK2, total JAK2, RAGE and P21 by AGE-RAGE interaction, by which AGE-induced production of ROS and generating transduction events may cause altered gene expression in endothelial cells through RAGE. Moreover it has been reported that AGEs interact with RAGE leading cell death as a result of ROS (Morita *et al*, 2013)

AGEs are associated in in the pathogeneses of several chronic conditions such diabetes and its complications. Inhibiting the production of AGEs and interfering with AGEs-induced oxidative stress are two an important practicable goals for improving medicinal plants against diabetic complications. In this study, MC extracts exhibit the potent capacity not only against glucose, methylglyoxal and AGEs-induced oxidative stress and cytotoxicity in BAEC, but also downregulated mRNA expression of pro-oxidative stress regulator genes and upregulated antioxidant enzyme genes. These findings propose potential usefulness of the MC extracts as antioxidant agents and provide an insight into the molecular mechanism underlying the anti-oxidative activity of MC extracts. To this effect, if data from this current study further extended *in vivo*, then it can be proposed that ingestion of MC extracts might be useful in the long-term management of diabetes.

Chapter 6. General discussion, conclusion and future work

6.1 Discussion

Advanced glycation endproducts result from non-enzymatic reactions between free amino groups of proteins, lipids, DNA and the carbonyl groups of reducing sugars. They contribute to the development and progression of several chronic conditions such as the vascular complications of diabetes (Yamagishi, 2011). However, the main causes responsible for the development of these complications have not yet been fully understood. In this regards, glycation of protein is one of many pathways proposed to explain the relation between hyperglycaemia and the development of diabetic complications (Gul *et al*, 2008; Srikanth *et al*, 2011). The production and accumulation of AGEs in humans play a pivotal role in structural and functional changes in tissue proteins (Wu *et al*, 2009). A large number of studies have indicated that AGEs are a key factor in the pathogenesis of diabetic complications and that the amount of AGE build-up in tissues depends on the degree and duration of hyperglycaemia, the half-life of the modified protein, the amount and permeability of the cell to free glucose (Ahmed, 2005; Elosta *et al*, 2012). Long-lived proteins such as eye lens crystallin and collagen are particularly exposed to AGE modification (Berbaum *et al*, 2008).

Important novel findings have emerged from this study, which is discussed in relation to the publish literature described in the previous chapters. The reactions were studied under high-sugar concentrations to accelerate the reaction and reduce the incubation time. Different analytical techniques including the SDS-PAGE, spectrophotometer, ELISA were utilized to follow the glycation reaction. Long-term incubations of lysozyme with glucose cause the generation of cross-linked AGEs. The data obtained from this study indicated that HWE and CWE of MC have significant potential inhibition of the production of AGEs. This inhibition occurs in a dose-dependent manner and the inhibitory action of HWE is more potent than that of CWE of MC. Furthermore, several studies have reported that MC extracts contains amino acids that could reduce the formation of AGEs by the blockage of carbonyl groups of reducing sugar (Yuwai *et al*, 1999). Methylglyoxal is more reactive than the glucose and it can be produced by both fragmentation and dehydration of glucose and Amadori products. Methylglyoxal changes the lysine and arginine residues in proteins (Ahmed, 2005), mediates excessive cross-linking and produces ROS during protein glycation (Yim *et al*, 2001). In this study, MC extracts prevented the generation of methylglyoxal-derived cross-linked AGEs (Figures 3.10 and 3.12). The extracts of MC may act by blocking the alteration of dicarbonyl

intermediates to AGEs. Even though with utilising high concentrations of MC extracts, methylglyoxal was able to generate significant dimer. The excessive cross-linking induced by methylglyoxal due to its highly reactive nature may elucidate this. Previous studies have reported that methylglyoxal levels were increased in diabetic patients, and this resulted in the production of ROS (Artenie *et al*, 2002; Vander Jagt & Hunsaker, 2003). ROS increases the risk of oxidative stress and cellular impairment (Dizdaroglu *et al*, 2002). Therefore, preventing the formation of methylglyoxal-derived AGEs by MC extracts may decrease ROS production, and reduce the risk of oxidative stress in diabetic patients. ELISA experiments have demonstrated that the incubation of lysozyme with methylglyoxal resulted in the increased production of AGEs. The data obtained from this study indicated that HWE and CWE of MC have significant potential inhibition of the production of AGEs. This inhibition occurs in a dose-dependent manner and the inhibitory action of HWE is more potent than that of CWE of MC (Figure 3.21). AGE production is a very complicated process; to understand the inhibition mechanism of AGEs generation induced by MC extracts requires further investigation. Hung *et al* (1999) suggested that MC contains of several amino acids that could reduce the formation AGEs by blockage carbonyl molecules. This may result in reducing the production of AGEs (Botau *et al*, 2011; Yuwai *et al*, 1991).

A large and growing body of literature has reported that the medicinal plants possess great antioxidant potential. Antioxidants decrease the oxidative stress in cells and are therefore useful in curing many human diseases (Dhar *et al*, 2012; Tripathi & Chandra, 2009). The literature reveals that natural antioxidants within medicinal plants represent a potentially side-effect free alternative to synthetic antioxidants in the food processing industry and for the use in preventive medicine (Krishnaiah *et al*, 2011). MC has been used as a traditional medicine in different part of the world, throughout centuries for alleviating the symptoms of several diseases including diabetes (Leung *et al*, 2009). Each plant contains several components, only a few of which may be therapeutically effective. Different parts of the plants such as roots, seeds, fruits and bark contain different active ingredients (Krishnaiah *et al*, 2011). Furthermore, different active compounds may be obtained by using different extraction methods (Prabhakar & Mukesh, 2011). Owing to the role of medicinal plants with antiglycation properties, significant protection against the production of AGEs could be provided, since AGEs may play a pivotal role in the progression and development of diabetic complications. Using hot water as the extract solvent improves the ability of small components within the plant cells to dissolve readily into the medium solution as compared to cold water solvent (Tsai *et al*, 2006). It may

be that the potency of hot water solvent is related to the influence of the temperature (Pitipanapong *et al*, 2007), which enhances the solubility of the small components within the plant cells. The results obtained from this study are similar to the previous study stated that hot water extract of MC provides high levels of total phenols and potent antioxidant activity (Budrat & Shotipruk, 2008). A similar study on *Picralima nitida* medicinal plant has indicated that hot water extract contains higher amounts of active ingredients than the cold water extract (Nkere & Iroegbu, 2005). Moreover, previous work which indicated that generally HWE and CWE are most commonly used in the traditional method of preparing folk medicines (Ponnusamy *et al*, 2010), for its safety and bioavailability.

Results from this study demonstrated that MC extracts with high polyphenolic content represent potent antiglycation properties; this may leads to the differences in antioxidant and antiglycation ability between HWE and CWE of MC. Electron transfer-based antioxidant assays measure the capacity of antioxidants in the reduction of an oxidant, which changes the colour of the mixture when reduced (Apak *et al*, 2007). HWE of MC have shown more potent reducing activity effects than the CWE of MC. Recent evidence suggests that the antioxidant and free radical scavenging activities of phenolic molecules are generally derived from their redox properties, which may act as hydrogen atom donors to their phenolic hydroxyl groups (Siddhuraju, 2006). However, despite MC containing polyphenolic compounds, it also contains other substances such as ascorbic acid which may contribute to the antioxidant capacity of polyphenols. However, there are a considerable number of studies suggested that natural polyphenolic antioxidant molecules extracted from plants could be substitute for the synthetic antioxidants (Qader *et al*, 2011).

In this study, pro-antioxidant enzyme activity of MC extract was investigated for the first time in the presence of low concentrations of AGEs. Even though the mechanism behind this activity is not fully understood, it may be related to the presence of polyphenolic molecules such as catechine, quercetine, trans-chalone, caffeine, caffeic acid and gallic acid compounds, which possess higher antioxidant and free radical scavenging properties (Nerurkar *et al*, 2011). The mechanism of antioxidant enzymes and antioxidant compounds in the inhibition of free radical formation may involve breaking the chain reactions, chelating the transition metal catalysts and decreasing the concentrations of ROS. Furthermore, elevating the expression of genes encoding the antioxidant enzymes SOD, CAT and GPx, may result in upregulated endogenous antioxidants (Halliwell & Gutteridge, 1999; Semiz & Sen, 2007). SOD, CAT and ROS assays are widely utilised to measure the antioxidant capacity of plant extracts due to their simplicity,

low cost, stability and reproducibility (Hwang *et al*, 2013). The results demonstrated that MC extracts significantly increased SOD and CAT antioxidant activity. A previous study indicated that MC extracts significantly increased antioxidant enzymes such SOD and CAT in diabetic rats (Semiz & Sen, 2007). SOD and CAT account for the most potent free radical scavenging enzymes that have been shown to be decreased in a variety of chronic diseases like diabetes (Cohen & Heikkila, 1974). This activity of MC extract may related to its free radical scavenging properties, which could play a central role against pathophysiological modifications caused by the presence ROS. These results confirm a similar previous study (Chiang *et al*, 2006), which found that antioxidative effects exerted by rice black extracts are induced via decreases in free radical formation as well as elevations in SOD and CAT activities both *in vitro* and *in vivo*. The antioxidant effect of MC extracts might induce its antioxidant effects via suppression of ROS and the induction of antioxidant enzyme activities. These findings clearly showed the pro-antioxidant activity of MC extracts in glucose, methylglyoxal and AGE-induced oxidative stress in BAEC, although further work is needed to determine the active compounds in MC extracts that are responsible for its pro-antioxidant activity

Oxidative stress accelerated by hyperglycaemia-induced production of ROS largely influences the development and progression of diabetic complications such as retinopathy, neuropathy, nephropathy and delay in wound healing (Gao & Mann, 2009). At the cellular level, oxidant insult evokes several responses including proliferation, apoptosis and growth arrest (Martindale & Holbrook, 2002). The present study has shown that at low concentrations of AGEs, methylglyoxal and glucose decrease BAEC proliferation and viability, which are supposed to play a central role in the inhibition of angiogenesis in diabetic complications. In addition, AGEs production may cause changes in the functions of macrophages and biological activities of endothelial cells which are involved in angiogenesis, hence this leads, at least in part, to the elevated susceptibility of diabetic patients to complications (Liu *et al*, 1999). Nevertheless, in this regards, pro-angiogenic action of HWE and CWE of MC was investigated in the presence of glucose, methylglyoxal and AGEs. It was found that in the presence of toxic glycated protein, MC extracts results in significant stimulation of cell proliferation and protective activity on glucose, methylglyoxal and AGE-induced toxicity. In this regards the activity MC extracts are not fully understood, however, the results suggest that MC extracts may act as an inhibitor of oxidative stress through the extracellular inhibition of AGE formation as well as intercellular ROS scavenging in BAECs.

To the best of our knowledge, this is the first study to evaluate the relationship between the inhibitory effects of MC extracts and pro-antioxidant enzyme and pro-oxidative mRNA expression genes in BAEC exposed to AGEs. Figure 5.16 illustrated that the MC extracts inhibit pro-oxidative stress enzyme activity by reducing *HOX1, NOX5, NOX4, P47^{phox}, P22^{phox}* mRNA gene expression. In contrast, MC extracts upregulated pro-antioxidant enzymes *SOD* and *CAT* mRNA expression genes. Recently, a similar study using the medicinal plant *Nigella sativa* extracts has shown the effect in oxidant-induced renal injury in rats (Bayrak *et al*, 2008). The AGE-RAGE interaction could play a significant role in the pathogenesis of diabetic complications such retinopathy and agents capable of attenuating RAGE signalling could provide an advantage in preventing these complications (Zong *et al*, 2010). AGEs can bind and stimulate RAGE, thus resulting in intercellular signal transduction associated with varieties of chronic disorders such diabetic vascular complications (Yamagishi, 2011). The interaction between AGEs and RAGE results in the generation of oxidative stress and activation of several signalling pathways including NADPH oxidase, p.JAK2/STAT3, MAPK and, most importantly, NF-kB through activation of the p21ras protein (Gasic-Milenkovic *et al*, 2003). Previously, a study has indicated that NADPH oxidase-mediated activation of AGEs/ RAGE signalling plays an important role in regulating oxidative stress and endothelial dysfunction in diabetes mellitus. NADPH oxidase such as NOX4 and NOX5, JAK2, total p-JAK2, p21 and RAGE were studied to understand the molecular mechanism and cellular consequence behind the action of MC extracts (Figure 5.17-5.30). This result confirms other research work and verifies the inhibitory effect of MC extracts on oxidative stress-signalling pathways genes in BAEC (Cheng *et al*, 2012; Nerurkar *et al*, 2011). In addition, it has been well established that AGEs interact with RAGE generating programmed cell death with the contribution of ROS (Morita *et al*, 2013). Moreover, down regulation *HOX1, NOX5, NOX4, P47^{phox}, P22^{phox}* and upregulated pro-antioxidant enzymes *SOD* and *CAT* mRNA expression genes by MC extracts required more investigation. The current study suggested for the first time MC extracts inhibited activation of NOX4 and NOX5, JAK2, total p-JAK2, p21 signalling through RAGE. These activities of MC extracts may be related to its multiple activities of the extract, which derived from its active ingredients. As noted by (López-Alarcón & Denicola, 2013), antioxidant activity is not only involved in scavenging free radicals, but also includes upregulation of antioxidant, detoxifying enzymes, modulation of redox cell signalling and gene expression.

The promising results obtained in this study with HWE and CWE of MC *in vitro* encouraged us to carry out an *in vivo* study.

6.2 Conclusion

The findings of the present study have shown that AGEs causes structural changes, which fundamentally influence the physical and chemical properties of proteins. These data support the contribution of protein glycation and oxidation in the development and progression of diabetic complications. The results have demonstrated antiglycation activity of MC extracts on glucose and methylglyoxal-induced AGEs generation *in vitro*. Moreover, MC extracts provides antiglycation activity by the prevention of AGE accumulation. Such inhibitory effects are mainly contributed to the antioxidant activities. This finding suggests that the daily consumption of MC extracts is considered to be of potential benefit for the prevention of chronic complications such those associated with diabetes. MC extracts have been shown to be rich sources in polyphenol compounds, which are mainly responsible for their antioxidant activities. Based on these results and previous studies, this study can conclude that the extracting solvent markedly influenced the antioxidant and total phenolic compounds of the MC extracts. Clearly, there was a significant difference between HWE and CWE of MC, thus, HWE possess more potent antioxidant activity and contain more total phenolic compounds. Moreover, MC extracts have increased antioxidant enzymes and reduced ROS *in vitro*. In addition to the already published findings, MC extracts inhibit pro-oxidative stress enzyme activity by reducing *HOX1, NOX5, NOX4,* $P47^{phox}$*,* $P22^{phox}$ mRNA gene expression. In contrast, MC extracts upregulated pro-antioxidant enzymes *SOD* and *CAT* mRNA expression genes. MC extracts have demonstrated an inhibitory effect on oxidative stress-signalling pathways proteins such NOX4, NOX5, JAK2, total p-JAK2, p21 and RAGE in BAEC. MC extracts may be a potentially novel medicinal plant to reduce hyperglycaemia and protects against the development of diabetic complications.

6.3 Future work

Further studies related to the current project may be carried out in the following areas:

- Matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALD-TOF-MS) will be used to study formation, qualitative determination of AGEs and antiglycation property of *Momordica charantia*.

- Administration of MC extracts to animal models that may be revealing the effect on *in vivo* AGEs and progression of diabetic complications. Further clinical exploration in diabetic retinopathy rats to investigate these experimental findings and to improve *Momordica charantia* as potential cataract therapy for the management of experimental diabetic retinopathy.

- Protein micro-array will be utilised for a broad-spectrum investigations of signalling pathways induced by AGEs and MC extracts. The investigations of the cytoplasmic proteins that are involved in AGEs and MC extracts induced cell signal transduction will provide a new/deeper insight into mechanisms of how MC extracts against AGEs-induced oxidative stress.

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Chapter 8. Appendex

Manchester Metropolitan University

Antiglycation property of *Momordica harantia*

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Introduction

Diabetes mellitus (DM) is a multifactorial disorder characterized by hyperglycemia. Hyperglycemia may lead to diabetic complications such as nephropathy, neuropathy, retinopathy and delayed wound-healing via increased formation of advanced glycation endproducts (AGEs) and glycation-derived free radicals. A diversity of plants are utilized worldwide as traditional medications for the treatment of diabetes mellitus including *Momordica Charantia* (MC). Momordica charantia contains several active compounds, which are present in the leaves, fruits and stems of the flora.

Aims

The aims of this study is to investigate antiglycation property of hot water extracts (HWE) and cold water extracts (CWE) of MC *in vitro*.

Methods

Fresh MC was obtained from the local Asian supermarket. Two separate extracts of MC were prepared using hot water extraction (HWE) and cold water extraction (CWE). Using lysozyme as a model protein and two different carbohydrate (glucose and methylglyoxal), samples were incubated with different concentration of MC extracts under *in vitro* conditions. The formation and inhibition of AGEs were determined using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), and enzyme linked immunosorbent assay (ELISA).

Results

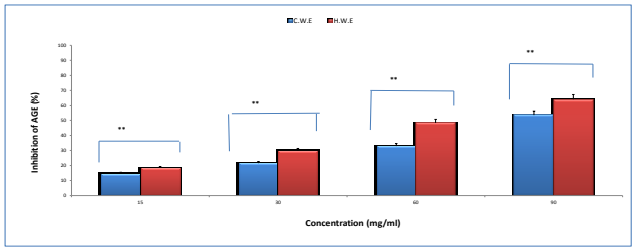

Figure 1: The effect of MC on AGEs production as measured by an ELISA. The graph demonstrates the inhibition effect of HWE and CWE of MC at different concentrations on AGEs levels. Each value represents the mean ± SD (n=3) from three independent experiments, **$p < 0.01$.

SDS-PAGE analysis of AGEs inhibition by HWE of MC in lysozyme and methylglyoxal system

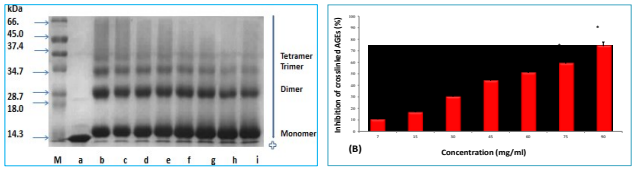

Figure 2: (A) SDS-PAGE analysis of AGEs prevention by HWE of MC in lysozyme and methylglyoxal system. (B) The bar graph demonstrates scanning of SDS-PAGE gel to show the effect on methyglyoxal-lysozyme (10 mg/ml) with different concentration of HWE of MC in 0.1 M sodium phosphate buffer of pH 7.4 at 37°C for one week. Each value represents the mean ± SD (n = 3), * $p < 0.05$.

SDS-PAGE analysis of AGEs inhibition by CWE of MC in lysozyme and methylglyoxal system

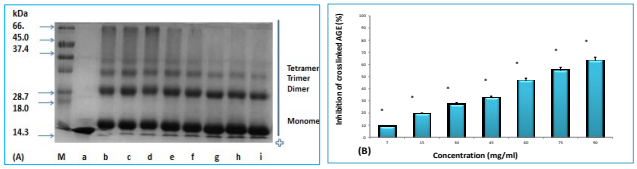

Figure 3: (A) SDS-PAGE analysis of AGE prevention by CWE of MC in lysozyme and methylglyoxal system. (B) The bar graph demonstrates scanning of SDS-PAGE gel to show the effect on methyglyoxal-lysozyme (10 mg/ml) with different concentration of HWE of MC in 0.1 M sodium phosphate buffer of pH 7.4 at 37 °C for 1 week. Each value represents the mean ± SD (n = 3), * $p < 0.05$.

SDS-PAGE analysis of AGE inhibition by HWE of MC in lysozyme and Glucose system.

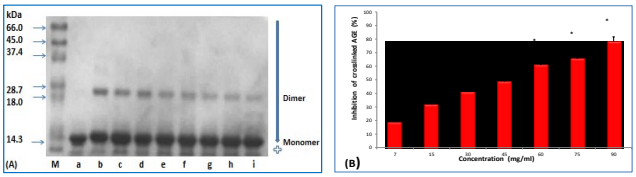

Figure 4: (A) SDS-PAGE analysis of AGE prevention by HWE of MC in lysozyme and glucose system. (B) The bar graph demonstrates scanning of SDS-PAGE gel to show the effect on glucose-lysozyme (10 mg/ml) with different concentration of HWE of MC in 0.1 M sodium phosphate buffer of pH 7.4 at 37 °C for 1 week. Each value represents the mean ± SD (n = 3), * $p < 0.05$.

SDS-PAGE analysis of AGE inhibition by CWE of MC in lysozyme and Glucose system.

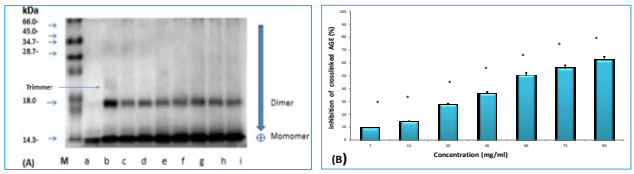

Figure 5: (A) SDS-PAGE analysis of AGE prevention by CWE of MC in lysozyme and glucose system. (B) The bar graph demonstrates scanning of SDS-PAGE gel to show the effect on glucose-lysozyme (10 mg/ml) with different concentration of HWE of MC in 0.1 M sodium phosphate buffer of pH 7.4 at 37 °C for 1 week. Each value represents the mean ± SD (n = 3), * $p < 0.05$.

Conclusion

The results have demonstrated that HWEs and CWEs of MC have antiglycation activities. The Elisa analysis showed both extracts were effective at inhibiting AGE production; the highest concentration at 90mg/ml of HWE and CWE of MC managed to inhibit AGE by over 64% and 53% respectively, compared to the positive control. The SDS-PAGE analysis demonstrated that the dose-dependent inhibition occurred in both lysozyme-methylglyoxal and Lysozyme-glucose AGE systems by HWE and CWE of MC. In both methods, HWE of MC have shown greater inhibitory effects than the CWE of MC. One possible explanation of this result is that MC is an important source of phenolic compounds which possess strong antioxidant activity (Krishnaiah *et al*., 2011). Moreover, hot water is a suitable and environmentally benign solvent that could enhance the total phenolic compounds extracted from this plant. The amount of active ingredient within the extract depends on the methods and the solvent used for extraction (Platel and Srinivasan, 1995; Budrat and Shotipruk, 2009; Kumar *et al*., 2009).

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THE PLANT EXTRACT MOMORDICA CHARANTIA REDUCES EXPRESSION OF VASCULAR CALCIFICATION MARKERS IN A SMOOTH MUSCLE CELL CULTURE MODEL

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The onset of vascular calcification in type-2 diabetic patients is one of the major health burdens in the industrialised world due to the associated increase in comorbidities and mortality. The fruits of the subtropical vine *Momordica Charantia* are thought to have potent anti-diabetic effects due to the anti-hyperglycaemic, anti-oxidant and anti-inflammatory properties of its 200+ constituents, some of which may mimic the effect of insulin.

The aim of this study was to investigate the inhibitory effect of *Momordica Charantia* extract on vascular calcification pathways in a cell culture model. Vascular smooth muscle cells (VSMC) were isolated and cultured from femoral arteries following amputation from diabetic patients. Osteogenic differentiation was induced using β-glycerophosphate (β-GP), methylglyoxal (MGO) and additional advanced glycated end-products (AGEs). The cells were subsequently treated with varying concentrations of *Momordica Charantia* extract to determine the anti-calcification effects *in vitro* using qPCR, Alkaline phosphatase (ALP) activity and Alizarin red staining.

We demonstrate that *Momordica Charantia* reduced gene expression of a range of biomarkers linked with vascular calcification after 4 days in a dose-dependant manner, including osteocalcin, BMP-2, C-MET and NOX-1, compared with osteogenic controls. ALP activity was also reduced in treated cells compared with osteogenic controls. *Momordica Charantia* extract shows promise as a potential therapeutic intervention to reduce calcification. Several small-scale clinical studies have shown the benefit of dietary supplementation in reducing the effects of diabetes, however further work is required to identify the specific component responsible for the effects, elucidate the mechanisms involved and validate the response *in vitro*.

The effect of *Momordica charantia* fruit extract on advanced glycation endproducts (AGEs) (oral presentation)

Niazi Nazhad (School of Healthcare Science, Manchester Metropolitan University). Manchester, UK.

Abstract

DM is a multifactorial disorder characterised by hyperglycaemia. DM caused complications such as nephropathy, neuropathy, cataracts and delayed wound-healing. Non-enzymatic reaction between reducing sugar and protein produce advanced glycation endproducts (AGEs). Non-enzymatic reaction between reducing sugar and protein produce advanced glycation endproducts (AGEs).Accumulation of toxic AGEs can permanently alter the structure and function of body proteins in diabetes mellitus. A variety of plants are used worldwide as traditional medicines for the treatment of DM especially in developing countries. *Momordica charantia* has been used to treat diabetes mellitus as a potent folk medicine. *Momordica charantia* contains several active compounds, which are present in the leaves, fruits and stems of the plant. Phenolic and flavonoids (e,g Charantin) compounds believe to be responsible for antiglycation antioxidant properties of MC (Snee *et al.,* 2011; Joseph and Jini, 2013). The aim of the current study is to investigate the antiglycation and antioxidant effects of hot water extract (H.W.E) and cold water extract (C.W.E) of *Momordica charantia* (MC).

The effect of *Momordica charantia* fruit extract on glycation protein (oral presentation)

Niazi Nazhad (School of Healthcare Science, Manchester Metropolitan University). Manchester, UK.

Abstract

Diabetes mellitus is a multifactorial disorder characterised by hyperglycaemia and this leads to form advanced glycation endproducts (AGEs) and this cause diabetes complications. Plant extract of *Momordica charantia* is used worldwide as traditional medicines for the treatments of diabetes mellitus especially in developing countries. The aim of this study is to investigate the antiglycation and antioxidant effects of hot and cold water extracts of *Momordica charantia* on protein glycation.

Advanced glycation endproducts formation and inhibition were monitored by a number of methods including sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), enzyme linked immunosorbent assay (ELISA), and fluorescent spectrometry. Furthermore, superoxide dismutase assay (SOD) and catalase assay (CAT) were used to study antioxidant properties of *Momordica charatnia* on bovine aortic endothelial cell in a cell culture model,

The results have shown that hot and cold water extracts of *Momordica charantia* inhibit the production of AGEs in a dose-dependent manner. Additionally, the extraction efficiency of the hot water extracts was found to be highly influenced by temperature. Advanced glycation endproducts leads to creation of free radical species in all types of cells. These may contribute towards diabetic complications. Thus the aqueous extract of *Momordica charantia*, an edible vegetable, may have therapeutic potential in the management of diabetes mellitus.

Antiglycation and antioxidant properties of *Momordica charantia*

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Abstract

Diabetes mellitus is a multifactorial disorder characterised by hyperglycaemia and leads to complications such as nephropathy, neuropathy, cataracts and delayed wound –healing. The complications are caused by advanced glycation endproducts (AGEs) that form via protein glycation as a consequence of hyperglycaemia. A variety of plants are used worldwide as traditional medicines for the treatments of diabetes mellitus especially in developing countries. A considerable amount of literature has been published on *Momordica charantia* as a potent folk medicine for diabetes mellitus. *Momordica charantia contains* more than 230 active compounds, which are present in the leaves, fruits and steam of the plant.

The aim of this current study is to investigate the antiglycation and antioxidant effects of hot and cold water extracts of *Momordica charantia* on protein glycation.

Model protein such as lysozyme were glycated using sugars (glucose and methylglyoxal and ribose) as glycating agents both in the presence and absence of *Momordica charantia* extracts. Advanced glycation endproducts formation and inhibition were monitored by a number of methods including sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), enzyme linked immunosorbent assay (ELISA), and fluorescent spectrometry. Furthermore, superoxide dismutase assay (SOD) and catalase assay (CAT) were used to study antioxidant properties of *Momordica charania* on bovine aortic endothelial cell in a cell culture model,

The results have shown that hot and cold water extracts of *Momordica charantia* inhibit the production of AGEs in a dose-dependent manner.

Moreover, hot water extracts of *Momordica charantia* showed more potent antiglycation and antioxidant properties in contrast to the cold water extracts. Additionally, the extraction efficiency of the hot water extracts was found to be highly influenced by temperature. Advanced glycation endproducts leads to creation of free radical species in all types of cells. These may contribute towards diabetes complications. Thus the aqueous extract of *Momordica charantia* , an edible vegetable, may have therapeutic potential in the management of diabetes mellitus.