Protective Effects of Dietary Coumarins on Disease Conditions Associated with Oxidative Stress: Neurodegenerative Diseases and Cancer

By

Sudhakar Raja Subramaniam

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Strathclyde Institute of Pharmacy and Biomedical Sciences

University of Strathclyde, Glasgow, UK

This thesis is the result of author's original research. It has been composed by the author and has not been previously submitted for the examination which has lead to the award of a degree.

Sudhakar Raja Subramaniam

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Abstract

Oxidative and nitrosative stress has been related to various pathological conditions in humans including neurodegenerative diseases, cancer, cardiovascular diseases and aging. The involvement of oxidative stress in disease conditions has stimulated huge interest in novel antioxidant strategies for therapeutic treatment. In this work, the protective effects of dietary coumarin compounds against oxidative stress-induced neurotoxicity and hepatotoxicity were investigated using *in vitro* and *in vivo* models. Dietary coumarin compounds showed neuroprotective effects in neuronal cell lines against oxidative stress induced by hydrogen peroxide and 1-methyl-4-phenyl-pyridinium (MPP^+). In addition, dietary supplementation of coumarins to C57BL/6J mice reduced MPTP-induced dopaminergic neurotoxicity. These neuroprotective effects of coumarins appear to be through their ability to maintain intracellular GSH levels, reduce ROS levels and peroxynitrite production, which lowered JNK activation and apoptosis. Treatment with coumarins lowered MPTP-induced elevation of Bax levels, inhibited the consequent Cytochrome-c release, decreased caspase-3 induction in dopaminergic neurons and up-regulated expression of NRF2/ARE-dependant protective enzymes. Moreover, esculetin (6,7-dihydroxycoumarin) showed a powerful protective effect against hydrogen peroxide-induced oxidative stress in human hepatoma HepG2 cells lines. Further, the supplementation of coumarin compounds in diet protected Wistar rats against NDEA-induced hepatotoxicity. Treatment with coumarin compounds increased cell survival, maintained redox status, reduced hepatic injury, decreased lipid peroxidation, and maintained cell architecture and histological characteristics of hepatocytes in rats. Furthermore, the coumarin compounds enhanced the antioxidant defence systems both *in vitro* and *in vivo* in different tissues including liver, brain and kidneys in mice and rats, by the induction of NRF2/ARE-dependant antioxidant genes such as NQO1, HO1 and GST. In conclusion, the present study shows that coumarin compounds protect cells from oxidative damage, and this protective effect may be due to their ability to maintain the cellular redox homeostasis and enhance endogenous antioxidant defence systems, in addition to their known free radical scavenging properties.

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List of abbreviations

%	percent
×g	times gravity
µl	micro litre
µM	micro molar
4-HNE	4-hydroxynonenal
AKR	aldoketo reductase
AKT/PKB	protein kinase B
APS	ammonium persulfate
ARE	antioxidant response element
ATCC	American Type Culture Collection
Bax	Bcl_2-associated X protein
Bcl_2	B-cell lymphoma 2 protein
Bcl_{XL}	B-cell leukaemia XL protein
BHA	butylated hydroxyanisole
BHT	butylated hydroxyl toluene
casp	caspase
CBP	CREB binding protein
CDNB	1-chloro-2,4-dinitrobenzene
DCF-DA	dichlorofluorescin diacetate
DCPIP	2,6-dichlorophenolindophenol
dH_2O	distilled water
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
DTNB	5,5'-dithiobis-2-nitrobenzoic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ERK	extracellular signal-regulated kinases
Esc	esculetin
FAD	flavin adenine dinucleotide
FBS	fetal bovine serum

g	grams
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GRed	glutathione reductase
GSH	reduced glutathione
GSSG	glutathione disulfide (oxidised glutathione)
GST	glutathione S-Transferase
H_2O_2	hydrogen peroxide
HAM-F12	Ham's nutrient mixture F-12
HepG2	human hepatocellular carcinoma cell line
HO1	hemeoxygenase 1
h/hrs	hours
IgG	Immunoglobulin G
IP	Intraperitoneal
JNK	c-Jun NH_2-terminal kinase
Keap-1	Kelch-like ECH-associated protein 1
LDH	lactose dehydrogenase
LSB	Laemmeli's sample buffer
Maf	musculoaponeurotic fibrosarcoma protooncogene
MAPK	mitogen-activated protein kinase
MEM	modified Eagle's medium
min	minutes
ml	milli litre
mM	milli molar
MPP^+	1-Methyl-4-phenylpyridinium ion
MPTP	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAC	N-acetylcysteine
NAD	Nicotinamide adenine dinucleotide
NADPH	reduced form of Nicotinamide adenine dinucleotide phosphate
NDEA	N-Nitroso diethylamine
nm	nanometres
nM	nanomolar

NQO1	NADPH: quinone oxidoreductase 1
NRF2	nuclear factor-E2-related factor 2
^{o}C	degree Celsius
OD	optical density
p-AKT	phosphorylated protein kinase B
PBS	phosphate buffer saline
PCR	polymerase Chain Reaction
p-ERK	phosphorylated extracellular signal-regulated kinases
PI3K	phosphatidylinositol 3-kinase
p-JNK	phosphorylated c-Jun NH_2-terminal kinase
p-NBA	p-nitrobenzaldehyde
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
SH-SY5Y	human neuroblastoma cell line
SOD	superoxide Dismutase
SSA	sulfosalicylic acid
TEMED	NNN-N'-tetramethylethylenediamine
TNB	5-thio-2-nitrobenzoic acid
Umb	umbelliferone
v/ v	volume/ volume
w/ w	weight/ weight
γ-GCS	gamma-glutamylcysteine synthetase

CHAPTER 1

INTRODUCTION

1. Introduction

The importance of free radicals in biology and medicine was realised when two major scientific advances were made: the discovery of CuZnSOD by McCord and Fridovich in 1968 and the report by Bernie Babior in 1973 that indicated that activated neutrophils produce superoxide for microbicidal action as a part of the immune response. After these findings, research on free radicals gained momentum particularly in the last few decades, which has led to many interesting discoveries including the hypothesis that free radicals play an active dual role in both beneficial and deleterious effects on the biological system.

It was found that reactive species (including free radicals and non-radicals) are products of normal cellular metabolism and are involved in various physiological processes. At normal low cellular concentrations the reactive species (RS) exert beneficial effects to the biological system, which include phagocytosis, enzyme catalysis, electron transport, signal transduction and gene expression, and activation of transcription factors. (Babior et al., 2002, Shapiro, 2003, Bernhagen, 2005), (Eklund et al., 2001, Knappe and Wagner, 2001, Whittaker, 2005, Dunford, 1995, Sumimoto et al., 2005, Brandes and Kreuzer, 2005, Edens et al., 2001). In contrast, overproduction of RS such as reactive oxygen species (ROS) and reactive nitrogen species (RNS), when not successfully compensated by the antioxidant defence system, leads to cell injury and is related to various pathological conditions in humans including cancer, neurodegenerative diseases, cardiovascular diseases, diabetes, rheumatoid arthritis and aging (Halliwell B., 2007, Valko et al., 2007).

The following sections give a general background on free radicals in biological systems and examine the role of oxidative stress in cancer and neurodegenerative diseases. The sections provide a mechanistic insight into various cellular and molecular aspects of oxidative stress and disease conditions. Endogenous antioxidant defence systems and novel antioxidant strategies for therapeutic treatment of diseases with an underlying oxidative stress are also discussed in detail.

1.1 Free Radicals in Biology

Oxygen appeared in earth's atmosphere about 2.2 billion years ago, under an atmosphere containing mainly nitrogen and carbon dioxide. Living organisms appeared much earlier to this period and they were anaerobes. It is believed that oxygen was introduced to earth's atmosphere by anaerobic bacteria called cyanobacteria, as a by-product of its photosynthesis to produce energy. From there on, the oxygen content in the earth's atmosphere significantly increased that it became toxic to living organisms, which forced them to the evolutionary path of 'adapting' to the high oxygen levels. Many organisms were killed by its toxicity, some adapted by 'restricting' themselves to oxygen free environments (anaerobes) and the others instead began to evolve 'antioxidant defence systems' to survive (aerobes). In addition, the aerobes including complex multi-cellular organisms such as mammals used oxygen for beneficial purposes such as metabolic transformations through enzymes and energy production by the electron transport chains and oxidative phosphorylation. One question that arose was 'what causes the toxicity of oxygen'? In 1954, Rebecca Gershmann and Daniel L. Gilbert (Gershman, 1954) proposed that the toxic effect of oxygen is due to the 'free oxygen radicals' that impair key enzymes and biomolecules in the cells.

Halliwell and Gutteridge (Halliwell and Gutteridge, 2007) defined a free radical as 'any species capable of independent existence that contains one or more unpaired electrons'. However, toxicity is not only due to free radicals that are present in the biological system but also non-radicals. Reactive species (RS) is a collective term used to denote free radicals and non-radical derivatives. The RS of interest in biology and medicine are reactive oxygen species (ROS) and reactive nitrogen species (RNS).

1.1.1 Reactive Oxygen Species (ROS)

ROS are free radicals derived from oxygen and are considered to be the most important class of free radicals generated in the living systems (Miller et al., 1990).

ROS includes free radicals such as superoxide radical, hydroxyl radical, peroxyl and alkoxyl radicals. Various forms of ROS and their formation pathways are outlined in Fig 1.1.

1.1.1.1 Superoxide Radical

Superoxide anions are produced endogenously mostly within the mitochondria of a cell (Cadenas and Sies, 1998). Superoxide radicals are generated when there is an electron leak during energy transduction in the mitochondrial electron transport chain. The superoxide radicals evolved through this process are known as 'primary ROS', and can interact with other molecules to form 'secondary ROS, through enzyme or metal mediated catalysis (Valko et al., 2005).

1.1.1.2 Hydroxyl Radical

The hydroxyl radical is produced in the biological system mainly by the breakdown of hydrogen peroxide (H_2O_2) by transition metals (Fe^{2+}, Cu^{+} and others) through the Fenton reaction. This radical has a short half-life and is highly reactive which makes it a dangerous radical (Pastor et al., 2000). Due to its high reactivity it reacts with polyunsaturated fatty acids (PUFAs) to initiate lipid peroxidation process, which results in formation of toxic products that can damage cellular components.

1.1.2 Reactive nitrogen Species (RNS)

Nitric oxide and peroxynitrite are the biologically important free radicals derived from nitrogen known as reactive nitrogen species. Nitric oxide is an important radical, which is produced in the tissues by the enzyme nitric oxide synthase (NOS) during the metabolic conversion of arginine to citrulline. Nitric oxide was acclaimed to be 'molecule of the year' in 1992 Science magazine (Koshland, 1992) due to its

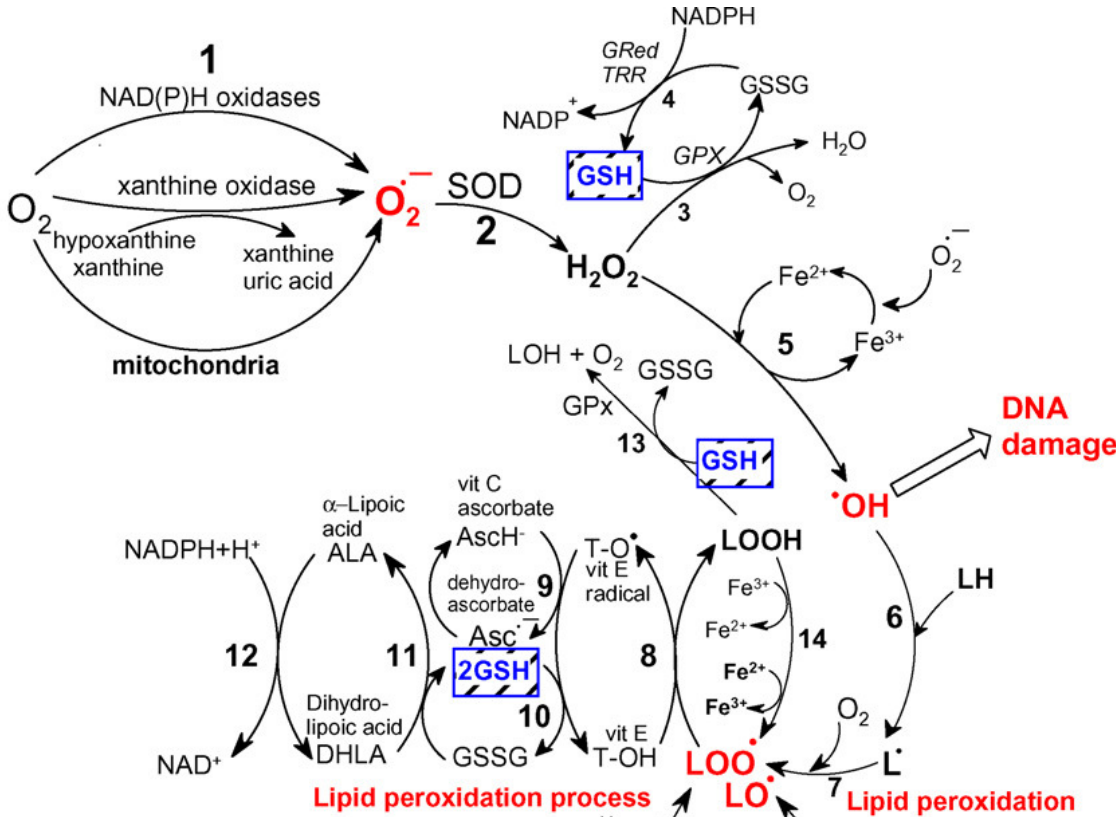


Fig. 1.1 - Pathways of ROS formation, the lipid peroxidation process and the role of glutathione (GSH) and other antioxidants (Vitamin E, Vitamin C, lipoic acid) in the management of oxidative stress (equations are not balanced) (Valko et al., 2007). *Reaction 1:* Superoxide free radical is generated by reduction of molecular oxygen, by the enzymes xanthine oxidase and NAD(P)H oxidases or non-enzymatically by redox-reactive compounds of the mitochondrial transport chain. *Reaction 2:* Superoxide radical is dismutated by superoxide dismutase (SOD) to hydrogen peroxide (H_2O_2). *Reaction 3:* H_2O_2 is converted to water and oxygen by glutathione peroxidase in the presence of reduced glutathione (GSH) as electron donor. *Reaction 4:* Oxidised glutathione (GSSG) is converted back to GSH by glutathione reductase using NADPH as electron donor. *Reaction 5:* Transition metals such as Fe^{2+} and Cu^{+} can react with H_2O_2 (Fenton's reaction) to form highly reactive hydroxyl radical ($^{\bullet}OH$). *Reaction 6:* Hydroxyl radical can abstract an electron from polyunsaturated fatty acids (PUFA's) to form lipid radical ($^{\bullet}L$). *Reaction 7:* Lipid radical can then interact with molecular oxygen to generate lipid peroxyl radical ($LOO^{\bullet}$), which if not effectively scavenged by antioxidants leads to lipid peroxidation process. *Reaction 8:* Lipid peroxyl radical is reduced within the membrane by (reduced) vitamin E (T-OH). *Reaction 9:* Vitamin E is regenerated from vitamin E radical ($T\text{-}O^{\bullet}$) by vitamin C (ascorbate). *Reaction 10:* Vitamin E is regenerated from vitamin E radical ($T\text{-}O^{\bullet}$) by GSH. *Reaction 11:* GSSG and ascorbyl radical ($Asc^{\bullet -}$) are reduced back to GSH and ascorbate ($AscH^{-}$) by dihydrolipoic acid (DHLA). *Reaction 12:* DHLA is regenerated from α-lipoic acid (ALA) using NADPH.

extraordinary properties of ‘oxidative biological signalling’. This radical is involved in various physiological process-signalling including neurotransmission, blood pressure regulation, defence mechanisms, smooth muscle relaxation and immune response (Bergendi et al., 1999). The combination of superoxide radical and nitric oxide generates peroxynitrite radical, which is a potent oxidant. The production of peroxynitrite radical occurs in the immune cells during the ‘oxidative burst’ triggered during inflammation. High levels of peroxynitrite radical can cause DNA fragmentation and lipid peroxidation in the biological systems (Carr et al., 2000).

1.2 Oxidative stress

Oxidative stress is a condition in which more oxidants are produced than can be scavenged and leads to an imbalance in the oxidant-antioxidant equilibrium in the biological system. Persistent oxidative stress causes harm to cellular structures including nucleic acids, lipids and proteins resulting in oxidative damage. Elevated ROS causes cell injury mainly by damaging cellular components such as DNA, proteins and polyunsaturated lipids. ROS can further react with nitric oxide and other radicals to initiate lipid peroxidation processes, resulting in production of toxic compounds such as MDA (malondialdehyde) and 4-HNE (4-hydroxynonenal), which is discussed later in this chapter. The antioxidant defence system is very essential for protection from oxidative damage and comprises of endogenous substances and dietary components which function in combination. However, unfortunately in certain environmental, pathological, dietary and lifestyle conditions the antioxidant defence system fails and the resulting accumulation of ROS results in severe cellular damage. Hence, oxidative stress has been related in various pathological conditions in humans including cancer, neurodegenerative diseases, cardiovascular diseases, diabetes, rheumatoid arthritis and aging (Halliwell B., 2007, Valko et al., 2007).

1.3 Cellular response to oxidants

The extent of oxidative damage to cellular components depends on the severity of the attack and the cell type. Therefore the cellular response to oxidants may also vary accordingly (Fig. 1.2). According to Halliwell (Halliwell B., 2007), it may lead to

i. *Increased proliferation:* many cells respond to mild oxidative stress by increased proliferation.
ii. *Adaptation:* cells tolerate mild to moderate oxidative stress by up-regulation of the defence systems, which may completely or partially protect them against the damage.
iii. *Cell injury:* it involves damage to cellular targets such as DNA, lipids, protein and carbohydrate.
iv. *Senescence:* the cells survive but no longer divide and grow.
v. *Cell death:* cell injury, especially to DNA, may trigger cell death by apoptosis or necrosis or by any other intermediatary mechanisms.

1.4 Cellular consequences of oxidative damage

The biomolecular damage caused by the attack of reactive species upon constituents of the living organisms is known as oxidative damage.

1.4.1 Damage to nucleic acids

Damage to the DNA usually leads to cell death via apoptosis but otherwise increases mutation rate (Evans et al., 2004, Neeley and Essigmann, 2006), misincorporation (Henderson et al., 2001) and changes in gene expression. Hydroxyl radical and LPO products like MDA, HNE, isoprostanes and acrolein, can react with components of the DNA molecule, damaging the purine and pyrimidine bases, and also the backbone of the DNA (Hu et al., 2002, Marnett, 1999, Marnett, 2000). Damage

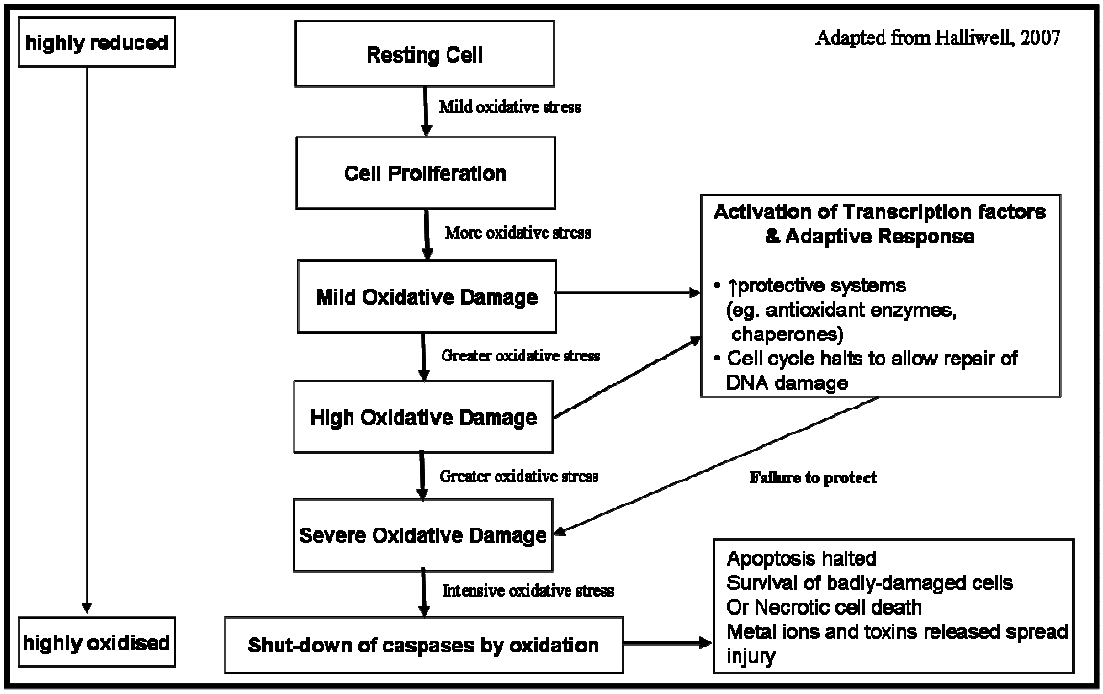


Fig. 1.2 – Cellular response to oxidants. Adapted from (Halliwell B., 2007).

to DNA usually prevents DNA replication and cell division, by mechanisms involving p53. High oxidative damage to DNA may lead to p53-mediated apoptosis and/or NAD^+ depletion via the nuclear enzyme poly (ADP-ribose) polymerase (PARP) (Masutani et al., 2000, Matalon et al., 2003). Alternatively, genotoxicity can occur, leading to increase in mutation rate of DNA (Neeley and Essigmann, 2006).

1.4.2 Damage to lipids: Lipid Peroxidation (LPO)

ROS and RNS may attack cellular components containing polyunsaturated fatty acids (PUFAs) such as membrane lipids, fat carrying lipoproteins and dietary lipids, leading to a chain of reactions known as lipid peroxidation. Lipid peroxidation (LPO) leads to membrane fluidity and enzyme/receptor/ion channel inactivation. PUFAs are extremely sensitive to oxidation and upon reacting forms lipid peroxidation products such as lipid hydroperoxides, isoprostanes and isoketals, hydrocarbons, malondialdehyde (MDA), 4-hydroxy-2-trans-nonenal (HNE) and acrolein

(Esterbauer et al., 1991, Roberts and Fessel, 2004, Marnett, 2000, Uchida et al., 1998). Although some of these compounds are reported to exert certain physiological functions, many of these compounds are reported toxic to the cells, especially HNE (Hu et al., 2002, Chung et al., 2000) and MDA (Marnett, 1999, Marnett, 2002).

1.4.2.1 Lipid Peroxidation Process

Lipid peroxidation (LPO) is defined as the oxidative destruction of polyunsaturated fatty acids (PUFAs). PUFAs are compounds that contain two or more carbon-carbon double bonds and are more susceptible to oxidation. The LPO process is a chain reaction and may occur in the biological system. The lipid peroxidation process occurs in three different steps: initiation, propagation and termination (Fig. 3). The process of 'initiation' can be triggered by the addition of any reactive species. The reactive species abstracts a $H^{\bullet}$ from the methylene group of the PUFA, resulting in the generation of unstable carbon free radical ($R^{\bullet}$). The carbon unstable radical undergoes molecular rearrangement and takes in oxygen (O_2) to form the peroxyl radical ($ROO^{\bullet}$). The 'propagation' of this process starts when the peroxyl radical further abstracts hydrogen from adjacent fatty acid side chains. This chain reaction continues. Peroxyl radical ($ROO^{\bullet}$) after abstracting hydrogen forms lipid hydroperoxide or lipid peroxide ($ROOH^{\bullet}$). Thus a single initiation can generate numerous lipid peroxide molecules by chain reaction (Gardner, 1989, Pinchuk et al., 1998, Kohen and Nyska, 2002). The process of LPO can be accelerated by the presence of singlet oxygen (Girotti and Kriska, 2004).

1.4.2.2 Targets of LPO

The main targets of LPO are membrane lipids and proteins, lipid carrying lipoproteins and dietary lipids. The biological membrane can be easily attacked by lipid peroxidation products since these membranes are composed of lipids (containing PUFAs) and proteins. Lipoproteins involved in the transport of lipids (e.g. low-density lipoprotein, LDL) are also vulnerable to the oxidation *in vivo*,

contributing to atherosclerosis. The fatty acids in the circulating system are also prone to oxidation. Moreover, the dietary lipids if oxidised already before getting into the biological system can be a potential target of oxidation *in vivo*.

1.4.2.3 Effects of LPO

The deleterious effects of lipid peroxidation are decrease in membrane fluidity, damage to membrane proteins, inactivation of enzymes and ion channels (Richter, 1987). Lipid peroxidation in the endoplasmic reticulum or golgi apparatus can decrease the ability to synthesise and export proteins. The peroxyl and alkoxyl radicals, aldehydes and singlet oxygen can cause damage to receptors, enzymes and potassium channels. The damage to the K^+ channels can lead to irregularity in heart beat and neuronal cell death (Duprat et al., 1995). Mitochondrial lipid peroxidation can cause damage to matrix enzymes, ubiquinone and mitochondrial DNA (Bindoli, 1988).

1.4.2.4 Products of LPO

Lipid peroxide is the primary product of LPO process and once formed they can be rearranged by cyclisation reactions, with the final product being malondialdehyde (MDA) (Marnett, 1999). Simultaneously, lipid peroxide can undergo decomposition by metal ions or by heating to produce epoxides, aldehydes (4-hydroxy-2-trans-nonenal (HNE), acrolein), ketones and hydrocarbons (Esterbauer et al., 1991, Roberts and Fessel, 2004, Marnett, 2000, Uchida et al., 1998). Meanwhile the peroxidation of phospholipids generate compounds called isoprostanes (IsoPs), which can be derived from linolenic acid, arachidonic acid (F_2-isoprostanes), eicosapentaenoic acid (F_3-isoprostanes) and docosahexaenoic acid (F_4-isoprostanes or neuroprostanes) (Roberts and Fessel, 2004). In addition, the oxidation of cholesterol in the membranes and lipoproteins during the process of LPO generate a mixture of products called cholesterol oxidation products (COPs) (Girotti and Kriska, 2004).

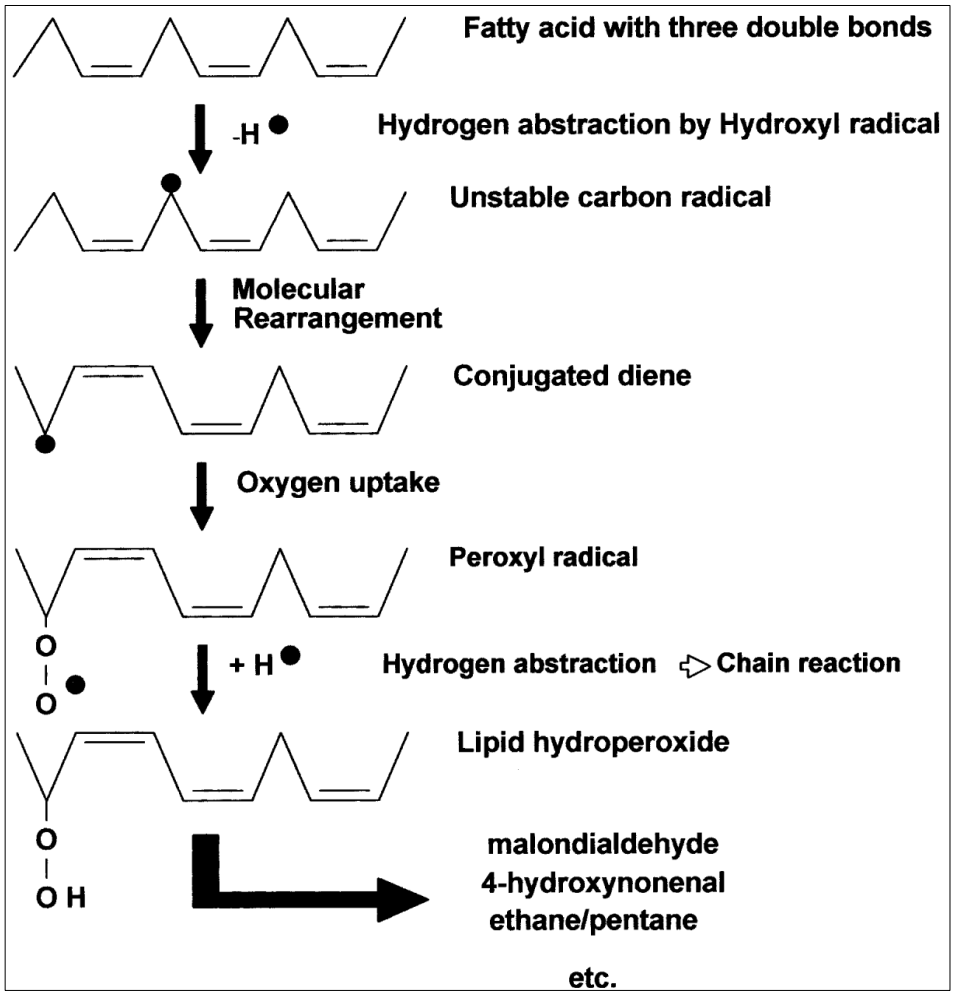


Fig. 1.3 - Lipid peroxidation process (Young and McEneny, 2001)

1.4.3 Damage to proteins and carbohydrates

The oxidative attack of cellular proteins by ROS result in impaired function of antibodies, enzymes, receptors, signal transduction and transport proteins. Reactive species attack proteins either directly or produce secondary damage involving damage by lipid peroxidation products, like isoketals, MDA and HNE. Proteins can also be damaged by glycation. The side chains of all amino acid residue in the proteins, particularly cysteine and methionine residues are prone to oxidative damage by the reactive species (Stadtman, 2004). Advanced glycation end products (AGEs) are a class of complex products that are formed by the reaction between carbohydrates and free amino acid group of proteins. Most of AGEs are very unstable and very complex. AGEs so far reported in human are pentosidine and carboxymethyl lysine (Dalle-Donne et al., 2005).

1.5 Reactive Oxygen Species and Redox Regulation

As mentioned above ROS are produced in normal cellular metabolism and serve in several physiological functions. These operations take place at low but measurable concentrations within the cell. This 'steady state' concentration is determined by their rate of production and rate of removal by various antioxidants. Thus each cell has its own concentration of electrons, stored in various cellular components. This is called the 'redox state' of that cell and the cell functions according to the variation in the redox state (Schafer and Buettner, 2001).

1.5.1 Glutathione and Thioredoxin

The redox environment of a cell is mainly maintained by glutathione (GSH) and thioredoxin (TRX), and both act as a redox buffer within the cell. Specifically, glutathione (reduced and oxidised forms, 2GSH/GSSG couple) reflects the redox environment of the cell (Droge, 2002, Schafer and Buettner, 2001). These redox

reactions are now believed to control intracellular signalling (e.g. in communication between nucleus and mitochondria, in response of the cell to hormones and growth factors), intercellular signalling (e.g. nitric oxide, cytokines) and regulate the response to oxidative stress. In addition to GSH and TRX systems, other low molecular mass compounds including amino acids, peptides and proteins also contribute to significant ROS scavenging activity (McEligot et al., 2005, Sies, 1993).

1.5.2 Redox signalling

When the cellular concentration of the reactive oxygen species raise, glutathione is oxidised and GSSG (oxidised glutathione) content increases. GSSG, a disulphide, then reacts with cellular proteins to increase the total protein mixed disulphides in the cell. Many signalling proteins (such as receptors, protein kinases and some transcription factors) have critical thiol groups, and the formation of protein mixed disulphides can alter their function. Thus GSSG acts as a non-specific signalling molecule. A regulatory process in which signal is delivered through such redox reactions are referred to as redox signalling. Redox signalling requires steady state of 'redox balance', which can be disturbed by ROS/RNS formation or reduction in antioxidant defence systems. Regulated increase in ROS level can build the redox state to be much oxidised and that explains the physiological basis of redox regulation. The signalling mechanisms that respond to thiol/disulphide concentration involve (i) transcription factors AP-1 and NF-κB; (ii) protein tyrosine phosphatases; (iii) Src family kinases; (iv) JNK and p38 MAPK signalling pathways; (v) insulin receptor kinase activity, and others (Droge, 2002, Galter et al., 1994, Hehner et al., 2000, Kuge and Jones, 1994, Aslund et al., 1999). Under different pathological conditions, abnormally high concentrations of ROS may lead to permanent alteration in signal transduction and gene expression, which is characterised in different disease conditions. The redox environment of the cell is also reported to influence the onset of apoptosis and necrosis. Apoptosis is induced by moderate oxidising stimuli while necrosis is induced by intense oxidising effect (Cai and Jones, 1998, Evens, 2004, Voehringer et al., 2000).

1.6 Reactive Oxygen Species and Signal Transduction

Cells communicate with each other and respond to external stimuli through biological mechanisms called 'cell signalling' or 'signal transduction' (Poli et al., 2004). Signal transduction enables information to be transmitted from outside the cell (by mediators such as hormones, growth factors, cytokines and neurotransmitters) to the functional components inside the cell. Signalling to transcription machinery inside the cell for expression of certain genes is carried out by proteins called transcription factors. These transcription factors bind to specific DNA sequences and regulate the activity of RNA polymerase II resulting in transcription of the gene and production of specific desired protein. The signal transduction process is the basic mechanism behind physiological processes such as cell growth, gene expression and neurotransmission. (Thannickal and Fanburg, 2000).

Reactive oxygen species, apart from inflicting oxidative damage to cells, are also responsible for many physiological processes or intracellular signalling and regulation (Droge, 2002). Most cells are reported to generate low concentrations of ROS through small oxidative burst when stimulated by cytokines, growth factors, hormones, e.g. interleukin-1β (IL-1β), interleukin 6 (IL-6), interleukin 3 (IL-3), tumour necrosis factor-α (TNF- α), angiotensin II (ANGII), platelet derived growth factor (PDGF), nerve growth factor (NGF), transforming growth factor- β1 (TGF-β1), granulocyte-macrophage colony-stimulating factor (GM-CSF), and fibroblast growth factor (FGF-2) (Thannickal and Fanburg, 2000). ROS can thus play an important role in signal transduction as secondary messengers. The following are some of the signalling pathways that are related to ROS.

i. Cytokines and growth factor signalling
ii. Non-receptor tyrosine kinases
iii. Protein tyrosine phosphatases
iv. Serine/theronine kinases

The most significant effect of ROS is observed and reported in mitogen-activated protein kinase (MAPK) pathways (Sun and Oberley, 1996) (Fig. 1.4). While ROS is predominantly related to cell damage, they also play an important role in intracellular signalling and regulation (Lowenstein et al., 1994).

1.6.1 ROS and MAPK pathway

Mitogen-activated protein kinases (MAPKs) relay signals generated by exogenous and endogenous stimuli to intracellular space via phosphorylation of proteins. MAPKs interact with up-stream mediators such as growth factor receptors, G-proteins, tyrosine kinases and down-stream mediators such as nuclear transcription factors (Lopez-Ilasaca et al., 1997). A number of studies have reported that MAPKs can be induced and regulated by ROS. The mammalian MAPK families include: extracellular regulated kinases (ERKs), c-jun-NH_2-terminal kinase (JNKs) and p38 MAPK. These MAPK families are reported to be involved in cell proliferation, differentiation, survival, apoptosis and transformation (Kyriakis and Avruch, 2001). Activation of MAPKs phosphorylate various substrate protein including transcription factors such as Elk1, c-Jun, ATF2, and p53. The JNK and p38 signalling pathways are activated by pro-inflammatory cytokines such as tumour necrosis factor (TNF-α) and interleukin (IL-β) in response to cellular stress including genotoxicity, hypoxia or oxidative stress. In JNK and p38 pathways, a MAP3K, apoptosis signal regulating kinase 1 (ASK1) is activated as a result of variety of stressors including reactive oxygen species (Nagai et al., 2007) leading to apoptosis (Fig. 1.4).

1.7 Oxidative stress and human diseases

Oxidative stress has been implicated in various pathological and disease conditions including cancer, neurodegenerative diseases, cardiovascular diseases, diabetes, other diseases and aging (Halliwell B., 2007, Valko et al., 2007). Oxidative stress-related diseases can be classified into two groups:

i. Diseases characterised by pro-oxidants shifting the thiol/disulfide redox state and impairing glucose tolerance, known commonly as mitochondrial oxidative stress. E.g. Cancer, diabetes mellitus
ii. Diseases characterised by inflammatory oxidative conditions and enhanced activity of NADPH oxidase (atherosclerosis and chronic inflammation) or xanthine oxidase-induced generation of ROS (ischemia and reperfusion injury).

In addition, aging also plays an important role due to the damaging effects of ROS considering the decline in antioxidant defence system (Harman, 1956).

1.7.1 Oxidative stress and cancer

ROS are produced during normal metabolic process mainly inside mitochondria and effectively countered by endogenous antioxidant defence systems. Despite the presence of antioxidant defence systems, oxidative damage accumulates during the life period, and damages are inflicted on DNA, proteins and lipids, which are related to age-related diseases including cancer. In this section, the evidence available for the involvement of ROS in imposing damage to cellular components and carcinogenesis are examined.

1.7.1.1 Metal induced oxidative stress and cancer

Several lines of evidence emphasise the implication of metal-induced ROS and RNS generation in biological systems to their toxicity and carcinogenicity. Free radicals mediated by metals are involved in modifications of DNA bases, enhanced lipid peroxidation, and changes in calcium and sulfhydryl homeostasis. The metals which are believed to induce ROS resulting in cancer include iron, copper, chromium, cobalt, vanadium, cadmium, arsenic and nickel, reviewed by Valko and co-workers (Valko et al., 2006).

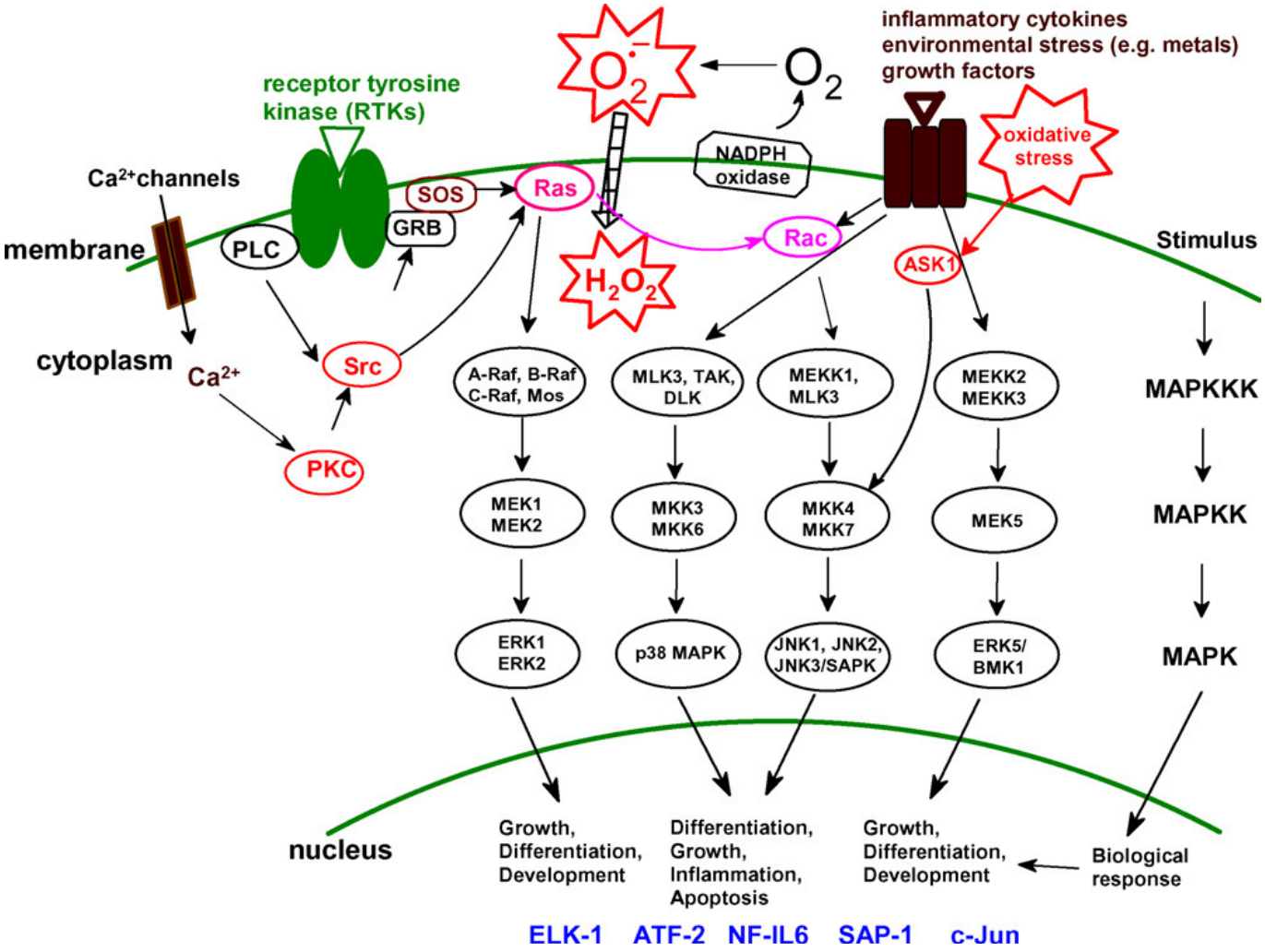


Fig. 1.4 – ROS-induced MAPK signalling pathways (Valko et al., 2007)

1.7.1.2 DNA damage and cancer

The hydroxyl radical has been reported to react with DNA bases resulting in the damage of both purine and pyrimidine bases, and also the deoxyribose backbone (Dizdaroglu et al., 2002). This oxidative damage leads to permanent modifications in the genetic material and represents the first step involved in mutagenesis, carcinogenesis and aging. To date, more than 100 products resulting from oxidation of DNA have been identified. Damage to DNA may result either in arrest or induction of transcription, signal transduction pathways, replication errors and genomic instability (Marnett, 2000, Cooke et al., 2003). An example of the formation of 8-hydroxyguanine (8-OH-G) is illustrated in Fig 1.5, a biomarker for oxidative DNA damage that was first reported to be identified in urine.

In addition to hydroxyl radical, peroxynitrite, formed by the combination of superoxide radical and nitric oxide, has also been implicated in DNA damage (Brown and Borutaite, 2001). Peroxynitrite reacts with guanine base to form 8-nitroguanine; however the relationship between 8-nitroguanine and carcinogenesis is not yet completely understood. Moreover, damage to mitochondrial DNA has also been reported to be involved in the carcinogenesis process in neoplasia (Inoue et al., 2003). Hydrogen peroxide and other ROS have also been involved in mitochondrial DNA damage.

1.7.1.3 Lipid peroxidation and cancer

Oxidative stress induced imbalance in the redox environment of the cell is considered to be related to the oncogenic stimulation. Oxidative damage to DNA is the primary step in mutagenesis and carcinogenesis. Apart from oxidative DNA damage, lipid peroxidation has also been observed to play its role in the mechanism of carcinogenesis. The lipid peroxidation products including lipid hydroperoxides, decomposition products such as aldehydes, and polymeric materials exert cytotoxic and genotoxic effects (Esterbauer, 1993). The lipid peroxidation product

malondialdehyde (MDA) can form adducts with DNA bases G, A and C (M_1G, M_1A and M_1C respectively) (Marnett, 1999) (Fig 1.6). The M_1G adduct of MDA-DNA was detected in human breast cells (Wang et al., 1996). M_1G was reported to be mutagenic in *E. coli* (Fink et al., 1997, Mao et al., 1999). The adducts produced from other lipid peroxidation products such as etheno-dA, etheno-dC and etheno-dG have been reported to have high genotoxicity but are less mutagenic in *E. coli* (Fedtke et al., 1990). Hydroxypropanodeoxyguanosines (HO-PdGs) were reported to be present in human DNA that might be derived from the reaction between DNA and acrolein or crotonaldehyde (Marnett, 2000). Deoxyguanosine adducts of HNE-DNA were detected in rat and human endogenous DNA from tissues (Chung et al., 2000). Recent studies conclude that HNE preferentially forms DNA adduct at codon 249 of human p53 gene, which is the hot spot for mutation in hepatocellular carcinoma. These results also suggest that HNE may be the etiological agent for human cancer that has mutation at codon 249 of human p53 gene (Hu et al., 2002).

1.7.1.4 ROS, signalling pathways and cancer

Reactive oxygen species play a major role in intracellular signalling and it has been established that ROS can interfere with the expression of several genes and signal transduction pathways (Thannickal and Fanburg, 2000). At low concentrations they can act as secondary messengers but at high physiological concentrations they can prove detrimental. The major pathway that is known to be influenced by oxidative stress is MAPK pathway. Several studies indicate that oxidants can regulate the kinases of MAPK family including JNK and p38 (Fig 1.4). In addition, carcinogenic materials have also been shown to activate MAPK pathways (Leonard et al., 2004).

1.7.1.5 Oxidative stress and mechanism of carcinogenesis

Carcinogenesis is a complex multi-step process from a healthy to a precancerous state and then to an early stage of cancer. Epidemiological and animal studies show that the process of carcinogenesis is characterised by multi-stage-mechanism known

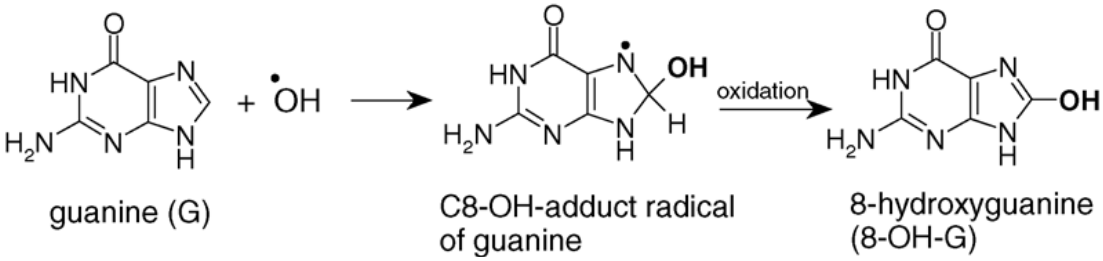


Fig. 1.5 - Reaction of guanine with hydroxyl radical (Valko et al., 2006)

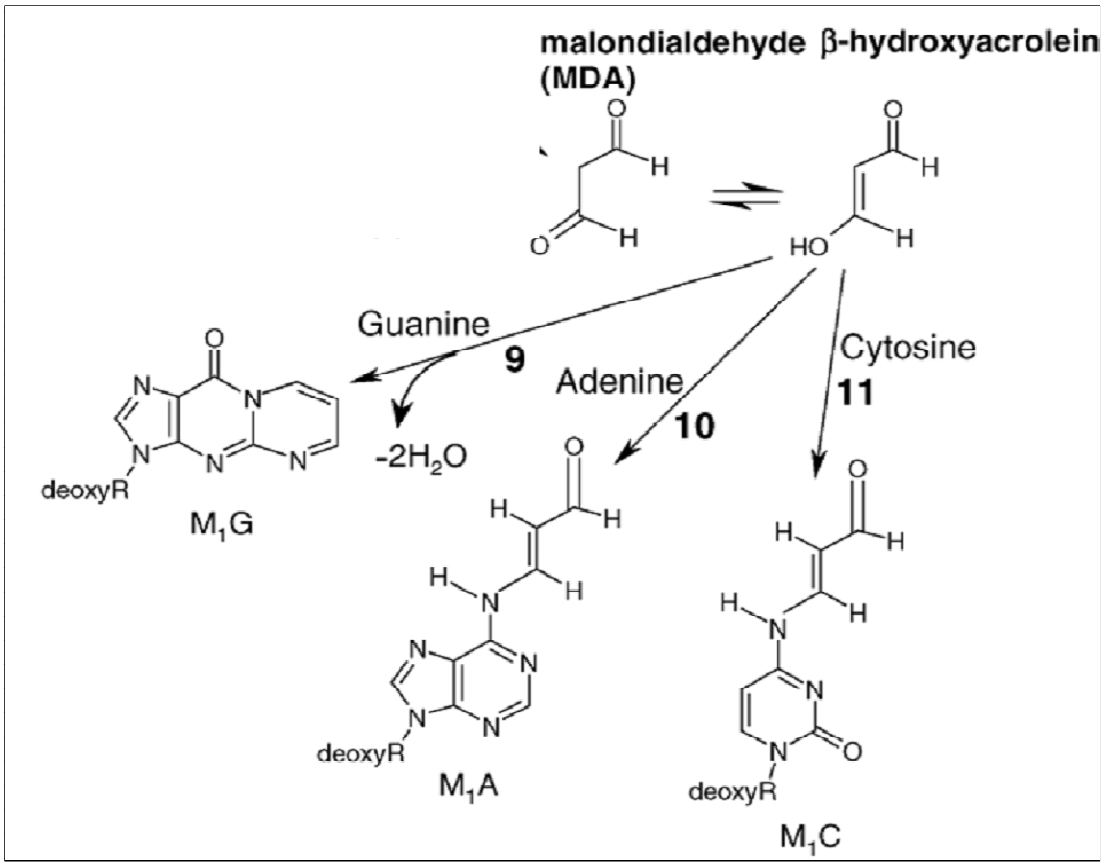


Fig. 1.6 - Formation of DNA adducts by malondialdehyde (Valko et al., 2006)

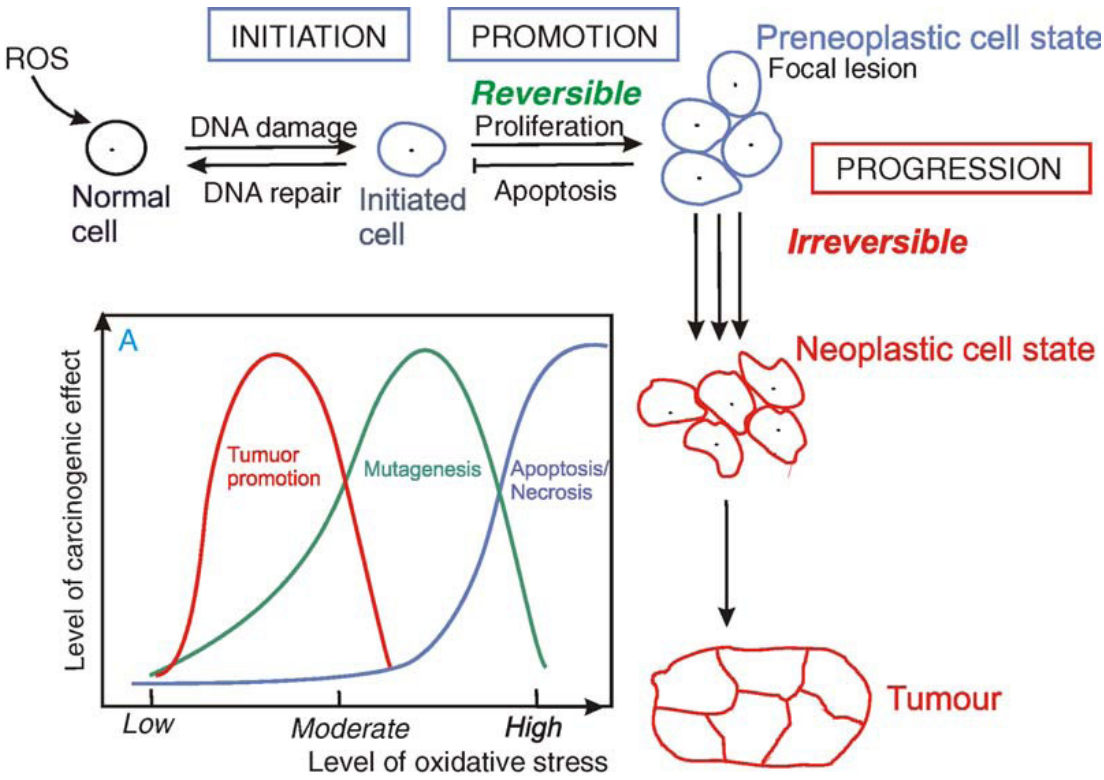


Fig. 1.7 – Initiation-Promotion-Progression model of carcinogenesis and the role of oxidative stress at different stages of disease progression (Inset A) (Valko, 2006).

Cancer development is a cumulative action of multiple events on a single cell and can be described in three stages. Stage 1: Initiation: Persistent oxidative stress to cells by ROS (e.g. hydroxyl radical) can induce DNA damage or mutation. Initiation process involves a non-lethal mutation in DNA that produces an altered cell followed by at least one cycle of DNA synthesis to fix the damage produced during initiation. Stage 2: Promotion: This stage is characterised by clonal expansion of initiated cells by the induction of cell proliferation and/or inhibition of programmed cell death (apoptosis). This process results in the formation of identifiable focal lesion. This stage requires continuous presence of tumour promotion stimulus and therefore a reversible process. Stage 3: Progression: This is the final stage of carcinogenic process and involves cellular and molecular changes that occur from the preneoplastic to the neoplastic state. This stage is characterised by accumulation of additional damage to genomic DNA, leading to the transition of the cell from benign to malignant. This stage is irreversible and is also characterised by genetic instability and disruption of chromosome integrity. *Inset A:* The level of oxidative stress alters the carcinogenic process. Low level of oxidative stress causes tumour promotion and an increase to moderate stress leads to mutagenesis. High oxidative stress results in apoptosis or necrosis of the cell.

as the initiation-promotion-progression model (Klaunig and Kamendulis, 2004, Trueba et al., 2004). This process is summarised in Fig 1.7.

1.7.2 Oxidative stress and neurodegenerative diseases

Neurodegenerative diseases are heterogeneous group of disorders characterised by gradually progressive, selective loss of anatomically or physiologically related neuronal systems (Lin and Beal, 2006). The human brain is extremely vulnerable to oxidative damage because of their low glutathione content, high PUFAs in membranes, high oxygen utilisation and presence of redox-catalysing metals (Fe, Cu, Zn) (Christen, 2000). As mentioned previously, aging causes increase in oxidative stress as a result of increase in accumulation of ROS and decline in cellular antioxidant defence system. Consequently aging and associated oxidative stress are major risk factors of various neurodegenerative diseases. Apparently, genetic mutations and environmental exposure may also induce oxidative stress and neurodegeneration. A huge body of evidence suggests that ROS and RNS resulting from mitochondrial dysfunction, neuroinflammation or toxins are responsible for oxidative stress-induced neuronal cell death that underlies various neurodegenerative disorders including Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS) and many others (Beal, 2007, Halliwell, 2006, Hensley et al., 2006, Tansey et al., 2008, von Bernhardi, 2007). This section provides available evidence suggesting the role of oxidative stress in Alzheimer's disease and Parkinson's disease.

1.7.2.1 Oxidative stress and Alzheimer's disease (AD)

AD is characterised clinically by progressive cognitive decline, and pathologically by the presence of senile plaques composed primarily of amyloid-β-peptide (Aβ) and neurofibrillary tangles made up of hyper-phosphorylated tau protein (Lin and Beal, 2006). Nearly, 5-10% cases of AD are familial and three proteins are associated with these cases: amyloid precursor protein (APP), presenilins 1 and 2 (PSEN1 and

PSEN2). Many lines of evidence suggest that mitochondrial dysfunction and oxidative stress are involved in AD. It is reported that Aβ induced oxidative damage is the cause for neurodegeneration in AD (Butterfield and Lauderback, 2002). The toxic Aβ peptide may induce direct intralysosomal oxidative stress resulting in the release of proteolytic cathepsins into the cytoplasm, ultimately causing toxic protein aggregation (Pivtoraiko et al., 2009). AD brains are reported to have experienced oxidative damage much before the onset of plaque pathology (Nunomura et al., 2001). Hydrogen peroxide treatment in fetal guinea pig neurons has been shown to increase intracellular Aβ levels (Ohyagi et al., 2000). In APP-mutant transgenic mice, deficiency of mitochondrial antioxidant enzyme MnSOD markedly elevated brain Aβ levels and plaque deposition. Oxidative stress-induced pathways such as JNK and p38 have also been involved in AD pathology. Oxidative stress increased the expression of β-secretase through activation of JNK and p38 pathways and increased aberrant tau phosphorylation (Tamagno et al., 2005, Lovell et al., 2004). In addition, oxidant-induced destruction of critical molecules can also be detrimental. For instance, prolyl isomerase PIN1 was found susceptible to oxidative damage and which in turn affects APP and tau processing in brain (Sultana et al., 2006, Pastorino et al., 2006).

Aβ induces lipid peroxidation products-mediated (HNE and acrolein) damage to cellular proteins by introducing carbonyl groups into them. Aβ also causes accumulation of hydrogen peroxide and lipid peroxides in AD brains (Behl et al., 1994). In AD the levels of oxidised lipoproteins were found to be elevated in the cerebrospinal fluid (Schippling et al., 2000). Reports illustrate that the neurodegenerative diseases are not only associated with the oxidation of proteins but also the generation of lipid peroxidation products (Markesbery, 1997, Butterfield and Lauderback, 2002, Poon et al., 2004, Pratico and Delanty, 2000). The amount of LPO biomarkers MDA (Dib et al., 2002), conjugated dienes (Schippling et al., 2000), isoprostanes (Pratico et al., 2000, Quinn et al., 2004), 4-HNE (Zarkovic, 2003), degradation products of phospholipids (Pettegrew et al., 2001), acrolein (Lovell et al., 2001, Uchida et al., 1998), cholesterol oxidation products (COPs) (Nelson and

Alkon, 2005) and advanced glycation end products (AGEs) (Dei et al., 2002, Smith et al., 1994, Vitek et al., 1994) were reported to be elevated in AD brains.

1.7.2.2 Oxidative stress and Parkinson's disease (PD)

Parkinson's disease is clinically characterised by progressive bradykinesia and tremor, and pathologically by loss of pigmented neurons in the substantia nigra and the presence of Lewy bodies (distinctive cytoplasmic inclusions containing α-synuclein and ubiquitin) (Lin and Beal, 2006). Parkinson's disease (PD) is the second most prevalent age-related neurodegenerative disorder affecting 0.3% of entire population, and more than 1% of humans over 60 years of age (de Lau and Breteler, 2006).

Evidence for the involvement of ROS in PD is derived from the post mortem analysis of brain tissues from PD patients that reveals increased levels of oxidized proteins, lipids and nucleic acids (Dexter et al., 1986, Spencer et al., 1994, Carney and Carney, 1994). In addition, nitric oxide (NO), synthesized by nitric oxide synthase (NOS), and peroxynitrite, formed by the interaction of NO with superoxide anion, are known to be involved in neurodegeneration (Ebadi and Sharma, 2003). Both ROS and reactive nitrogen species (RNS) are known to inhibit mitochondrial complex I (Chinta and Andersen, 2010). Evidence shows that mitochondrial complex I deficiency and glutathione depletion in substantia nigra was found in idiopathic PD patients and patients with pre-symptomatic PD (Schapira et al., 1989). The deficiencies in mitochondrial complex I observed in idiopathic PD patients may result in ROS generation, and these defects may induce neurodegeneration (Swerdlow et al., 1996, Schapira et al., 1998). The ROS-induced damage to cell organelles can further induce damage to mitochondria, ultimately triggering Bax-dependent Cytochrome c release and apoptosis (Knott and Bossy-Wetzel, 2009) (Fig 1. 8).

Genes associated with PD have been related to mitochondria in disease pathogenesis. Currently, at least nine nuclear genes have been identified to cause PD or increase

PD risk which include α-synuclein, parkin, ubiquitin carboxy-terminal hydrolase L1, DJ-1, phosphatase and tensin homologue (PTEN)-induced kinase 1 (PINK1), leucine-rich-repeat kinase 2 (LRRK2), the nuclear receptor NURR1, HTRA2 and tau. Among these nuclear genes α-synuclein, parkin, DJ-1, PINK1, LRRK2 and HTRA2 are reported to be directly involved in mitochondria (Lin and Beal, 2006).

In transgenic mice, α-synuclein overexpression resulted in mitochondrial dysfunction, elevation of oxidative stress and increase of nigral pathology (Song et al., 2004). A study overexpressing A53T mutant α-synuclein in mice showed the presence of α-synuclein inside degenerating mitochondria, suggesting that α-synuclein mutation may directly affect mitochondrial dysfunction (Martin et al., 2006). Deficiency or mutations in parkin gene has been shown to lead to oxidative stress and mitochondrial dysfunction. Parkin-null drosophila (Pesah et al., 2004) and mouse (Palacino et al., 2004) strains showed increase in oxidative stress and mitochondrial dysfunction, and patients with parkin mutations also exhibited selective impairment in complex-I activity (Muftuoglu et al., 2004). The function of the gene DJ-1 seemed to be associated with protection against cell death, especially against oxidative stress-induced cell death (Canet-Aviles et al., 2004). Other nuclear genes associated with PD such as PINK1 (Silvestri et al., 2005), LRRK2 (West et al., 2005) and HTRA2 (Martins et al., 2004) have also been linked to mitochondrial dysfunction leading to oxidative stress.

Thus mitochondrial dysfunction, generation of ROS and free radical NO, and peroxynitrite production have all been implicated in degenerative neuronal cell death in PD (Fig 1.9) (Thomas, 2009, Chinta and Andersen, 2010).

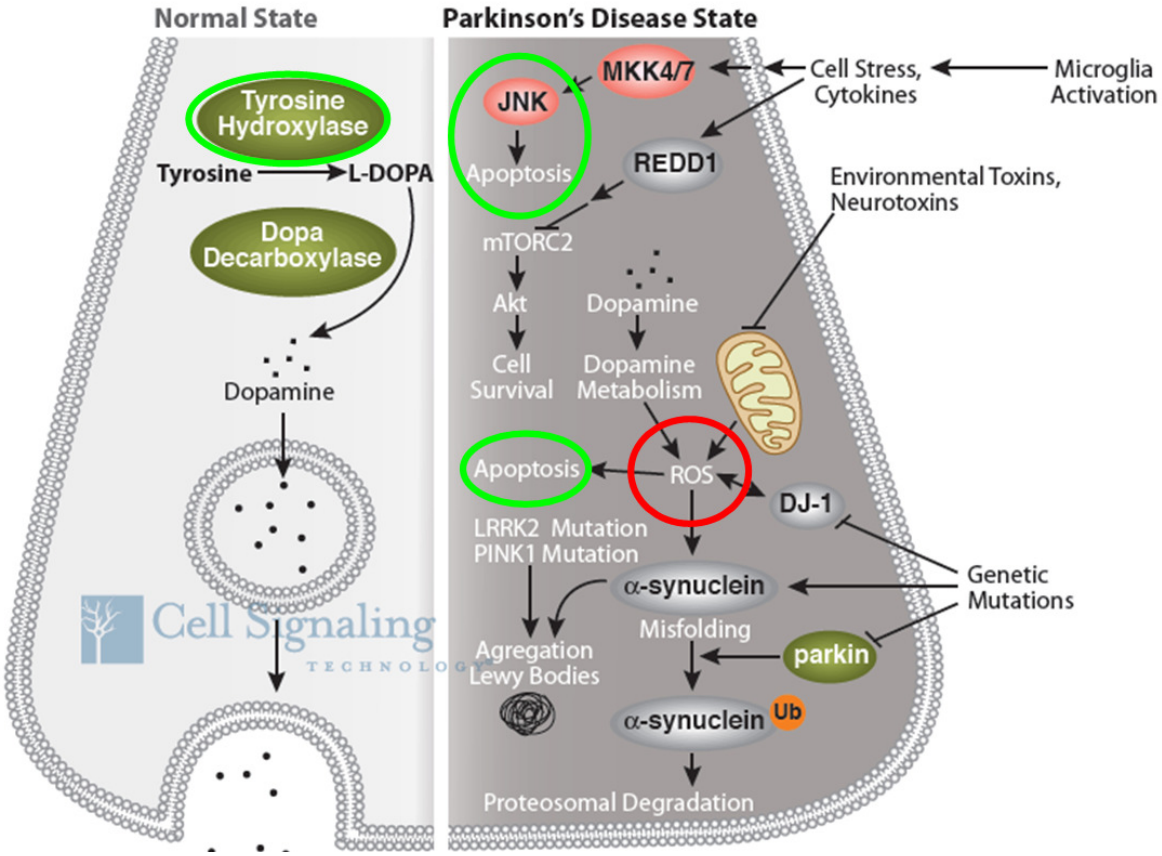


Fig. 1.8 - Pathophysiology of Parkinson's disease (courtesy, Cell Signalling Technology Ltd., UK).

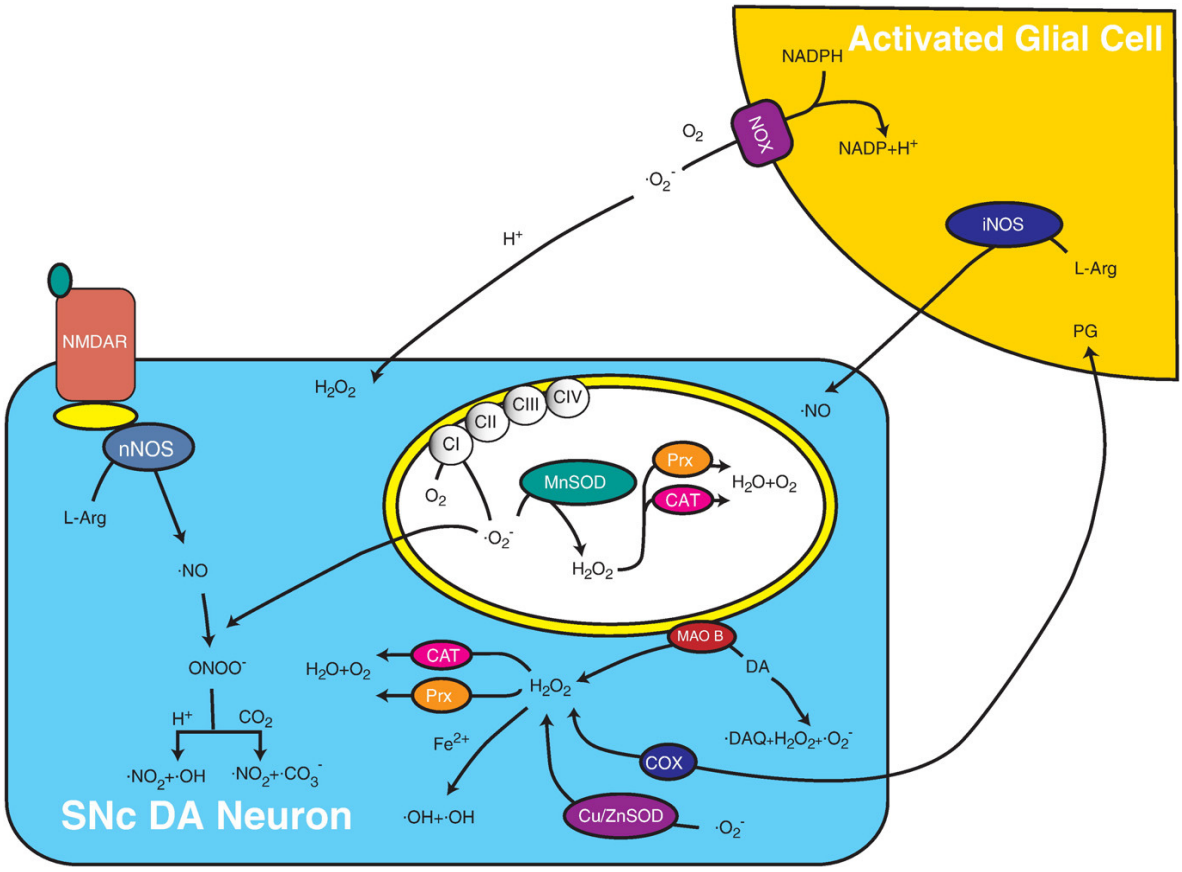


Fig. 1.9 - Generation of ROS and RNS in dopaminergic neurons in Substantia Nigra par compacta (SNpc) (Tsang and Chung, 2009).
Leakage of electrons from mitochondrial electron transport chain, especially complex I, leads to partial reduction of molecular oxygen to superoxide ($^{\bullet}O_2^-$) which is dismutated by SOD into hydrogen peroxide (H_2O_2). H_2O_2 can be converted to highly reactive hydroxyl radical ($^{\bullet}OH$) by the catalysis of Fe^{2+} through Fenton's reaction. Excessive stimulation of N-methyl-D-aspartate receptor (NMDAR) leads to activation of neuronal nitric oxide synthase (nNOS) resulting in production of nitric oxide in excess (NO). NO can react with $^{\bullet}O_2^-$ to form highly reactive peroxynitrite ($ONOO^-$). Dopamine (DA) catabolism by monoamine oxidase (MAO) can generate H_2O_2. In addition, oxidation of DA also produces ROS and DA quinone radical ($^{\bullet}DAQ$) that can modify protein directly. Glial cells are activated under neuroinflammatory conditions and activated glial cells can generate ROS by NADPH oxidase (NOX) and NO by inducible nitric oxide synthase (iNOS) which induce oxidative and nitrosative stress to SNpc DA neurons. SNpc DA neurons express cycloxygenase (COX), which produces prostaglandin (PG) and generates H_2O_2 as by-product.

1.8 Antioxidant Defence System

The increasing levels of oxygen in earth's atmosphere and exposure to various other free radicals led the organisms to develop antioxidant defence systems. An antioxidant is any substance that delays, prevents or removes oxidative damage to a target molecule. Antioxidants may be endogenous i.e. synthesised *in vivo* or taken in from the diet.

The antioxidant defence system comprises (1) substances that catalytically remove RS e.g. superoxide dismutase, catalase, peroxidase enzymes (2) agents that decrease RS formation e.g. transferrins, albumin, heme oxygenases (3) proteins that protect biomolecules by other mechanisms e.g. chaperones (4) agents that physically quench RS e.g. quenching of singlet oxygen by carotenoids (5) molecules that replace biomolecules sensitive to oxidative damage e.g. fumarase C of *E. coli* (6) agents that sacrifice themselves to prevent oxidative damage of important biomolecules e.g. GSH, α-tocopherol, ascorbate, bilirubin, urate and plasmogens. (Halliwell B., 2007).

1.8.1 Endogenous Antioxidant defence System

Agents that are produced within the biological system, which delay, prevent or remove oxidative damage are referred to as endogenous antioxidants. They are usually enzymes, metal sequestering agents and low molecular mass compounds produced *in vivo*. Enzymes catalytically remove reactive species in the system and they include superoxide dismutase (SOD) (Fridovich, 1995), superoxide reductase (SOR) (Niviere and Fontecave, 2004), catalase (Chance et al., 1979), glutathione peroxidise family (Chance et al., 1979, Brigelius-Flohe, 1999), NAD(P)H: oxidoreductases (Nioi and Hayes, 2004, Jaiswal, 2000), heme oxygenase 1 (HO1) (Prestera et al., 1995), glutathione-S-transferases (GSTs) (Rushmore and Pickett, 1990), aldo-keto reductases (AKRs) (Ellis et al., 2003), γ-glutamylcysteine synthase (γ-GCS) (Wild and Mulcahy, 1999) and other peroxidases such as Cytochrome C peroxidase (CCP) and NADH oxidase. Antioxidant protection is also rendered by

low molecular mass compounds that are synthesised *in vivo* such as bilirubin, α-ketoacids, melatonin, lipoic acid, coenzyme Q and uric acid. (Halliwell B., 2007).

1.8.2 Induction of antioxidant defence system

The two families of enzymes that are involved in the metabolism are phase 1 and phase 2 enzymes. Phase 1 enzymes catalyse asynthetic reactions which functionalise compounds through oxidation and reduction. Phase 2 enzymes promote synthetic conjugation of phase 1 products with endogenous ligands such as glutathione (GSH), glucuronic acid and amino acids, leading to water-soluble and excretable products. Over the last few decades researchers have revealed that these two groups of enzymes can be induced by several synthetic and natural compounds, and are regulated by distinct and separate mechanisms. Induction of phase 2 enzymes and their mechanisms seemed to have therapeutic importance and scope in the treatment of pathological conditions. It has been revealed that Phase 2 enzymes can be

i. Co-ordinately induced by various synthetic and natural compounds (glutathione S-transferases (GSTs), NAD(P)H: quinone oxidoreductases (NQOs or QRs) and heme oxygenases (HOs)) (Talalay, 2000).
ii. Regulated by similar mechanisms and may involve common promoter elements (e.g. Antioxidant Responsive Element, ARE) (Jaiswal, 1994, Rushmore et al., 1991).
iii. Responsible for catalysis of a wide variety of reactions that protect cells against toxicity of electrophiles and reactive oxygen species by converting them to less toxic products (Kensler, 1997, Talalay et al., 1995).

Induction of phase 2 enzymes can protect from electrophile and reactive oxygen species toxicities by a wide variety of mechanisms. These mechanisms include conjugation with endogenous ligands, modification of reactive molecules that can damage cellular components and generation or augmentation of cellular antioxidants (Talalay, 2000). Induction of phase 2 antioxidant enzymes appears to be part of a

generalised mechanism of protection against electrophiles and reactive oxygen species, thereby ameliorating the risk of damage to cellular components. Antioxidant responsive element (ARE) or electrophile responsive element (EpRE) is involved in triggering the transcription of a battery of genes encoding phase 2 detoxification enzymes (Rushmore et al., 1991, Rushmore and Pickett, 1990). The genes that are driven by ARE and induced co-ordinately include NAD(P)H: oxidoreductases (Nioi and Hayes, 2004, Jaiswal, 2000), heme oxygenase 1 (HO1) (Prestera et al., 1995), glutathione-S-transferases (GSTs) (Rushmore and Pickett, 1990), aldo-keto reductases (AKRs) (Ellis et al., 2003) and γ-glutamylcysteine synthetase (γ-GCS) (Wild and Mulcahy, 1999).

1.8.2.1 NQO1

NADPH: quinone oxidoreductase (NQO1) is an enzyme that catalyzes the reduction of quinones to hydroquinones, thereby preventing the one electron reduction of quinones that would otherwise produce reactive oxygen species within the cell (Vasiliou et al., 2006). At least two different forms of NAD(P)H: oxidoreductases have been identified including NQO1 and NQO2 (Wu et al., 1997, Zhao et al., 1997). NQO1 is the most extensively studied and its main function is to catalyse two-electron reduction and detoxification of quinones and its derivatives (Radjendirane et al., 1997, Ernster, 1987, Talalay et al., 1995, Riley and Workman, 1992). It has been shown that the two-electron reduction of quinones competes with one-electron reduction catalysed by Cytochromes P450 and P450 reductase and produces comparatively stable hydroquinone that is removed by conjugation with glutathione, and UDP-glucuronic acid (Joseph and Jaiswal, 1994, Lind et al., 1982). NQO1 is present ubiquitously in all the tissues (Lind et al., 1990). It is known that tumour tissues and cells of hepatic and colon origin express higher levels of NQO1 gene compared to normal tissues and cells of similar origin (Radjendirane et al., 1997, Riley and Workman, 1992). The NQO1 gene expression is induced in response to oxidants, antioxidants, xenobiotics, heavy metals and UV light (Radjendirane et al., 1997).

1.8.2.2 HO1

Heme oxygenase (HO) is a protective enzyme that catalyses the conversion of heme to biliverdin, which is enzymatically reduced to bilirubin. Heme is pro-oxidant and bilirubin is a potent antioxidant, thus heme oxygenase may protect cells against oxidative damage (Stocker et al., 1987a, Stocker et al., 1987b). Heme oxygenase exists in two forms HO1 and HO2. Although, both forms are expressed in variety of tissues, HO2 is constitutively expressed and HO1 is highly inducible (Maines, 1992). HO1 is induced by various agents such as phenolic antioxidants and isothiocyanates (Lin et al., 2004, Soane et al., 2010).

1.8.2.3 GST

Glutathione S-transferases (GST) are enzymes that are involved in catalysing the nucleophilic attack by reduced glutathione on non-polar compounds containing an electrophile, thereby resulting in detoxification (Hu et al., 2006, Nair et al., 2006). In addition, GST can also detoxify products of biological oxidation. GSTs reduce many hydroperoxides by selenium-independent peroxidase function and reduce hydroperoxides of DNA bases and lipids (Berhane et al., 1994). These transferases effectively scavenge adenine and thymine propenals of DNA bases (Berhane et al., 1994) and 4-hydroxyalkenals by combining with GSH (Hurst et al., 1998, Hubatsch et al., 1998). Moreover, GSTs also detoxify o-quinone metabolites of neurotransmitter catecholamines (dopamine, dopa, epinephrine, and nor-epinephrine) and consequently prevent their oxidative recycling and ROS production (Segura-Aguilar et al., 1997). This evidence implies that the primary function of GSTs is preventing cells from oxidative stress.

1.8.2.4 NRF2-ARE signaling pathway

The induction of phase 2/cytoprotective enzymes in response to oxidative or chemical stress is regulated primarily at transcriptional level. This transcriptional

regulation is mediated by a cis-acting element known as ARE, initially found in the promoters of genes encoding glutathione S-transferase A2 (GST A2) and NQO1 (Rushmore and Pickett, 1990, Friling et al., 1990, Li and Jaiswal, 1992). ARE is reported to possess structural and biological features that characterise its unique responsiveness to oxidative stress (Rushmore et al., 1991). The alteration of the redox status of the cell due to elevated ROS or a reduced antioxidant capacity appears to be a vital signal for the transcriptional regulation of antioxidant/cytoprotective genes by ARE. Due to the constant attack on aerobes by electrophiles and ROS, ARE plays a critical role in the controlled transcription of cytoprotective genes and maintaining the redox homeostasis (Talalay, 2000).

Activation of the antioxidant responsive element (ARE) is mediated mainly by NRF2 (nuclear factor E2-related factor 2), a basic leucine zipper transcription factor that belongs to cap'n'collar family. NRF2 can be activated by at least two mechanisms (i) stabilisation of NRF2 via Keap1 cysteine thiol modification and (ii) phosphorylation of NRF2 by upstream kinases. Cytoplasmic expression of NRF2 is dependent on Kelch-like ECH-associated protein 1 (Keap1) (Posner et al., 1994), which sequesters NRF2 in the cytoplasm and controls its degradation (Prochaska and Talalay, 1988) by Cullin 3 Cul3-dependent ubiquitin ligase complex. ARE inducers such as pro-oxidants, electrophiles and some phase 2 enzyme inducers can modify critical cysteine residue(s) (e.g., Cys 151) of Keap1, which results in conformational change in Keap 1 and the inability of Cul3 to ubiquitinate NRF2. Then modified Keap1 undergoes proteosomal degradation, thereby allowing NRF2 to get stabilised and accumulated in nucleus (Fig. 1.10). On the other hand, activation of protein kinases such as PKC, JNK and ERK induces phosphorylation of NRF2, which may stimulate dissociation of NRF2 from its repressor Keap1 and subsequent translocation into the nucleus (Fig. 1.10) (Surh et al., 2008).

Initially, the involvement of NRF2 in expression of NQO1 was identified in cell-based transient transfection experiments (Fahey and Talalay, 1999). There on, similar observations of the role of NRF2 and ARE in the expression of other protective genes were also established (Duffy et al., 1998). Severe impairment in several ARE-

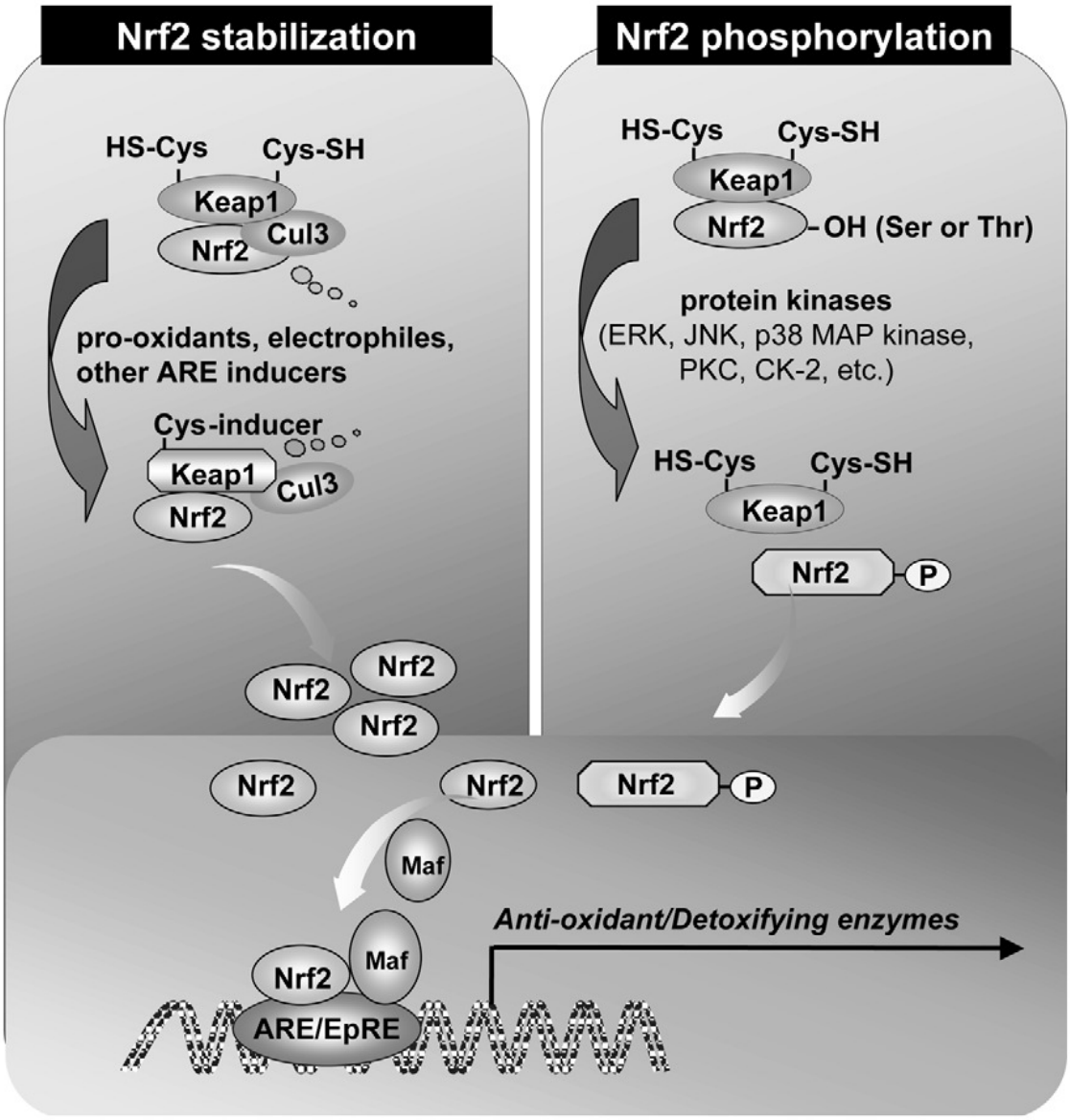


Fig. 1.10 – Proposed mechanism of NRF2-ARE signalling pathway (Surh et al., 2008).
NRF2 can be activated by two mechanisms: (i) stabilization of NRF2 via Keap1 cysteine thiol (Cys-SH) modification and (ii) phosphorylation of NRF2 by upstream kinases. Keap1 is an adaptor protein for Cullin 3 (Cul3)-dependent ubiquitin ligase complex; Maf: transcription factor Maf; ARE: antioxidant responsive element; EpRE: electrophile responsive element.

dependent cytoprotective enzymes were observed in NRF2 knock-out transgenic mice (Henderson et al., 1998, Hubatsch et al., 1998), and chromatin immunoprecipitation assays also demonstrated the endogenous interaction between NRF2 and ARE in H4IIE cells (Hurst et al., 1998).

1.8.3 Dietary Antioxidants

The diet derived antioxidants include the compounds rich in fruits and vegetables such as vitamins, carotenoids and polyphenols. Dietary antioxidants act by scavenging free radicals and inhibiting oxidative damage to cellular components.

Foods derived from plants (fruits, vegetables and whole grains), contain biologically active phytochemicals, and may provide several health advantages apart from nutrition, such in minimising the risk of chronic diseases (Liu, 2003). Phytochemicals are defined as non-nutrient plant compounds present in fruits, vegetables and other plant parts (Liu, 2004). More than 500 chemicals have been identified and their role as bioactive compounds are been studied. Numerous lines evidence suggests that the therapeutic benefits of phytochemicals may be even higher than currently proposed because of the involvement of free radicals in various chronic diseases (Block et al., 1992, Cantuti-Castelvetri et al., 2000, Reddy et al., 2003, Mathew et al., 2004).

Dietary phytochemicals may be classified into carotenoids, phenolics, alkaloids, nitrogen containing compounds and organo-sulphur compounds (Fig 1.11). Most of the phytochemicals are secondary metabolites, which are stored in different plant parts and perform several physiological functions in plants. In addition, these phytochemicals posses wide range of pharmacological properties, and can contribute significantly to the promotion and maintenance of good health. Among these phytochemicals carotenoids and phenolics are most studied for their antioxidant and protective properties.

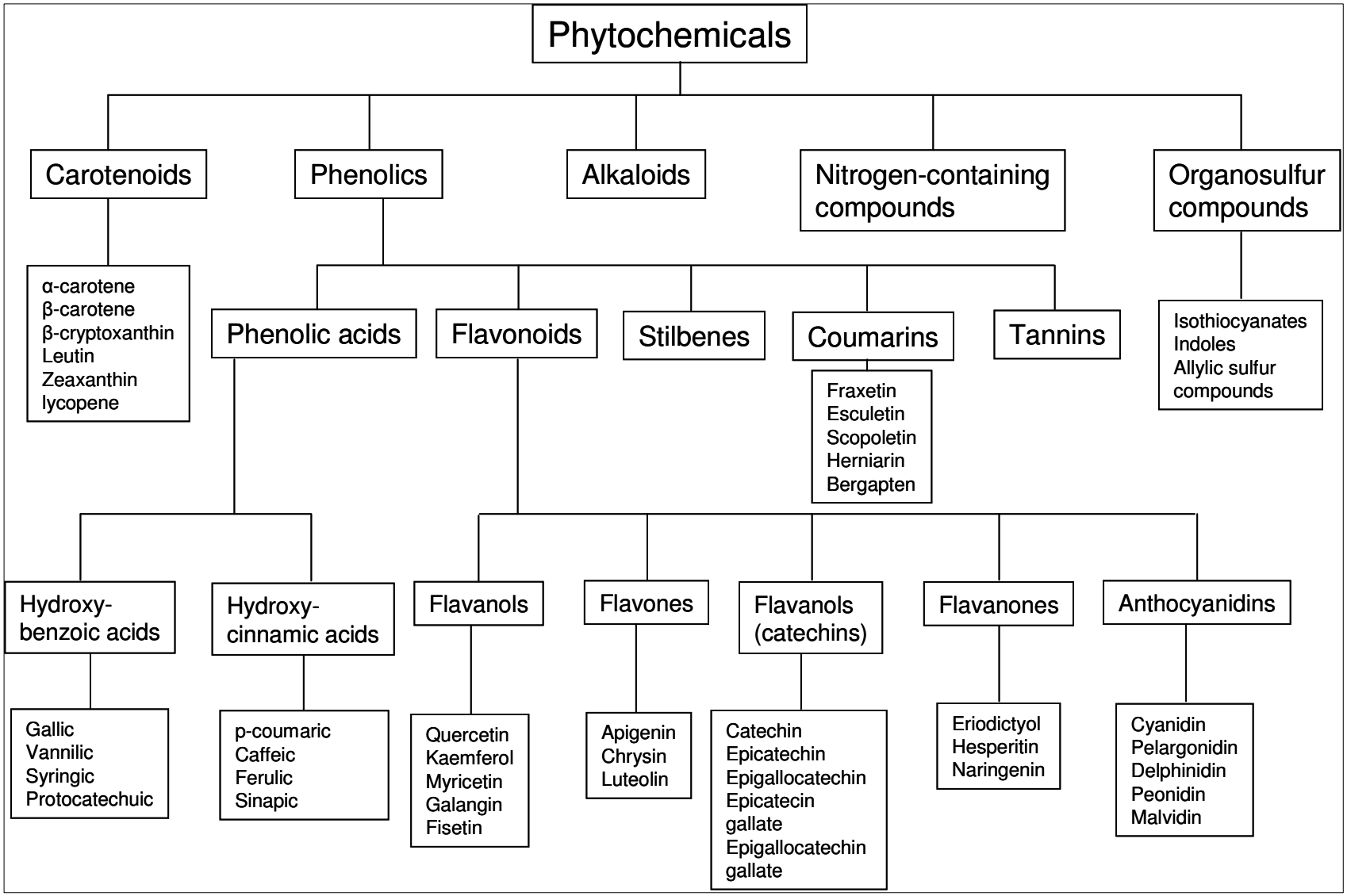


Fig. 1.11 – Classification of Phytochemicals (Liu, 2004)

1.9 Novel antioxidant strategies for diseases with an underlying oxidative stress

During the past few decades supplementation of antioxidants has been intensely studied in various animal models and clinical trials as a therapeutic possibility. The effects of antioxidants such as vitamin E, vitamin C, N-acetyl cysteine and glutathione have been investigated in several clinical trials for neuroprotection. However, most of these clinical trials did not show any potential beneficial effects on patients (Casetta et al., 2005). Recently, researchers have focused on novel antioxidant strategies to target particular organelles such as mitochondria that are known to generate huge amounts of ROS. Studies are being carried out on mitochondria-targeting antioxidant peptides, vitamin E and ubiquinone to enhance the benefits of antioxidant therapies (Kelso et al., 2001, Smith et al., 1999, Zhao et al., 2004). In this section, some of the novel antioxidant strategies that are being investigated are summarised.

1.9.1 Catalytic antioxidants

Compounds that mimic the actions of certain antioxidant enzymes such as superoxide dismutase, catalase, peroxidases are being studied in various *in vitro* and *in vivo* models (Golden and Patel, 2009). These compounds have an advantage over classical antioxidants that they can directly scavenge the free radicals rather than acting stoichiometrically to scavenge ROS.

1.9.2 Modulation of endogenous antioxidant transcriptional regulators

As discussed in the previous section, endogenous antioxidant defence system seems to be strictly regulated at transcriptional level. The most important transcription factors include NRF2 (nuclear factor E2-related factor 2) and PGC-1α (peroxisome proliferator-activated receptor-γ co-activator-1α).

1.9.2.1 NRF2/ARE

NRF2 induces the expression of wide variety of antioxidant, detoxifying and cytoprotective genes including NAD(P)H: oxidoreductases (Nioi and Hayes, 2004, Jaiswal, 2000), heme oxygenase 1 (HO1) (Prestera et al., 1995), glutathione-S-transferases (GSTs) (Rushmore and Pickett, 1990), aldo-keto reductases (AKRs) (Ellis et al., 2003) and γ-glutamylcysteine synthase (γ-GCS) (Wild and Mulcahy, 1999). Several novel approaches are being tested to stimulate the NRF2 activity such as administration of small molecule activators of NRF2, overexpression of NRF2, or disruption of the Keap1 activity: the endogenous repressor of NRF2.

1.9.2.2 PGC-1α

PGC-1α is pleiotropic transcriptional co-activator which triggers few of the battery of genes induced by NRF2. In addition, PGC-1α regulates some of the antioxidant proteins within the mitochondria (specific peroxiredoxin isoforms) that are not regulated by NRF2 (Clark and Simon, 2009). A similar approach, as mentioned above for NRF2, to increase the activity of PGC-1α may result in an antioxidant response that will be beneficial in various pathological conditions.

1.9.3 Natural products

Natural products derived from diet are gaining more importance as potential therapeutic agents for oxidative stress related diseases. Several lines of evidence propose that an increased consumption of fruits and vegetables is a rather simple but realistic strategy for protection against oxidative damage and to decrease the occurrence of chronic diseases and age related disorders. Many epidemiological studies suggest a positive association between the consumption of a diet rich in fruits and vegetables and a lower occurrence of chronic diseases (Block et al., 1992, Cantuti-Castelvetri et al., 2000, Reddy et al., 2003, Mathew et al., 2004). The protective effects of fruits and vegetables are attributed to the presence of

phytochemicals such as vitamins, polyphenols and carotenoids. The rationale behind the protective effects of flavonoids and coumarins are their strong antioxidant effects and it is also reported that polyphenols may also boost the endogenous antioxidant defence systems. Natural compounds such as resveratrol, sulforafane and quercetin are reported to activate NRF2-dependent transcription of endogenous antioxidant genes (Linseman, 2009, Vincent et al., 2009, Tanigawa et al., 2007). In addition, green tea polyphenols such as epigallocatechin 3-gallate (EGCG) have been shown to enter mitochondria and protect neurons from oxidative stress and neurodegeneration (Schroeder et al., 2009). This evidence shows that natural products provide an abundant source of therapeutic agents that could be used to mitigate oxidative stress related diseases.

In the following section, details about the phenolic compounds present in diet especially coumarin derivatives and their known pharmacological properties are summarised.

1.9.3.1 Plant Phenolics

The phenolic compounds can be further categorised as phenolic acids, flavonoids, coumarins, stilbenes and tannins. The fruits such as cranberry, apple, red grape, strawberry, pineapple, banana, peach, lemon, orange, pear and grape fruit are rich in phenolics (Sun et al., 2002). Among vegetables broccoli, spinach, yellow onion, red pepper, carrot, cabbage potato, lettuce, celery and cucumber have high phenolic content (Chu et al., 2002). Flavonoids are the major constituents of phenolics followed by phenolic acids and coumarins. Flavonoids and coumarins are widely distributed in natural plants and they have recently attracted much attention due to their manifold pharmacological activities.

1.9.3.2 Plant Coumarins

Coumarins are aromatic lactones of phenolic compounds that are distributed widely in the plant kingdom. Coumarins comprise a benzene ring fused with α-pyrone ring and called benzopyrones. More than 1300 coumarins have been identified mainly

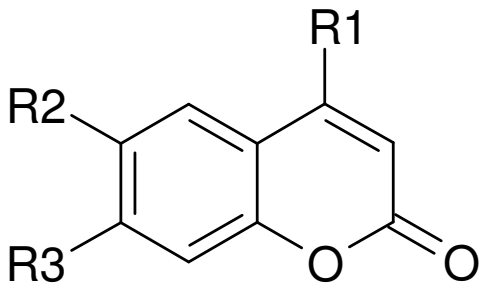
from plants and some from fungi and bacteria (Murray, 1991, Murray, 1989, Murray et al., 1982). Coumarins based on their structures can be classified into simple hydroxycoumarins (Fig. 1.12A), furanocoumarins (Fig. 1.12B) and pyranocoumarins.

1.9.3.3 Pharmacological properties of plant coumarins

Coumarins are reported to possess various pharmacological and biochemical properties, and their activity is dependent on the pattern of substitution in their chemical structure (Hoult and Paya, 1996). The coumarins are reported to exhibit antimicrobial (Dini et al., 1992, Kwon et al., 1997, Sardari et al., 1999), antiviral (Fuller, 1994), antimalarial (Yang et al., 1992), antinflammatory (Chen et al., 1995, Okada et al., 1995, Hiermann and Schantl, 1998, Hsiao et al., 1998, Garcia-Argaez et al., 2000, Fylaktakidou et al., 2004), antioxidant (Ng et al., 2000, Chang et al., 1996, Fylaktakidou et al., 2004) and antitumor promoting properties (Khan et al., 2004, Okuyama et al., 1990, Mizuno et al., 1994, Kofinas et al., 1998, Fujioka et al., 1999). The coumarin derivatives esculetin, fraxetin and daphnetin inhibit enzymes such as lipoxygenase, cycloxygenase (Hoult et al., 1994, Liu et al., 1998, Resch et al., 1998, Fylaktakidou et al., 2004) and monoamine oxidase (Huong et al., 1999). Coumarins were also reported to inhibit protein kinases *in vitro* (Yang et al., 1999).

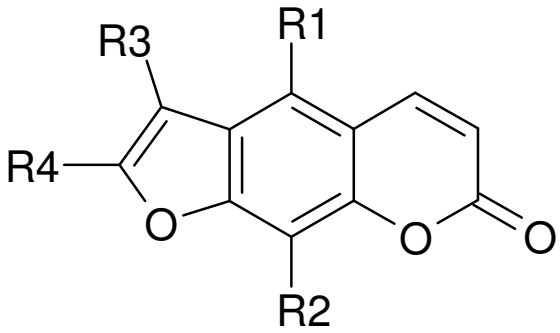
Plant coumarins protect cells from oxidative stress (Whang et al., 2005) and also act as neuroprotective agents (Molina-Jimenez et al., 2004). The consumption of diet rich in coumarin was reported to be chemopreventive against aflotoxin B_1-induced hepatocarcinogenesis in rats (Kelly et al., 2000). The coumarin derivatives esculetin, fraxetin and daphnetin were also observed to inhibit the generation of neutrophil-dependent superoxide anion (Ivanovska et al., 1994, Paya et al., 1994, Chang and Chiang, 1995, Chang et al., 1996, Fylaktakidou et al., 2004). Simple coumarins such as fraxetin and 4-methyl daphnetin inhibit lipid peroxidation and scavenge superoxide radicals. Another coumarin derivative, 5, 7-dihydroxy-4-methylcoumarin also inhibits lipid peroxidation and scavenges alkylperoxyl and superoxide radicals without pro-oxidant effect (Hoult and Paya, 1996). A new coumarin analogue from *Hibiscus syriacus* was observed to inhibit lipid peroxidation and the activity was

A



Simple coumarins	R1	R2	R3
Coumarin	H	H	H
Herniarin	H	H	OCH_3
Methyl-umbelliferone	CH_3	H	OH
Scopoletin	H	OCH_3	OH
Umbelliferone	H	H	OH
Esculetin	H	OH	OH

B



Furanocoumarins	R1	R2	R3	R4
Bergapten	OCH_3	H	H	H
Psoralen	H	H	H	H
Xanthotoxin	H	OCH_3	H	H

Fig. 1.12 – Structure of dietary coumarin derivatives

comparable to vitamin E. Some coumarin derivatives were potent inhibitors of microsomal lipid peroxidation (Paya et al., 1992a, Raj et al., 1998).

1.9.3.4 Pharmacokinetics of coumarins

To my knowledge the pharmacokinetics of coumarin derivatives have not been extensively investigated however numerous studies have reported the pharmacokinetics of the parent compound 'coumarin'. Following oral administration coumarin is rapidly absorbed from the gastrointestinal tract and is distributed through out the body (Pelkonen O. et al., 1997, Fentem and Fry, 1993). Coumarin appears to be extensively metabolised in all species with little unchanged coumarin being excreted. In humans, coumarin is completely absorbed from gastrointestinal tract after oral administration and extensively metabolised by the liver in the first pass, with 2-6 % reaching the systemic circulation intact (Ritschel et al., 1976, Ritschel and Hoffmann, 1981, Hardt and Ritschel, 1983). Coumarin is reported to be metabolised by hydroxylation at all six possible positions (i.e. at carbon atoms 3, 4, 5, 6, 7 and 8) and by opening of the lactone ring to yield various products. In majority of human subjects studied coumarin is extensively metabolised to 7-hydroxy coumarin (umbelliferone). In addition, 6, 7-dihydroxy coumarin (esculetin) is also reported to be one of the metabolites of coumarin. Generally, the half-life of coumarin is around 1-2 hours in humans and between 1-4 hours in other species including rodents (Lake, 1999).

1.9.3.5 Pharmacological properties of umbelliferone and esculetin

Umbelliferone (7-hydroxycoumarin; Umb) and esculetin (6,7-dihydroxycoumarin; Esc) (Fig 1.13) are phenolic compounds classified as coumarin derivatives that are found in plants with proven antioxidant and free radical scavenging properties (Paya et al., 1992b). Umb is present in plants including *Aegle marmelos* (golden apple) (Parmar and Kaushal, 1982) and *Citrus aurantium* (bitter orange) (Wu and Sheu, 1992). It is a promising antioxidant compound (Hoult and Paya, 1996, Thuong et al.,

2010), and reported to maintain redox status and prevent diabetic rats from erythrocyte lipid peroxidation (Ramesh and Pugalendi, 2005).

Esculetin is present in several plants such as *Artemesia scoparia* (Redstem Wormwood), *Artemesia capillaris* (Capillary Wormwood), *Ceratostigma willmottianum* (Chinese Plumbago) and in the leaves of *Citrus limonia* (Chinese lemon) (Chang et al., 1996, Yue and Xu, 1997). Esculetin is a proven antioxidant (Paya et al., 1992a, Lin et al., 2000) that has been shown to protect hamster lung fibroblasts (V79-4) from lipid peroxidation, protein carbonylation, and DNA damage induced by hydrogen peroxide (Kim et al., 2008). It has also been shown to suppress lipid hydroperoxide-induced oxidative damage to cellular DNA in human diploid fibroblasts (TIG-7) cells (Kaneko et al., 2003). In addition, esculetin can inhibit oxidative DNA damage and the formation of tumours induced by 1,2-dimethylhydrazine in rat colon (Kaneko et al., 2007). 6,7-di-O-glucopyranosyl-esculetin has been shown to protect SH-SY5Y neuroblastoma cells against dopamine-induced toxicity (Zhao et al., 2008).

1.10 Aims of the project

Plant coumarins are present in a wide range of fruits and vegetables, have a low toxic profile and are available in variety of daily food. Evidence shows that coumarin derivatives can effectively scavenge free radicals and inhibit lipid peroxidation, which make them potential candidates as therapeutic agents against oxidative stress-induced diseases. Despite these observations, little is known about the protective effects and mechanism of protection elicited by dietary coumarin derivatives on oxidative stress-induced damage.

Therefore, the aims of this project were:

- To study the protective effects of esculetin in HepG2 hepatoma cells against hydrogen peroxide-induced oxidative stress and investigate the mechanism of protection

- To determine the chemopreventive effects of esculetin and umbelliferone against NDEA-induced oxidative stress and hepatotoxicity in rat liver

- To investigate the protective effect of umbelliferone and esculetin against hydrogen peroxide and neurotoxin (MPP^+)-induced oxidative stress in SH-SY5Y neuronal cells

- To determine the neuroprotective effects of esculetin and umbelliferone against MPTP-induced dopaminergic toxicity in mouse substantia nigra and striatum, and elucidate the molecular mechanism involved

- To study the effects of dietary coumarin derivatives on up-regulation of NRF2-ARE dependant antioxidant, detoxification and cytoprotective genes in vital organs of rats and mice

CHAPTER 2

MATERIALS AND METHODS

2. Materials and Methods

2.1 Materials

2.1.1 Mammalian Cells

2.1.1.1 HepG2 Cells

Human hepatoma HepG2 cells (HB-8065) (Knowles *et al.*, 1980) were obtained from American Type Culture Collection (ATCC) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 1% non-essential amino acids, 1% sodium pyruvate, 100 U/ml penicillin and 100 µg/ml streptomycin in a humidified incubator with 5% CO_2 at 37°C. Cells were grown to 70% confluence before pre-treatment.

2.1.1.2 SH-SY5Y Cells

Human neuroblastoma SH-SY5Y cells were a gift from Dr. Eve Lutz and were maintained in 1:1 mixture consisting of Ham's F12 and Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% (v/v) heat-inactivated fetal bovine serum, 1% non-essential amino acids, 1% sodium pyruvate, 1% L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin in a humidified incubator with 5% CO_2 at 37°C. Cells were grown to 70% confluence before pre-treatment. All cells in the study where used at low passage numbers (< 20).

2.1.2 Cell Culture Media and Equipment

Cell culture media and reagents were obtained from Sigma (Poole, UK), Invitrogen (Renfrewshire, UK). The tissue culture hood was obtained from ICN Gelaire

(England) and the incubator from Heraeus (Germany). The cells were maintained at 37^{o}C at 5% carbon dioxide and 95% humidity.

2.1.2.1 Cell Culture Plasticware

Tissue culture flasks: EasyFlask 75 cm^2; 6-well, 12-well and 24-well plates were obtained from Fisher Scientific (UK). The 96-well plates were purchased from Greiner Bio-One (UK) and cell scrapers were obtained from BD biosciences (UK).

2.1.3 Animals

The use of animals in scientific procedures for this thesis work was approved by the Home Office (Science and Research), UK (PPL No. 60/4015). All the methods and procedures in the project licence were strictly followed.

2.1.3.1 C57BL/6J Mice

Male C57BL/6J 6-week old mice (Harlan, UK) were housed in cages in groups of six under controlled environmental conditions (19-23^{o}C; 40-60% humidity; 12h light/dark cycle) at the Biological Procedures Unit (BPU), University of Strathclyde.

2.1.3.2 Wistar Strain Rats

Male Wistar 10-week old rats (Charles River, UK) were housed in cages in groups of three under controlled environmental conditions 19-23^{o}C; 40-60% humidity; 12h light/dark cycle) at the Biological Procedures Unit (BPU), University of Strathclyde.

2.1.4 Chemicals and Reagents

Cofactors (NAD, FAD, NADPH), TEMED, ampicillin, coumaric acid, luminol, BHA, NAC, MPTP, MPP$^+$, NDEA, quercetin, umbelliferone, esculetin, fraxetin,

scopoletin, MTT, DCF-DA were supplied by Sigma (Poole, UK). Bio-Rad Protein Assay Reagents and pre-stained sodium dodecylsulphate (SDS) standards were supplied by Bio-Rad Laboratories (Hertfordshire, UK). Acrylamide was supplied by Kramel (Cramlington, UK). All other laboratory and analytical grade chemicals and reagents were purchased from Sigma (Poole, UK) or suppliers mentioned.

2.1.5 Antibodies

The antibodies used for Western blotting and immunohistochemistry are listed in table 2.1.

2.1.6 Oligonucleotides

The oligonucleotide primers used for Polymerase Chain Reaction (PCR) listed in table 2.2 were supplied by Eurofins MWG Operon (Ebersberg, Germany).

2.2 Cell Culture Methods

2.2.1 Treatment of Mammalian Cells

To study the toxicity of compounds and protective effects of plant coumarins, cells were plated in 96-well plates at a density of 2×10^4 cells per well (HepG2) and 5×10^4 per well (SH-SY5Y), or in 6-well plates at a density of 2×10^5 cells per well (HepG2) and 5×10^5 per well (SH-SY5Y), or in 24-well plates at a density of 1×10^5 cells per well (HepG2) and 3×10^5 per well (SH-SY5Y), and cultured for 24h in growth medium. Then cells were pretreated with/without coumarins in serum free medium for a specific time period (4 h or 8 h), then washed with PBS and exposed to toxic compounds in serum free medium for 24 h or 48 h.

Table 2.1 - Antibodies

Antibody	Suppliers	Cat. No	Source	Reactivity	MW (kDa)
NQO1	JH	-	Rabbit		30
SOD1	SCBT	sc11407	Rabbit	h, m, r	18
GAPDH	SCBT	sc25778	Rabbit	h, m, r	37
NRF2	SCBT	sc13032	Rabbit	h, m, r	68
LAMIN-B	SCBT	sc6216	Goat	h, m, r	71
HO1	SCBT	sc7695	Goat	h, m, r	35
GSTP1	JH	-	Rabbit	h, m, r	27
BAX	SCBT	sc493	Rabbit	h, m, r	20
TH	Abcam	ab49640	Mouse	h, m, r	56
3-NT	Abcam	ab61392	Mouse	h, m, r	1
CASPASE-3	SCBT	sc98785	Rabbit	h, m, r	17, 19
CYTOCHROME-C	SCBT	sc7159	Rabbit	h, m, r	11
pJNK	CST	4671S	Rabbit	h, m, r	46,54
JNK	CST	9252S	Rabbit	h, m, r	46,54
AKR1A3	JH	-	Rabbit	h, m, r	37
AKR7A1	JH	-	Rabbit	h, r	37
AKR7A4	JH	-	Rabbit	h, r	37
GSTY'8c/GSTA3	JH	-	Rabbit	h, r	26
Anti-rabbit IgG-HRP secondary Ab	BRL	166-2408EDU	Goat	-	-
Anti-goat IgG-HRP secondary Ab	SCBT	sc2033	Donkey	-	-
Anti-mouse IgG-Biotin secondary Ab	Abcam	ab6788	Goat	-	-

MW: molecular weight; SCBT: Santa Cruz Biotechnology, Inc., Germany; JH: Gift from Professor John Hayes, University of Dundee; Abcam: Abcam, UK; BRL: Bio-Rad Laboratories (Hertfordshire, UK); CST: Cell Signaling Technology, UK; h: human; m: mouse; r: rat

Table 2.2 – Oligonucleotide Primers

Primer	Sequence	Suppliers
SOD1Q_forward	5'-AGG GCA TCA TCA ATT TCG AGC -3'	MWG
SOD1Q_reverse	5'-GCC CAC CGT GTT TTC TGG A-3'	MWG
NQO1_forward	5'-ATG TAT GAC AAA GGA CCC TTC C-3'	MWG
NQO1_reverse	5'-TCC CTT GCA GAG AGT ACA TGG-3'	MWG
HO1_forward	5'-TGA TAG AAG AGG CCA AGA-3'	MWG
HO1_reverse	5'-TTT CCA GAG AGA GGG ACA-3'	MWG
β-actin_forward	5′-AGA GAG GCA TCC TCA CCC TGA AGT ACC-3′	MWG
β-actin_reverse	5′-GAC GTA GCA CAG CTT CTC CTT AAT GTC-3′	MWG
GAPDH_forward	5′-GGA GTC AAC GGA TTT GGT-3'	MWG
GAPDH_reverse	5'-GTG ATG GGA TTT CCA TTG-3'	MWG

MWG: Eurofins MWG Operon (Ebersberg, Germany)

2.3 Cell Viability Assay

2.3.1 MTT assay

Cell viability was evaluated as mitochondrial activity by the modified MTT dye uptake method (Mosmann, 1983). This tetrazolium dye colorimetric assay is used to monitor cell growth indirectly as indicated by the conversion of the tetrazolium salt to the coloured product, formazan, the concentration of which can be measured spectrophotometrically. In brief, cells were incubated in a 96-well plate in triplicate for 24h. Cells were then treated with coumarins and/or toxic compounds for specified time period. At the end of the incubation 20µl of MTT (1.2 mg/ml) was added to the cells and further incubated for 2h. Then MTT containing medium was removed and the intracellular formazan product was dissolved in DMSO, and the optical density (OD) at 540 nm was measured by means of a 96-well Labsystems iEMS plate-reader. Vehicle-treated cultures were used as controls. The following formula was used: Percentage cell viability = (OD of the experiment samples/OD of the control) x 100.

2.3.2 LDH leakage assay

The cytotoxicity of test compounds was determined as lactate dehydrogenase (LDH) leakage by commercial assay kit (Sigma, Cat. No. TOX7) (Decker and Lohmann-Matthes, 1988). This kit measures cytoplasmic LDH leakage as an indicator of the cell membrane integrity. In this assay, LDH reduces NAD^+, which is then used to convert a tetrazolium dye to a soluble coloured formazan derivative.

Cells were plated in 24-well plates and cultured for 24 h. The cells were treated with coumarins and/or toxic compounds in serum free medium for a specific time period. After incubation, one aliquot of medium (0.2ml) was taken out for extracellular LDH activity analysis. The total LDH activity was determined after the cells were disrupted thoroughly by sonication. The percentage of LDH leakage was then calculated from the ratio between the LDH activity in culture medium and that of the whole cell content to reflect the cytotoxicity.

2.4 Measurement of Reactive Oxygen Species (ROS)

Cellular reactive oxygen species was quantified by dichlorofluorescin diacetate (DCF-DA) assay using microplate reader (Wang and Joseph, 1999). After being oxidised by intracellular oxidants, DCF-DA will be converted to a fluorescence emitting compound, dichlorofluorescein which can be quantified. In brief, cells were incubated in a 96-well plate in triplicate for 24h. Cells were then treated with test compounds for 8h. After the treatments, cells were washed with PBS and incubated with 100μM DCF-DA for 30 min. Then the cells were washed twice with PBS to remove excessive probe. After washing out, 0.9mM H_2O_2 was added and the fluorescence was measured at 485 and 530 nm excitation and emission, respectively, for 30 min in a CytoFluor Series 4000 multi-well fluorescence plate reader (PerSeptive Biosystems Inc., Framingham, MA, USA) with temperature maintained at 37^{o}C. The percentage increase in fluorescence was calculated by the formula [(Ft_{30} – Ft_0)/ Ft_0*100], where Ft_{30} = fluorescence at time 30 min and Ft_0 = fluorescence at time 0 min.

2.5 Measurement of Total Cellular Glutathione

2.5.1 Preparation of cell extracts for total glutathione assay

Cells were plated in 6-well plates and treated with or without test compounds for 8h and/or with oxidants/lipid peroxidation products for 24h. After treatments, the cells were washed and scraped into PBS and centrifuged at 1000 rpm for 5 min at 4^{o}C. The pellet was re-suspended in PBS and protein concentrations of the samples were measured by Bradford's assay and normalised in 5% SSA.

2.5.2 Preparation of tissue homogenates for total glutathione assay

The snap-frozen tissues were homogenised in 3 vol. of 20mM sodium phosphate buffer pH 7.0, containing 1mM DTT and protease inhibitor cocktail using Teflon pestle. The homogenate was centrifuged at 10,000 ×g for 30 min at 4^{o}C and the supernatant was retained. Protein concentrations of the samples were measured by Bradford's assay and normalised in 5% SSA.

2.5.3 Total Glutathione assay

Total intracellular GSH was measured by an enzymatic recycling procedure (Eady et al., 1995). The sulfhydryl group of GSH reacts with DTNB (5,5'-dithiobis-2-nitrobenzoic acid, Ellman's reagent) and produces a yellow coloured 5-thio-2-nitrobenzoic acid (TNB). The disulfide that is produced is reduced by NADPH in the presence of GSH reductase. The rate of formation of TNB is measured at 412 nm, and GSH is quantified by reference to a standard curve. The specificity of this GSH assay is controlled by the very high specificity of glutathione reductase. A stock buffer of 100mM sodium phosphate and 1mM EDTA (pH 7.4) was used to prepare solutions of 0.4mM NADPH, 4.5mM DTNB, 1.9 units/ml GSH reductase (from Bakers Yeast, Sigma) and 5% sulfosalicylic acid (SSA). The standards of reduced GSH were prepared in 5% SSA. The samples (prepared as mentioned above) and standards were centrifuged at 10,000 rpm for 10 min. For each measurement, three wells on the 96-well plate contained 170µl reaction mixture (GSH reductase and NADPH), 20µl sample or GSH standard and 10µl DTNB. The mixture was incubated for 10 min at 37^{o}C and the absorbance was read at 412 nm. The concentration of total GSH in the samples was determined against the GSH standard calibration curve.

2.6 Protein Gel Electrophoresis

2.6.1 Preparation of Protein Extract from cell lines

Mammalian cells were grown in 6-well plates or 100mm dishes and treated with the test compounds for specific time period. The cells were washed with Phosphate Buffer Saline (PBS - 137 mM NaCl, 10mM sodium phosphate, 2.7mM KCl, pH 7.4), then detached by using 1x trypsin or 1x SSC (150mM NaCl, 15mM sodium citrate) and centrifuged for 5 min at 1000 rpm. Finally, the pellets were suspended in PBS, protein concentrations were determined, normalised to 1 mg/ml in 2× LSB and denatured by boiling for 10 min.

2.6.2 Preparation of protein extract from animal tissue

The harvested tissue was homogenised in 5 volumes of 20mM sodium phosphate buffer containing 1mM DTT and protease inhibitor cocktail using Teflon pestle. Finally, protein concentrations of the homogenates were determined, normalised with 2× LSB to yield 1 mg/ml solution and denatured by boiling for 10 min.

2.6.3 Sub-cellular fractionation of cell extracts and tissue homogenates

Sub-cellular fractionation of cell lines and tissue homogenates were carried out by differential centrifugation. Cells in 3 × 175 cm^2 flasks were rinsed with PBS once, scraped and centrifuged at 1,000 ×g for 3 min at 4^oC. Cell pellet or animal tissue (2g wet weight) were suspended in 1ml of homogenisation media (0.25M sucrose, neutralised to pH 7, 10mM HEPES, pH 7.5) and homogenised using a Teflon pestle. The homogenate was made up to 2 ml with homogenisation media and centrifuged at 2000 ×g for 10 min at 4^oC. The pellet was retained as nuclear fraction. The supernatant was removed and centrifuged at 9, 000 ×g for 10 min at 4^oC, and the pellet was retained as mitochondrial fraction. The resulting supernatant was removed and centrifuged at 34, 000 ×g for 30 min at 4^oC, and the pellet was retained as the

fraction enriched for Golgi. The supernatant was removed and centrifuged at 80, 000 ×g for 1h at 4°C to pellet the endoplasmic reticulum enriched fraction. The remaining supernatant was retained as cytosolic fraction. Protein concentration of the sub-cellular fractions was determined by Bradford's assay and normalised with 2× LSB (at least 2 vol. of fraction to 1 vol. of 2× LSB) to yield 1 mg/ml solution and denatured by boiling for 10 min.

2.6.4 Equipment and Buffers

Equipment: Electrophoresis was performed using the Atto mini gel system from Genetic Research Instrumentation Ltd., Dunmow, Essex, UK. One dimensional electrophoresis on SDS-acrylamide gels was used to separate proteins according to their molecular weight, using the buffer system described by Laemmli (Laemmli, 1970) (Appendix B).

2.6.5 Sample Loading and Electrophoresis

Samples were boiled in 2× LSB for 10 min to denature the proteins and 10µg protein was loaded in the well. The electrophoresis was carried out for 90 min at 125 volts to separate the proteins.

2.7 Western Blotting

2.7.1 Transfer and Detection

The resolved proteins were transferred to nitrocellulose membrane using a Bio-Rad minigel apparatus for 1.5h at 300 mA. Protein binding sites were blocked with 5% skimmed milk in TBST (20mM Tris-HCl, 150mM NaCl, 0.02% Tween-20) (Appendix B) for 1h. Then nitrocellulose membranes were incubated with primary antibodies (at 1:3000 dilution in TBST-skimmed milk) overnight at 4°C. After

hybridisation with primary antibodies the membranes were washed thrice with TBST, incubated with horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit at 1:3000 dilution in TBST-skimmed milk) for 2h and washed thrice with TBST. Finally antibodies were detected using enhanced chemiluminescence (ECL, Amersham). Images were taken using a LAS-3000 luminescent image analyzer (Fujifilm). Quantification of band intensities was performed using ImageJ analysis software.

2.8 Determining Protein Concentrations – Bio-Rad Protein Assay

The Bio-Rad Protein assay is based on the method of Bradford (Bradford *et al.,* 1976). Dilutions of Bovine Serum Albumin (BSA) protein standard, ranging from 0 to 20µg/ml were made in distilled water. 200µl of Bio-Rad reagent was added to 800µl of each of the protein standards, and the absorbance measured at 595 nm. A standard curve of OD_{595} *versus* concentration of protein standard was plotted. Unknown protein concentrations were calculated from the standard curve. The sample reaction mixture routinely included 0.2 ml of Bio-Rad reagent, 10µl of sample and 790µl of distilled water.

2.9 Enzyme Assays

2.9.1 Preparation of cell extracts for enzyme assays

Protein extracts from cell lines for enzyme assays were prepared by 'freeze-thaw' lysis method to preserve enzyme activity. The cells were washed with PBS once, detached in 1× SSC (150mM NaCl, 15mM sodium citrate) or 1× trypsin and centrifuged at 1000 rpm for 5 min at 4^{o}C. The resulting cell pellet was re-suspended in 250 mM Tris-Cl, pH 7.5. The cell suspension was frozen in liquid nitrogen for 5 min and transferred to 37^{o}C for 5 min. The freeze-thaw procedure was repeated twice again. Then the cell suspension was centrifuged at 1000 rpm for 5 min at 4^{o}C, and

the supernatant was used for enzyme assays. Protein concentrations were determined by using Bradford's method against BSA standard curve.

2.9.2 Preparation of tissue homogenates for enzyme assays

Frozen tissue was homogenised in 3 vol. of ice-cold 20mM sodium phosphate buffer pH 7.0, containing 1mM DTT and protease inhibitor cocktail using a Teflon pestle. The homogenate was centrifuged at 10,000 ×g for 30 min at 4^{o}C. The supernatant was used for enzyme assays. Protein concentrations were determined by using Bradford's method against BSA standard curve.

2.9.3 NAD(P)H: quinoneoxidoreductase (NQO1, QR) activity

The cell extract or tissue homogenate (20µl) was added to mixture containing 4mM NADPH, 1mM FAD, 160mM DCPIP (2,6-dichlorophenolindophenol), 4.6 mg/ml BSA, 0.2% Tween-20 in 25mM Tris-HCl buffer pH 7.4. The reduction of DCPIP was measured at 590 nm and NQO1 activity was expressed as nanomoles per minute per milligram of protein.

2.9.4 Glutathione-S-Transferase (GST) activity

The cell extract or tissue homogenate (20µl) was added to mixture containing 1mM reduced glutathione, 1mM CDNB (1-chloro-2,4-dinitrobenzene) and 3 mg/ml BSA in 10mM sodium phosphate buffer pH 6.5. The CDNB-GSH conjugate formed was measured at 340 nm and glutathione S-transferase activity was expressed as nanomoles per minute per milligram of protein.

2.9.5 Aldoketo Reductase (AKR) activity

The cell extract or tissue homogenate (10µl) was added to mixture containing 25 mM p-Nitrobenzaldehyde (p-NBA), 5mM NADPH in 100mM sodium phosphate buffer. The consumption of NADPH was measured at 340 nm and AKR activity was expressed as nanomoles per minute per milligram of protein.

2.9.6 Superoxide Dismutase (SOD) activity

The total superoxide dismutase activity was determined by manufacturer's protocol using SOD assay kit (cat. no. 19160) from Sigma-Aldrich Ltd. In this method, Dojindo's highly water soluble tetrazolium salt that produces a water soluble formazan dye upon reduction with superoxide anion was used. SOD linearly inhibits the formation of this coloured formazan dye. The SOD activity was quantified as inhibition activity by measuring the decrease in colour development of the formazan dye at 440 nm.

2.9.7 Glutathione Reductase activity

The cell extract or tissue homogenate (20µl) was added to reaction mixture containing 20mM GSSG in 50mM sodium phosphate buffer pH 7.0. The mixture was incubated at 37 ^{o}C for 3 min and 1.5mM NADPH (prepared in 0.1% sodium bicarbonate) was added. The consumption of NADPH was measured at 340 nm and glutathione reductase activity was expressed as nanomoles per minute per milligram of protein.

2.9.8 Glutathione peroxidise activity

The cell extract or tissue homogenate (20µl) was added to reaction mixture containing 10mM reduced glutathione, 2.4 U/ml glutathione reductase, 1.5mM

NADPH (prepared in 0.1% sodium bicarbonate) 1mM EDTA, 2mM sodium azide in 50mM sodium phosphate buffer. The mixture was incubated at 37^{o}C for 3 min and 2mM H_2O_2 was added. The consumption of NADPH was measured at 340 nm and glutathione peroxidase activity was expressed as nanomoles per minute per milligram of protein.

2.9.9 Caspase 3 activity

The cell extracts or tissue homogenates were prepared as mentioned above and the assay for Caspase 3 activity was performed according to manufacturer's protocol (Caspase-3/CPP32 colorimetric assay kit, BioVision, USA, Cat. No. K106-100). This kit assays the activity of caspases that recognise the sequence DEVD. The assay is based on the spectrophotometric detection of the chromophore p-nitroaniline (pNA) after cleavage from the labelled substrate DEVD-pNA. The pNA light emission was quantified using a micro-plate reader at 405 nm. Caspase 3 activity was calculated by comparing the absorbance of pNA from an apoptotic sample with uninduced control.

2.10 Lipid peroxidation assay

The assay for lipid hydroperoxide was performed according to manufacturer's protocol (Lipid hydroperoxide assay kit, Cayman Chemical Company, USA, Cat. No.705002). This kit measures the hydroperoxides directly utilizing the redox reactions with ferrous ions. Hydroperoxides are highly unstable and react readily with ferrous ions to produce ferric ions. The resulting ferric ions were measured using thiocyanate ion as the chromogen. A standard curve of OD_{500} *versus* concentration of lipid hydroperoxide standard was plotted and sample lipid hydroperoxide concentrations were calculated from the standard curve.

2.11 Fluorescence imaging for apoptosis and necrosis

After treatment with test compounds and toxins cells were analyzed for apoptosis or necrosis according to manufacturer's protocol (Annexin V-Cy3 Apoptosis Kit Plus, Cat. No. K202-25, BioVision, UK). The assay is based on the observation that soon after initiating apoptosis, cells translocate the membrane phospholipid phosphatidylserine (PS) from the inner face of the plasma membrane to the cell surface. Once on the cell surface, PS can easily be detected by staining with a fluorescent conjugate of Annexin V, a protein that has a strong natural affinity for PS. The SYTOX green dye, in the kit, is impermeant to live cells and apoptotic cells, but stains necrotic cells with intense green fluorescence by binding to cellular nucleic acids. After staining with annexin V-Cy3 and SYTOX Green dye, apoptotic cells show red fluorescence, dead cells show green fluorescence and live cells show little or no fluorescence. These populations were distinguished by Fluorescence microscopy using FITC and rhodamine filters.

2.12 RNA Methods

2.12.1 RNA Isolation from Mammalian Cells

Total RNA was isolated from HepG2 and SH-SY5Y cells from 100mm dishes using the Total RNA isolation kit (Promega) according to the manufacturer's instructions.

2.12.2 RNA Quantification

Purified RNA was diluted 1:100 in 10mM Tris, pH 7.5 and the OD measured at 260 nm and 280 nm to determine its concentration and purity. When calculating concentration, the conversion factor for RNA of 0.040µg/µl per OD_{260} unit was used. The ratio of the 260 nm measurement to the 280 nm measurement was used as an indicator of purity, and only samples with ratios of 1.8 to 2.1 were used for subsequent experiments.

2.12.3 Quantitative Reverse Transcription

2.12.3.1 Synthesis of First Strand cDNA using Reverse Transcriptase

1µg of random primer and 5µg of total RNA were mixed in a total volume of 11µl nuclease free water in a sterile RNase-free microcentrifuge tube. The mixture was heated to 70°C for 5 min followed by incubation on ice for 2 min. The tube was briefly centrifuged and the following components added: 5µl of M-MLV RT 5X buffer, 1.25µl of PCR nucleotide mix (10mM), 6.75µl of nuclease free water and 1µl of M-MLV reverse transcriptase (200U/µl). The contents were gently mixed by pipetting, and the tube was incubated at 25°C for 10 min, followed by incubation at 42°C for 50 min. Following incubation the reaction was inactivated by heating at 70°C for 15 min.

2.12.3.2 Quantitative PCR

The relative quantitation of expression levels were carried out with the LightCycler instrument (Roche Diagnostics) using a set of oligonucleotide primers to suit amplification under the specific cycling conditions for the LightCycler. Reactions were set up in 20µl volumes with 1µl of first strand cDNA, 1µl of each primer (5pmol/µl, final concentration of 0.25µM), 3µl of nuclease free PCR-grade water and 15µl of PerfeCta SYBR Green Fastmix (Quanta Biosciences) as detailed in the manual. The relative amount of cDNA synthesized in each RT-PCR was compared with GAPDH or β-actin mRNA levels detected using specific primers (Table 2.1)

2.13 Animal treatment and tissue isolation methods

2.13.1 General Diet and Drug Treatment

Animals were allowed to acclimatize for 2 weeks before being fed either a normal diet (powdered RM1 diet, Special Diet Services, UK) supplemented with 2% arachis

oil or diet containing test compounds with 2% arachis oil. Arachis oil was used as a vehicle for the uniform distribution of test compounds. The test compounds in animal diet were provided in the diet for 7 days in the following amounts: BHA, 0.75% (w/w); Umbelliferone, 0.5% (w/w); Esculetin, 0.5% (w/w); Fraxetin, 0.5% (w/w); and Scopoletin, 0.5% (w/w). During the experiment the animals were provided free access to food and water.

2.13.2 General Tissue Harvesting

After the treatment period the animals were sacrificed by cervical dislocation and organs such as brain, liver and kidneys were removed immediately, snap-frozen in liquid nitrogen and stored at -80^{o}C until analysis. Tissue slices were rinsed in physiological saline and immediately fixed in 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.4, for histological and immunohistochemical studies.

2.13.3 Histology

2.13.3.1 Tissue fixation and Processing

Tissue samples were rinsed in physiological saline and immediately fixed in 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.4 for 48 hrs. Fixed samples were then subsequently processed through a series of incubations in graded alcohol and HistoClear (xylene substitute; Fisher Scientific, UK) as described in Table 2.2.

2.13.3.2 Tissue Embedding and Sectioning

Fixed and processed tissue samples were embedded in blocks of paraffin wax and section of 10μm were cut using a rotary microtome (Leica, Wetzlar, Germany). Paraffin ribbons were placed in water bath at 40-45^{o}C and the sections were mounted

onto glass slides. The sections were allowed to air dry for 30 min and oven-dried overnight at 45-50°C.

For mouse brain MPTP-toxicity studies, serial 10µm-thick coronal sections containing the mid-striatum (+1.2 through -0.1 mm with respect to bregma) and midbrain (+3.4 through -3.64 mm with respect to bregma) were cut into paraffin ribbons using a microtome. Then sections were mounted on to the slides, air-dried for 30 min and baked overnight in an oven at 45-50^{o}C.

2.13.3.3 Immunohistochemistry

The sections were deparaffinised, hydrated (100%, 90% and 70% ethanol, distilled water) and antigen unmasking was carried out using 10mM citrate buffer, pH 6.0. The sections were then incubated with 3% hydrogen peroxide, washed with TBST (20mM Tris-HCl, 150mM NaCl, 0.01% Tween-20) and blocked with 5% horse serum in TBST for 1h. Then the sections were incubated with antibodies against tyrosine hydroxylase (1:100) and 3-nitrotyrosine (1:100) overnight at 4^{o}C. The sections were washed with TBST and incubated with biotinylated secondary antibody (1:1000) for 1h. Subsequently, sections were washed with TBST and incubated with avidin-biotin peroxidise complex for 1h at room temperature. Visualisation was performed using DAB solution containing 0.05% diaminobenzidine and 0.015% hydrogen peroxide in 10mM Tris buffer, pH 7.2. The sections were dehydrated as mentioned before and then cover-slipped using DPX mounting medium. Immunostaining images of the striatum and midbrain were collected and densitometric analysis was performed by using NIH ImageJ software. The mean threshold area of the brown-stained TH-positive or 3-NT-positive cells from 3 images of each animal was calculated and expressed as percentage density by comparing with vehicle treated control group.

Table 2.3 - Tissue fixation and processing

Solvent	Duration (Hours)
1. 70% Ethanol	1
2. 70% Ethanol	1
3. 80% Ethanol	1
4. 80% Ethanol	1
5. 95% Ethanol	1
6. 95% Ethanol	1
7. 100% Ethanol	1
8. 100% Ethanol	1
9. 100% Ethanol	1
10. HistoClear	1
11. HistoClear	1
12. HistoClear	1
13. Paraffin wax	1
14. Paraffin wax	1
15. Paraffin wax	1

2.13.3.4 Haematoxylin and Eosin (H&E) Staining

Mounted sections of tissues were subjected to H&E staining, which involved a series of stain and solvent incubations as described in Table 2.3. Then sections were dehydrated and mounted using DPX. The H&E stained images of the liver were collected and observed for cell morphology (loss of architecture and acute inflammatory cells with vacuolated cytoplasm).

2.13.3.5 Nissl Staining

Brain tissue sections were deparaffinised and hydrated as mentioned in table 2.4. Then the sections were stained with 0.1% crystal violet solution in water (containing 0.3% glacial acetic acid) for 10 min then washed quickly in distilled water. The sections were dehydrated and mounted using DPX (Table 2.4). The Nissl's stained images of the striatum and midbrain were collected and densitometric analysis was performed by using NIH ImageJ software. The mean threshold area of the blue-stained Nissl positive nuclei from 3 images of each animal was calculated and expressed as percentage density by comparing with vehicle treated control group.

2.14 Statistical Analysis

Experimental data were expressed as the mean ± S.D and statistically analysed by suitable statistical test, two-tailed Student's t-test or two-way ANOVA with Bonferroni's *post hoc* test using GraphPad Instat software. A value of $p < 0.05$ was considered as statistically significant.

Table 2.4 - Haematoxylin and Eosin Staining

Solvent	Duration (min)
1. HistoClear	5
2. HistoClear	5
3. HistoClear	5
4. 100% Ethanol	5
5. 100% Ethanol	5
6. 95% Ethanol	5
7. 80% Ethanol	5
8. Distilled Water	10
9. Haematoxylin	3
10. Distilled Water	rinse
11. Tap Water	5
12. 1% acid ethanol	Dip 10× (fast)
13. Tap Water	1
14. Tap Water	1
15. Distilled Water	2
16. 2% Eosin	5
17. 95% Ethanol	5
18. 95% Ethanol	5
19. 95% Ethanol	5
20. 100% Ethanol	5
21. 100% Ethanol	5
22. 100% Ethanol	5
23. HistoClear	15
24. HistoClear	15
25. HistoClear	15

Following H&E staining, Tissue samples were mounted in DPX mounting fluid (VWR International).

Table 2.5 - Nissl Staining

Solvent	Duration (min)
1. HistoClear	10
2. HistoClear	10
3. HistoClear	10
4. 100% Ethanol	5
5. 100% Ethanol	5
6. 95% Ethanol	3
7. 70% Ethanol	3
8. Tap water	rinse
9. Distilled Water	rinse
10. 0.1% cresyl violet (containing 0.3% glacial acetic acid)	3-10
11. Distilled Water	rinse
12. 95% Ethanol	2-30
13. 100% Ethanol	5
14. 100% Ethanol	5
15. HistoClear	5
16. HistoClear	5

Following Nissl staining, Tissue samples were mounted in DPX mounting fluid (VWR International).

CHAPTER 3

ESCULETIN PROTECTS HEPG2 CELLS AGAINST H_2O_2-INDUCED OXIDATIVE STRESS BY NRF2-MEDIATED INDUCTION OF NQO1 GENE

3. Esculetin-induced protection of human hepatoma HepG2 cells against hydrogen peroxide is associated with the NRF2-dependent induction of the NAD(P)H: quinone oxidoreductase 1 gene

3.1 Introduction

Several coumarin compounds as summarised in Chapter 1, have been shown to protect cells against oxidative damage (Kim et al., 2008) and can act as neuroprotective agents (Molina-Jimenez et al., 2005) as well as protecting against hepatotoxicity (Murat Bilgin et al., 2010). Coumarins therefore have the potential to protect against diseases in which reactive oxygen species (ROS) are elevated such as Alzheimer's, Parkinson's and cardiovascular disease (Riveiro et al., 2010).

Despite the promising pharmacological properties of esculetin mentioned in Chapter 1, little is known about its protective effect and the mechanism of protection. Its protective effects may be entirely due to its direct free radical scavenging activity, given that it can scavenge for the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, the hydroxyl radical and other intracellular ROS (Kim et al., 2008, Lin et al., 2000, Paya et al., 1992a). However, esculetin has also been reported to modulate the redox state of the cell, and can enhance reduced glutathione levels in the liver of C57BL/6J mice (Martin-Aragon et al., 1998), although it is unclear how this is achieved. Other mechanisms known to be initiated following esculetin treatment include the inhibition of signalling pathways (Pan et al., 2003), and the induction of apoptotic pathways (Chu et al., 2001, Yang et al., 2006). In addition, esculetin is known to inhibit lipoxygenase activity within platelets (Sekiya et al., 1982). Whether any of these mechanisms or pathways contributes to the antioxidant properties of esculetin is not known.

3.2 Aim of the chapter

The objective of the work described in this chapter was to characterize the protective effects elicited by esculetin against hydrogen peroxide toxicity in a well established cell line and to provide evidence for the mechanism of protection. The effect of esculetin on indicators of toxicity and markers for oxidative stress, and on the expression of the protective enzyme NADPH: quinone oxidoreductase 1 (quinone reductase, NQO1) and the role of NRF2 in mediating protection was investigated. In addition, the levels of antioxidant enzyme superoxide dismutase 1 (SOD1) was also determined. The protective effects of esculetin were compared with quercetin, an antioxidant compound which is known to protect cells from oxidative stress by NRF2-mediated induction of NQO1 (Tanigawa et al., 2007). The presence of esculetin in a range of medicinal plants and herbs underscores the significance of this work, and it has the potential to lead to the use of esculetin as a therapeutic against chronic diseases where there is an imbalance in redox status.

3.3 Materials and Methods

Human Hepatoma HepG2 cells were used for this study as it is a reliable model, well characterised and widely used to study the biochemical variation in antioxidant defence systems (Alia et al., 2005). In addition, the steady-state functioning of antioxidant defence system in HepG2 cells is higher compared to normal hepatocytes and other non-transformed cells. Therefore, human hepatoma HepG2 was used as a model to study the antioxidant and protective properties of esculetin against hydrogen peroxide induced oxidative stress.

All other materials and methods used in this Chapter are described in Chapter 2.

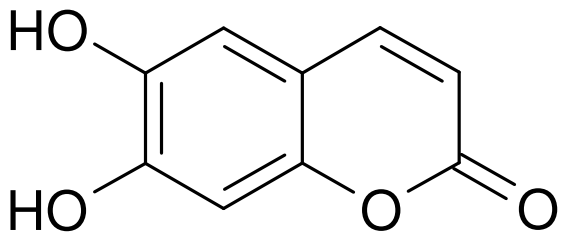


Fig. 3.1 – Structure of esculetin (6,7-dihydroxycoumarin)

3.4 Results

3.4.1 Determination of the sub-toxic concentration of esculetin

To determine the sub-toxic concentration of esculetin, HepG2 cells were plated and incubated for 24 hrs in 96-well plates followed by exposure to coumarins for further 24 hrs. HepG2 cells were exposed to different concentrations of esculetin ranging from 1μM to 2mM and the cell viability was measured using the MTT assay. Esculetin (IC_{50}=1.14mM) showed mild toxicity on HepG2 cells (Fig. 3.2), which may induce adaptive response and stimulate the endogenous antioxidant system.

Based on results and the nature of the toxicity curve, the sub-toxic concentration of esculetin was determined as 1-50 μM, and this concentration was used in subsequent experiments to study the protective effect of esculetin.

3.4.2 Toxic Effect of Hydrogen Peroxide on HepG2 cells

To study the toxic effects of hydrogen peroxide, HepG2 cells were plated and incubated for 24h in 96-well plates followed by exposure to the toxic compound at different concentrations for further 24h. Cell viability was calculated using the MTT assay, which showed hydrogen peroxide with an IC_{50} of 0.89±0.06mM was toxic in the range of 0.1mM to 1.2mM (Fig. 3.3).

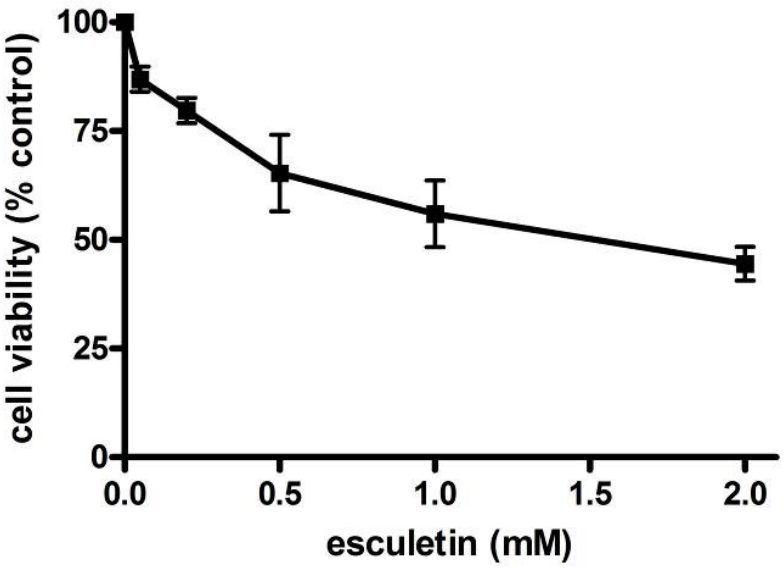


Fig. 3.2 – Determination of sub-toxic concentration of esculetin in HepG2 cells. Cells were treated with increasing concentrations of esculetin (0.01–2 mM) for 24 hrs and the level of toxicity was measured by MTT assay. Samples represent a mean ± SD of samples in triplicate (n=3) and cell viability was calculated as % untreated cells.

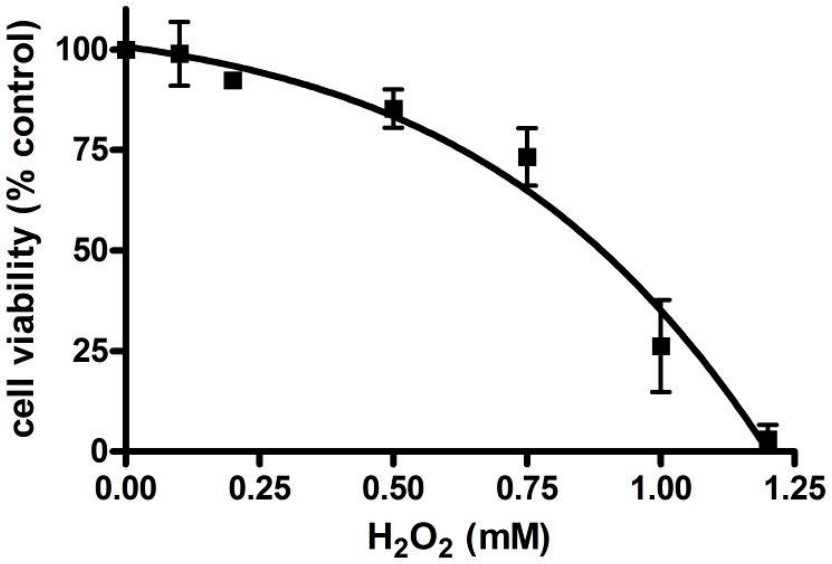


Fig. 3.3 - Cytotoxicity of H_2O_2 on HepG2 cells. Cells were treated with increasing concentrations of H_2O_2 (0.01–1.2 mM) for 24 hrs and the level of toxicity was measured by MTT assay. Samples represent a mean ± SD of samples in triplicate (n=3) and cell viability was calculated as % untreated cells.

3.4.3 Esculetin increases cell viability and maintains cell integrity during oxidative stress

In order to study the effects of esculetin in protecting cells against oxidants, HepG2 cells were pre-treated with esculetin (1-100 µM) and a positive control quercetin (40 µM), a potent antioxidant, for 8h and then exposed to hydrogen peroxide (0.9 mM) for further 24h. The effect on cells was assayed with MTT assay and LDH leakage assay.

The MTT assay results showed that esculetin pre-treatment increased cell viability by up to 40% in HepG2 cells and protected them from hydrogen peroxide induced cell damage. The dihydroxycoumarin enhanced cell viability by 10% more than the positive control, quercetin (40 µM). The protective effect of esculetin was found to be dose dependent and a linear increase in cell viability was observed in the range of 1-50 µM, which on further increase in concentration subsided (Fig. 3.4 and 3.5).

In addition, the LDH leakage assay revealed that esculetin treatment decreased LDH leakage by 20% and assisted the HepG2 cells to maintain cell integrity following hydrogen peroxide treatment. The reduction in LDH leakage by esculetin was virtually equal to the decrease shown by quercetin (40 µM). The decrease in LDH leakage was dose dependent and appeared to be linear in the range of 10-50 µM esculetin pre-treatment (Fig. 3.6).

These results show that esculetin protects HepG2 cells from hydrogen peroxide-induced toxicity in a dose dependant manner.

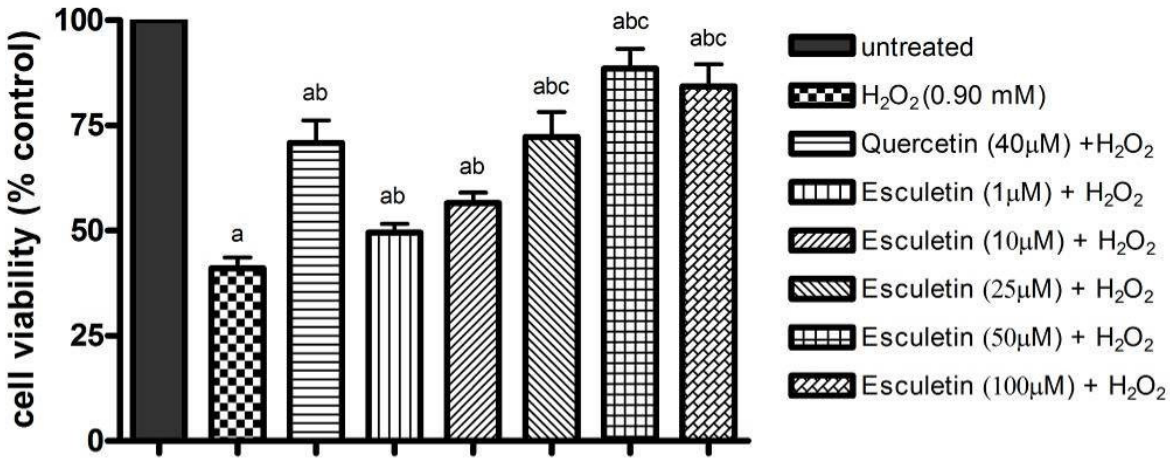


Fig. 3.4 - Esculetin protects cells against H_2O_2-induced cell damage. Cells were pre-treated with esculetin or quercetin for 8 hrs and further exposed to 0.9 mM H_2O_2 for 24 hrs. The level of toxicity was measured by MTT assay. Samples represent a mean ± SD of samples in triplicate (n=3) and cell viability was calculated as % untreated cells. $P < 0.05$ compared to untreated control (a), 0.9 mM H_2O_2 treated (b) and quercetin (c).

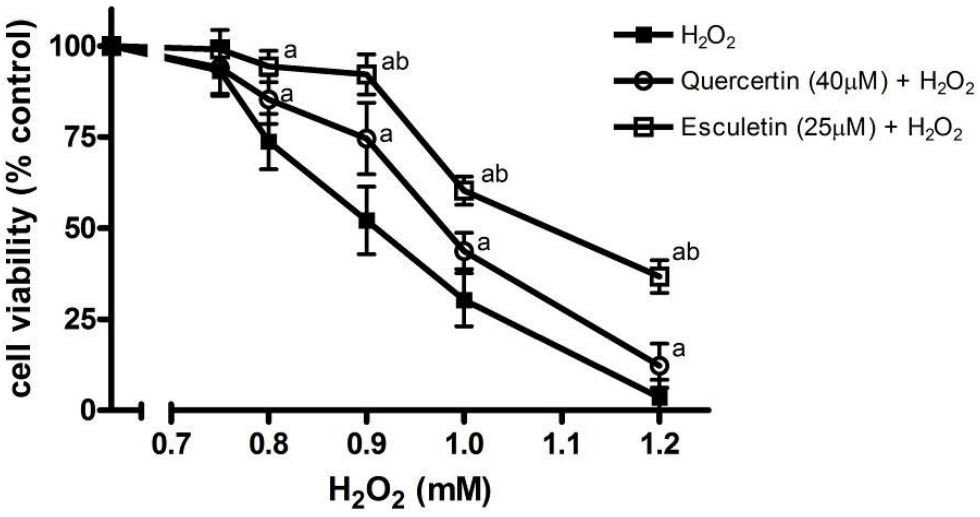


Fig. 3.5 - Esculetin protects cells against H_2O_2-induced cell damage. Cells were pre-treated with esculetin or quercetin for 8 hrs and further exposed to increasing concentrations of H_2O_2 (0.1–1.2 mM) for 24 hrs. The level of toxicity was measured by MTT assay. Samples represent a mean ± SD of samples in triplicate (n=3) and cell viability was calculated as % untreated cells. $P < 0.05$ compared to H_2O_2 treated (a) and quercetin (b).

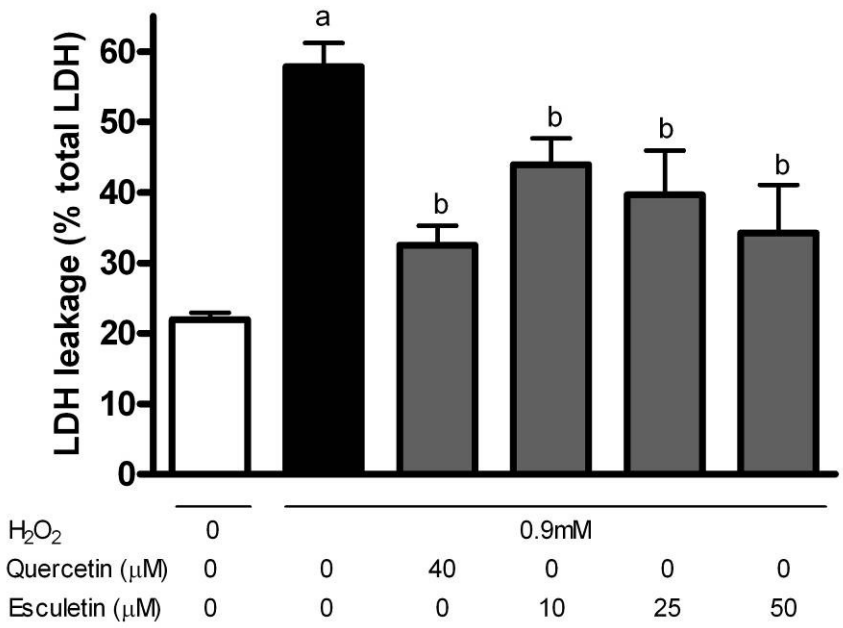


Fig. 3.6 - Esculetin protects cells against H_2O_2-induced cell damage. Cells were pre-treated with esculetin or quercetin for 8 hrs and further exposed to 0.9 H_2O_2 for 24 hrs. The level of LDH leakage was measured by Tox1 assay kit (Sigma). Samples represent a mean ± SD of samples in triplicate and % LDH leakage was calculated as ratio between LDH activity in culture medium and that of the whole cell content. $P < 0.05$ compared to untreated control (a) and 0.9mM H_2O_2 treated (b).

3.4.4 Esculetin-induced protection of HepG2 cells is mediated by antioxidant enzymes

To determine the role of antioxidant enzymes in the protective effect of esculetin on HepG2 cell lines, the cells were incubated with antioxidant enzyme inhibitors (15mM sodium diethyldithiocarbamate trihydrate and 13mM 3-amino-1,2,4-triazole) for 2 hrs prior to treatment with esculetin and H_2O_2 subsequently.

Results reveal that inhibition of the antioxidant enzymes reduced the protective effects of esculetin completely compared to control treatment with esculetin. Similarly, the inhibition showed an absolute decrease in the protective effects of quercetin (Fig. 3.7).

These results show that the protective effect exhibited by esculetin on HepG2 cells is partially dependent on antioxidant enzymes.

3.4.5 Esculetin Decreases Intracellular ROS Production

To evaluate the ability to scavenge ROS, HepG2 cells were pre-treated with esculetin for 8h and exposed to 0.9mM H_2O_2 for 30 min to measure the production of intracellular ROS.

The results showed a significant increase in ROS generation over time in the presence of 0.9 mM H_2O_2 as compared to unstressed control. Pre-treatment of cells with esculetin (10-25 µM) for 8 hrs greatly decreased production of intracellular ROS revealing the antioxidant properties of the dihydroxycoumarin. Moreover, esculetin (50 µM) decreased slightly more intracellular ROS generation compared to quercetin (40 µM), a proven antioxidant, which was used as a positive control (Fig. 3.8).

The results suggest that esculetin treatment decreases ROS levels in a superior manner compared to quercetin after exposure to hydrogen peroxide in HepG2 cells.

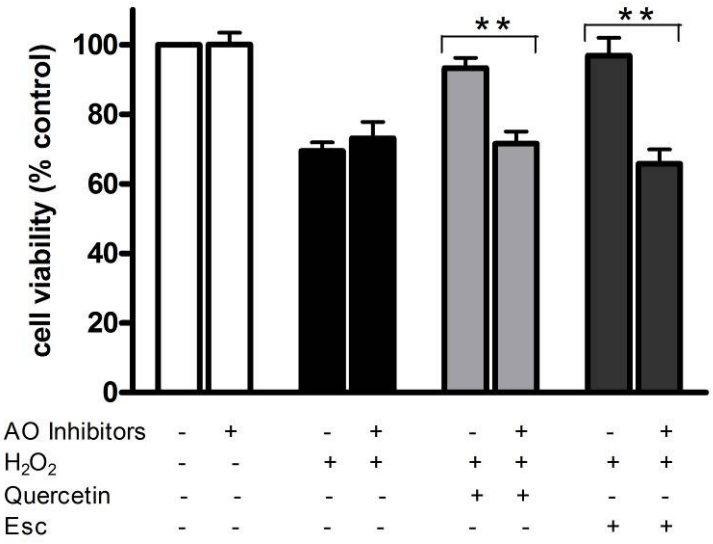


Fig. 3.7 - Esculetin-induced protection is mediated by antioxidant enzymes. Cells were incubated with antioxidant enzyme inhibitors (15 mM sodium diethyldithiocarbamate trihydrate and 13 mM 3-amino-1,2,4-triazole) for 2 hrs followed by pre-treatment with esculetin (25 μM) or quercetin (40 μM) for 8 hrs and further exposed to 0.9 H_2O_2 for 24 hrs. The level of toxicity was measured by MTT assay. Samples represent a mean ± SD of samples in triplicate and cell viability was calculated as % untreated cells. $P < 0.05$* compared to H_2O_2 treatment.

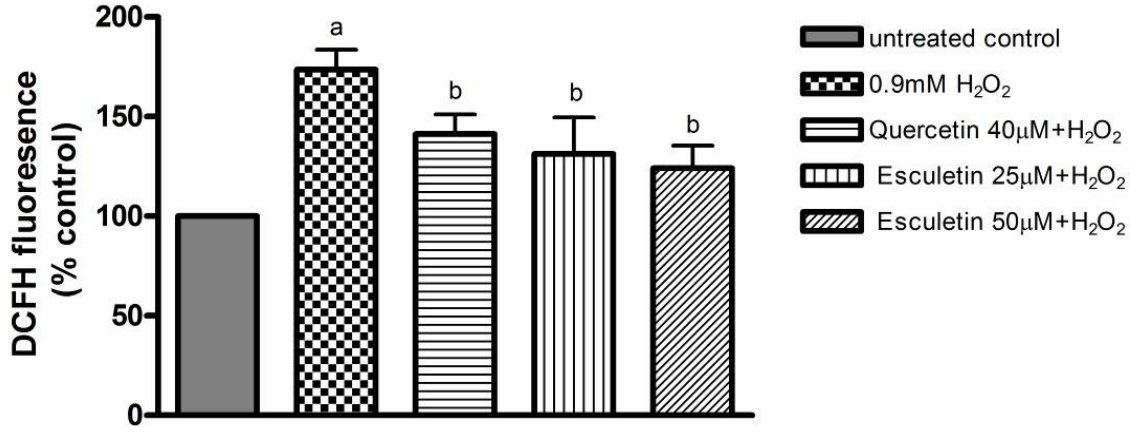


Fig. 3.8 - Esculetin decreases ROS generation. Cells were pre-treated with esculetin or quercetin for 8 hrs and incubated with 100 μM DCFH for 30 min. The cells were washed and then exposed to 0.9 H_2O_2 for 30 min and the level ROS generation was measured as fluorescence in a plate reader. Samples represent a mean ± SD of samples in triplicate and fluorescence was calculated as % of untreated cells. $P < 0.05$ compared to untreated control (a) and 0.9 mM H_2O_2 treated (b).

3.4.6 Esculetin increases intracellular GSH levels in HepG2 cells

Total intracellular glutathione levels were measured as an index of intracellular non-enzymatic antioxidant defence systems in HepG2 cells. HepG2 cells were exposed to 0.9 mM H_2O_2 for 24 hrs with or without pre-treatment with esculetin or n-acetyl cysteine (NAC, 250 mM), a potent inducer of glutathione.

Results revealed that exposure to H_2O_2 markedly decreased the intracellular glutathione levels. Pre-treatment with 25 µM esculetin and 250 mM NAC for 8 hrs showed a significant 2-fold and 4-fold increase in total glutathione levels, respectively. In addition, the esculetin pre-treatment prevented the decrease in total glutathione levels after H_2O_2 exposure and an increase of total glutathione was observed (Fig 3.9).

These results indicate that esculetin treatment increases GSH levels in HepG2 cells and prevents from GSH depletion after hydrogen peroxide exposure.

3.4.7 Esculetin inhibits H_2O_2-induced apoptosis and necrosis

The apoptotic and necrotic effects of exposure to hydrogen peroxide on HepG2 cells were determined by methods mentioned in materials and methods section.

Results show that exposure to 0.9 mM H_2O_2 induces apoptosis and necrosis in HepG2 cell lines. Pre-treatment with esculetin (25 µM) for 8 hrs prevented HepG2 cells from H_2O_2-induced apoptosis completely and necrosis partially. Treatment with quercetin (40 µM) significantly reduced cell death however failed to inhibit completely (Fig 3.10).

The results reveal that treatment with esculetin prevents HepG2 cells from hydrogen peroxide-induced apoptosis and necrosis.

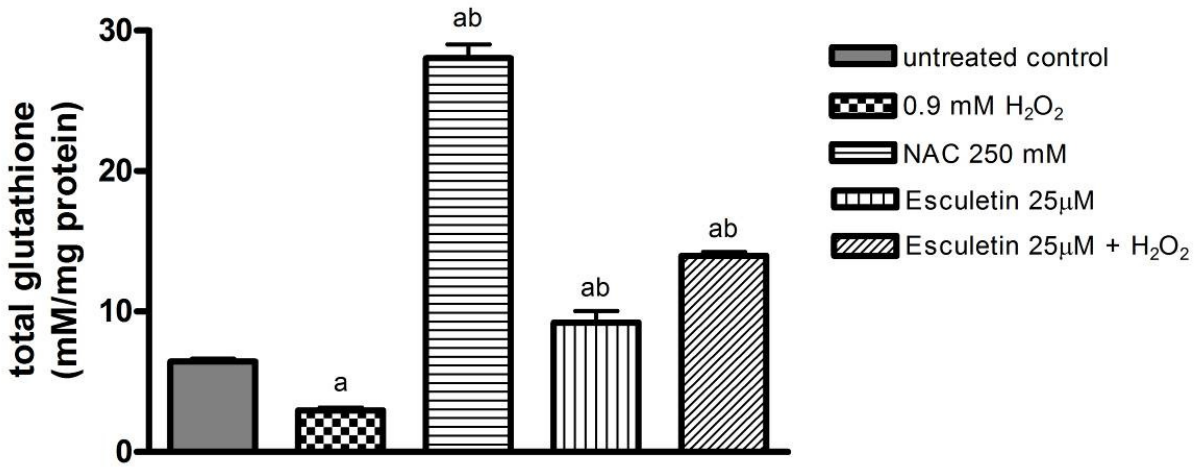


Fig. 3.9 - Esculetin increases total glutathione and inhibits decrease in glutathione levels during oxidative stress. Cells were pre-treated with esculetin (25 μM) or NAC (250 mM) for 8 hrs. Then cells were washed and exposed to 0.9 H_2O_2 for 24 hrs and glutathione levels were measured as absorbance by a colorimetric reaction with DTNB by spectrophotometer. Samples represent a mean ± SD of samples in triplicate and total glutathione was calculated per milligram of protein. $P < 0.05$ compared to untreated control (a) and 0.9mM H_2O_2 treated (b).

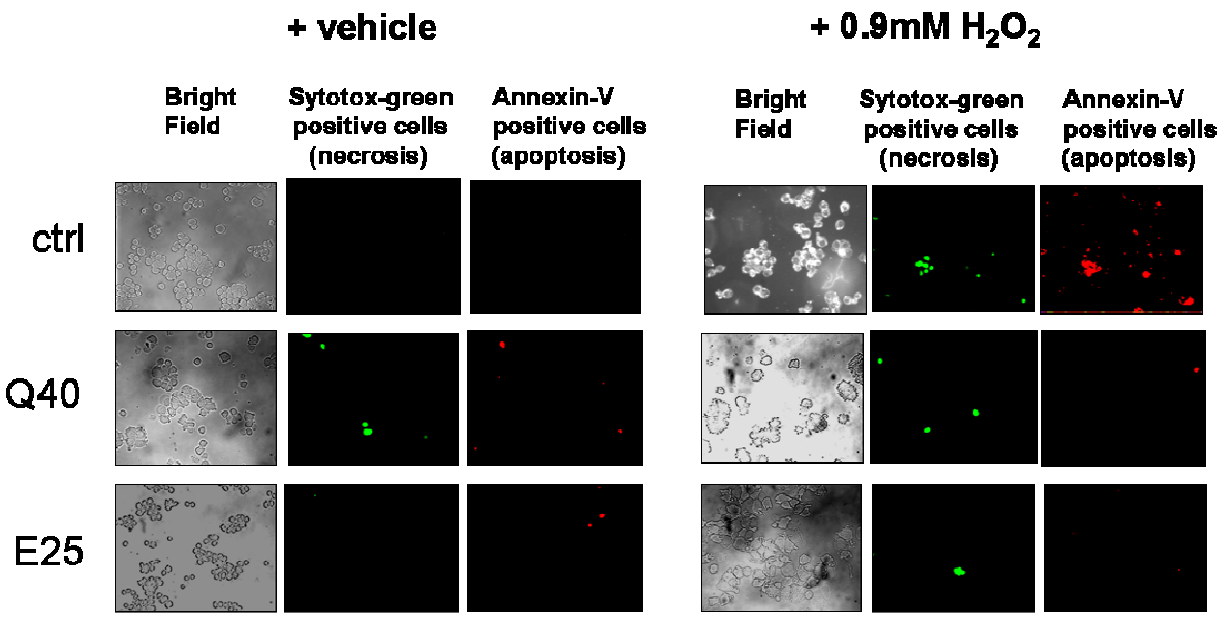


Fig. 3.10 - Esculetin inhibits H_2O_2-induced apoptosis and necrosis. HepG2 cells were pre-treated with quercetin (40 µM) or esculetin (25 µM) or umbelliferone (25 µM) for 8 hrs (20×). Cells were washed and exposed to 0.9 H_2O_2 for 24 hrs and analysed for apoptotic (Annexin-V positive, red fluorescence) and necrotic cells (Sytotox-green positive, green fluorescence) as mentioned in chapter 2 by using Annexin V-Cy3 Apoptosis Kit Plus (Cat. No. K202-25, Biovision, UK). Images represent at least 3 individual experiments.

3.4.8 Effect of esculetin on antioxidant enzyme activity

To study the effects of esculetin on the activity of antioxidant enzymes NADPH: quinone oxidoreductase (NQO1) and superoxide dismutase 1 (Cu/Zn SOD or SOD1), HepG2 cells were treated with esculetin and quercetin and the cell lysates were analysed for enzyme specific activity by kinetic assays.

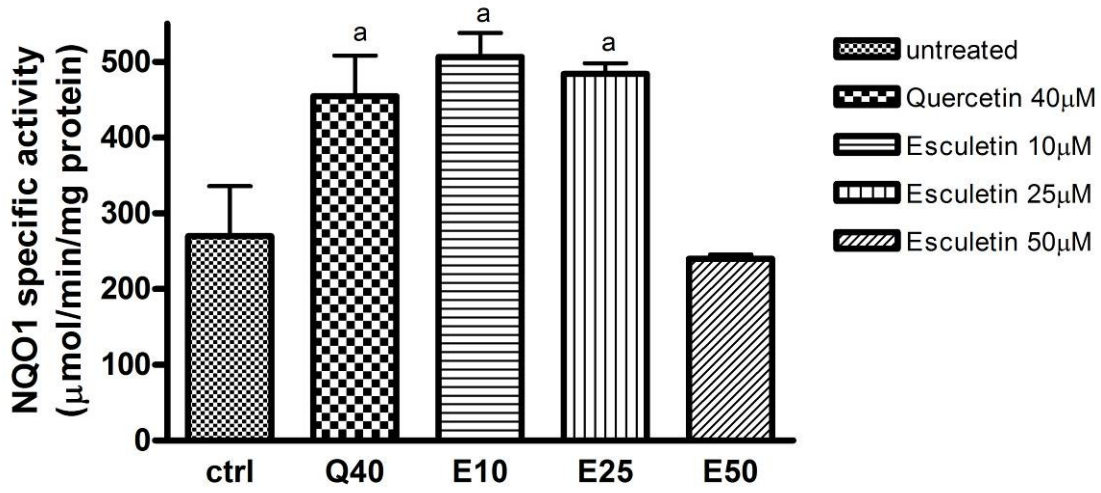
Results reveal that esculetin significantly increases NQO1 activity by 2-fold at 10µM and 25µM concentrations (Fig 3.11A). In contrast, no significant increase was observed in total SOD activity after esculetin treatment (Fig 3.11B).

3.4.9 Induction of expression of antioxidant enzymes by esculetin in HepG2 cells

Induction of the antioxidant enzymes NADPH: quinone oxidoreductase (NQO1) and superoxide dismutase 1 (Cu/Zn SOD or SOD1) were determined as protein expression by Western blotting. HepG2 cultures were treated for 8 hrs with esculetin (10-50 µM) and quercetin (40 µM) and the cell lysates were analysed for the protein expression.

The results showed that the expression of NQO1 and SOD1 proteins were significantly up-regulated by 25 µM esculetin treatment with a 12-fold and 20-fold increase respectively. The expression at 25 µM esculetin was the maximum and no linear increase in expression was observed on further increase in concentration (Fig. 3.12A). Esculetin (25 µM) showed a 3-fold increase in NQO1 and SOD1 expression compared to the positive control, quercetin (40 µM) (Fig. 3.12B).

A



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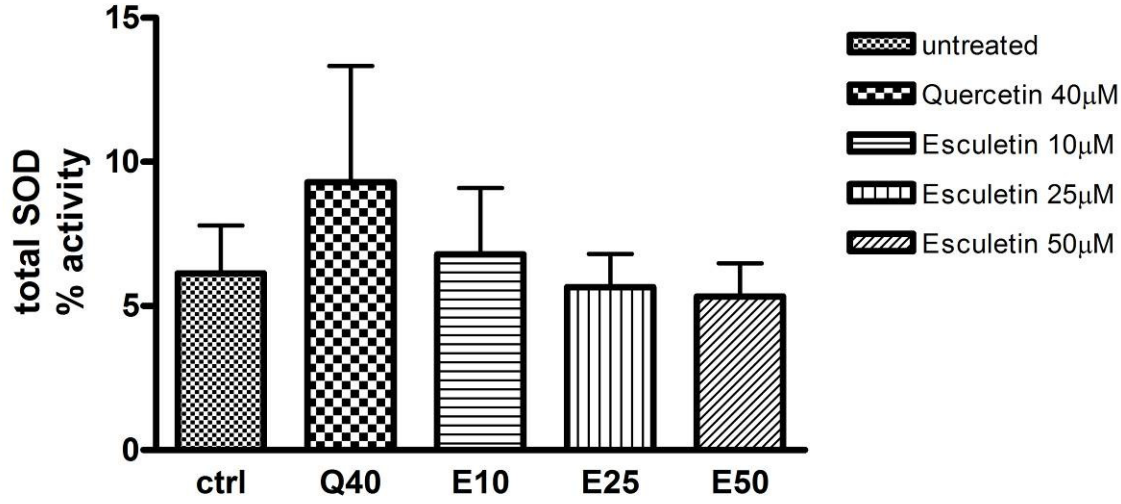


Fig. 3.11 - Effect of esculetin on NQO1 and total SOD activity. Cells were pre-treated with esculetin (10-50 μM) or quercetin (40 μM) for 8 hrs. Cells lysates were analysed for NQO1 (A) and total SOD (B) specific activity by kinetic assays. Samples represent a mean ± SD of samples in triplicate. $P < 0.05$ compared to untreated control (a).

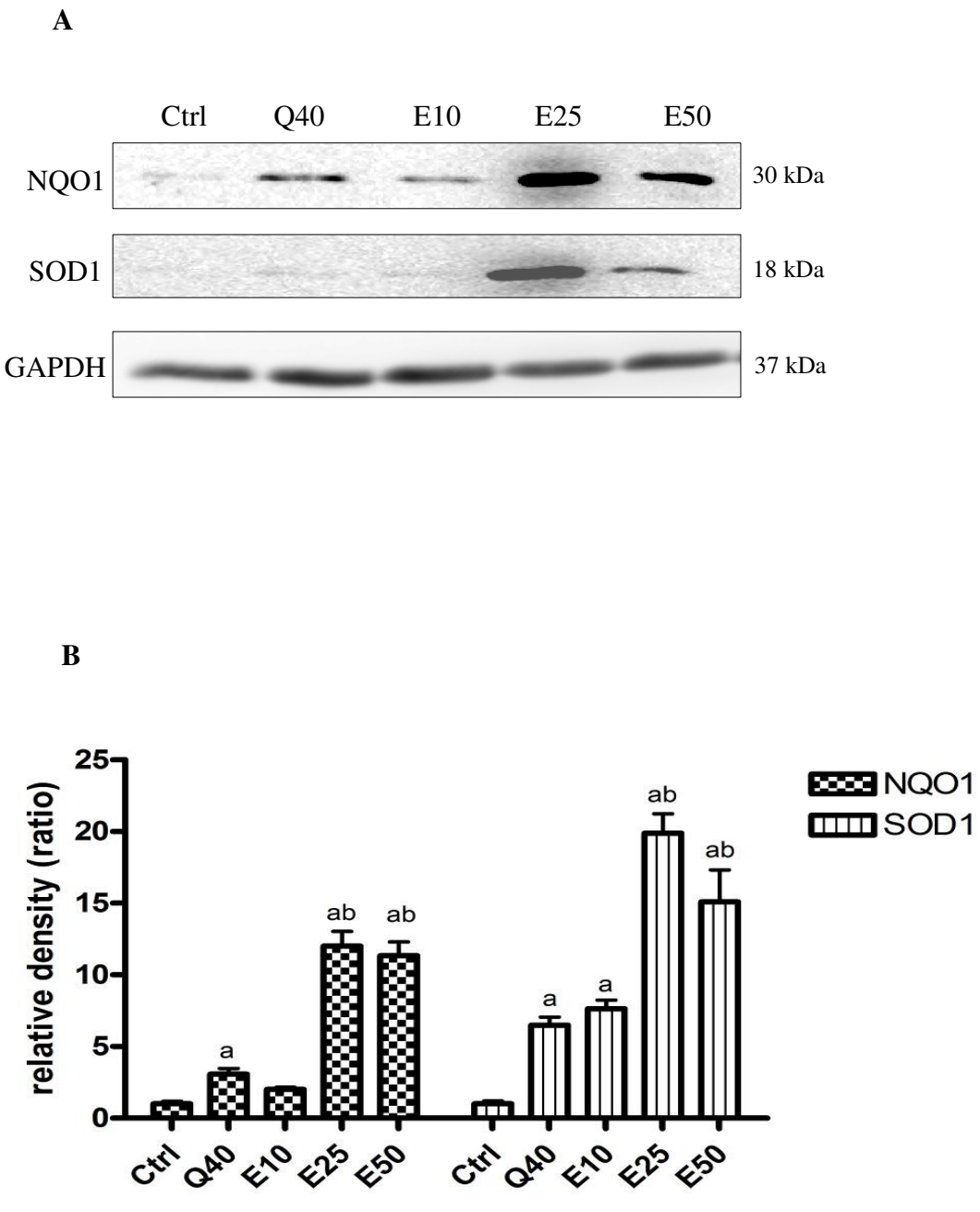


Fig. 3.11 - Induction of antioxidant enzymes NQO1 and SOD1 by esculetin. Cells were pre-treated with esculetin (10-50 µM) or quercetin (40 µM) for 8 hrs and the cell lysates were analysed by immunoblotting using specific antibodies or GAPDH as loading control. The blots represent experiments performed in triplicate (A). Histograms show the densitometric analysis of protein expression normalised to GAPDH by NIH ImageJ software (B). $P < 0.05$ compared to untreated control (a) and quercetin treated (b).

3.4.10 Esculetin induces NQO1 and SOD1 gene expression

To confirm the expression of NQO1 and SOD1 at the mRNA level HepG2 cells were treated with esculetin and quercetin, and analysed by RT-PCR. Total RNA was isolated after the drug treatments and cDNA was prepared according to the methods in chapter 2.

The results of RT-PCR with the NQO1 primers showed that esculetin significantly induced NQO1 by 15-fold at 25 µM treatment. Treatment with 10 µM esculetin showed no significant increase in mRNA levels of NQO1 (Fig 3.13A).

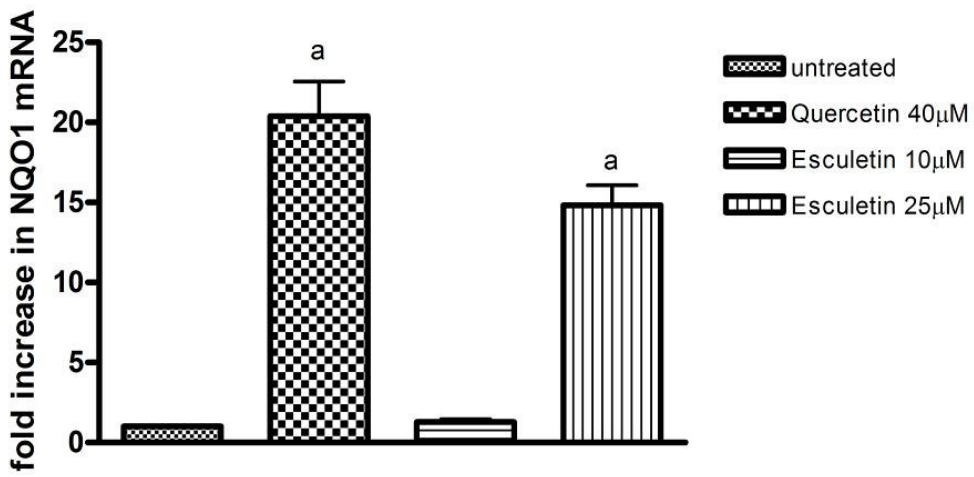
Esculetin treatment at 10 µM showed a marked increase in SOD1 mRNA levels by 15-fold compared to the untreated control levels. Treatment at higher dose (25 µM) also showed an increase in SOD1 mRNA expression although the increase was less than that observed at 10 µM (Fig 3.13B).

3.4.11 Effect of esculetin on Nuclear NRF2 accumulation

The expression of NQO1 is transcriptionally controlled by a cis-acting element known as antioxidant/electrophile response element (ARE/EpRE) (Nioi and Hayes, 2004, Jaiswal, 2000). Activation of ARE is mediated mainly by NRF2 (nuclear factor E2-related factor 2), a basic leucine zipper transcription factor (Fahey and Talalay, 1999).

To investigate the involvement of NRF2 in NQO1 expression, HepG2 cells were treated with esculetin (10-50 µM) or quercetin (40 µM) for 8 hrs and the nuclear accumulation of NRF2 was studied by Western blotting using specific antibodies. Treatment with esculetin significantly increased the levels of NRF2 in the nuclear fraction at 10 µM, 25 µM (7-fold) and 50 µM (8-fold). This compares with a 19-fold increase in nuclear NRF2 observed following quercetin (40 µM) treatment (Fig. 3.14).

A



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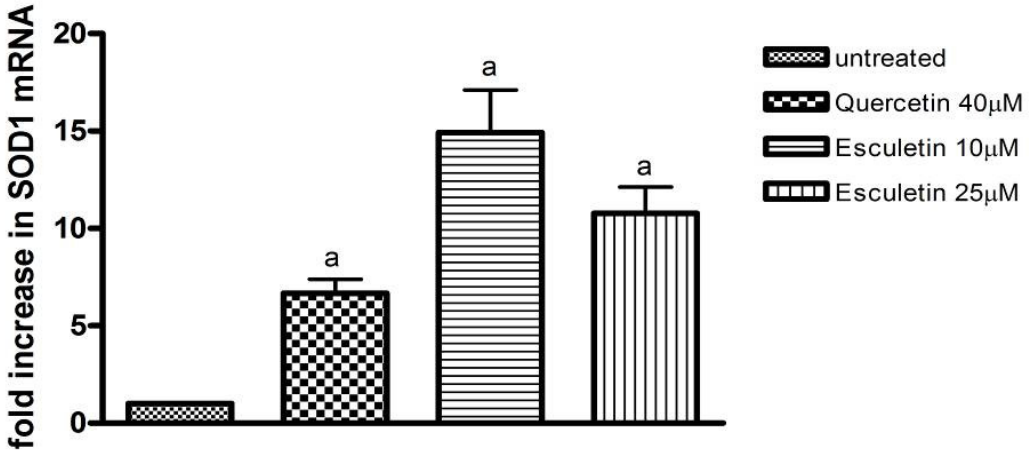
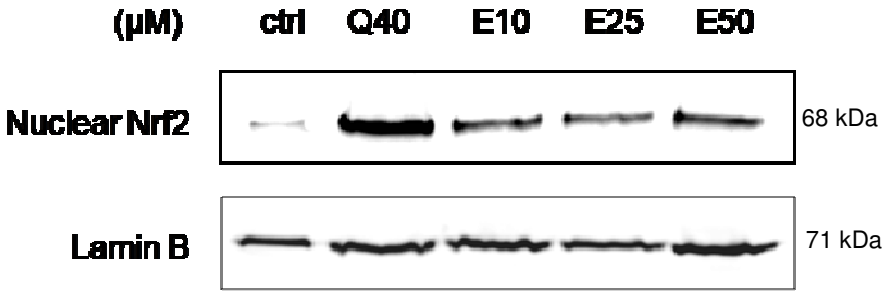


Fig. 3.12 - Induction of NQO1 and SOD1 mRNA levels by esculetin. Cells were pre-treated with esculetin (10-25 μM) or quercetin (40 μM) for 8 hrs and the mRNA levels were determined by RT-PCR using specific primers for NQO1 (A) or SOD1 (B) or GAPDH as loading control. Values represent mean ± SD of experiments performed in triplicates. Histograms show the fold increase in mRNA expression normalised to GAPDH. $P < 0.05$ compared to untreated control (a).

A



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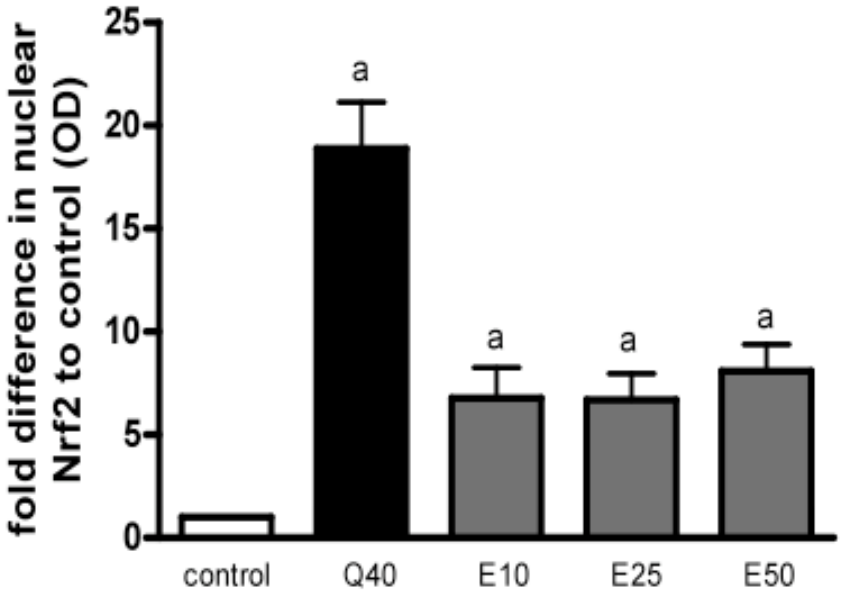


Fig. 3.13 - Effect of esculetin on nuclear NRF2 accumulation in HepG2 cells. (A) After treatment with esculetin (10-50 μM) or quercetin (40 μM) for 8 hrs, nuclear fractions of cell extracts were prepared as described in materials and methods. Equal amounts of the nuclear fractions were analysed by SDS-PAGE and Western blotting using antibodies to NRF2 or to Lamin B as loading control. Blots represent experiments performed in triplicate (n=3). (B) Histogram to show the densitometric analysis of NRF2 expression normalised to Lamin B, using ImageJ software. $P < 0.05$ compared to untreated control (a).

3.5 Discussion

In this chapter, oxidative stress was induced within HepG2 cells through treatment with hydrogen peroxide. This oxidant is known to cause significant molecular damage within cells, leading in particular to the peroxidation of lipids, which subsequently gives rise to the production of a range of ROS causing further damage to DNA, proteins and lipids (Esterbauer, 1993).

In the current study, esculetin pre-treatment for 8h protected HepG2 cells from hydrogen peroxide-induced toxicity and the cell viability was restored up to 90%, which was measured by using MTT assay. Esculetin pre-treatment also played an important role in increasing the cell integrity during oxidative stress, which was determined by LDH leakage assay. These results indicate that esculetin protected human hepatoma HepG2 cells from oxidative insult induced by hydrogen peroxide. Esculetin showed a protective effect equivalent to quercetin, a well-known free radical scavenger and protective compound (Tanigawa et al., 2007). These observations suggest that the increase in cell viability and cell integrity after esculetin pre-treatment may be attributed partly to the free radical scavenging property of esculetin (Lin et al., 2000, Paya et al., 1992a).

Non-enzymatic antioxidant defence system depends on redox state of the cell and the redox environment is maintained by glutathione and thioredoxin (Droge, 2002, Schafer and Buettner, 2001). Reduced glutathione is the main defence in this system which reduces different peroxides, hydroperoxides and radical within the cell (Vina, 1990). It is usually assumed that a reduction in reduced glutathione levels is an indication of oxidative stress. On the contrary, the cell is expected to prepare against oxidative insult by an increase in glutathione concentration (Rodgers and Grant, 1998, Myhrstad et al., 2002, Scharf et al., 2003). A number of physiological functions are also controlled by redox-responsive signalling pathways, which include redox regulated production of NO, ROS production by phagocytic NAD(P)H oxidase, ROS production by NAD(P)H oxidases in non-phagocytic cells, redox regulation of immune response and cell adhesion, ROS-induced apoptosis and other

mechanisms (Droge, 2002). In this Chapter, studies show that a marked decrease in total glutathione was restored completely by esculetin 25 μM treatment for 8 hrs. Increase in intracellular glutathione by esculetin has been previously reported in C57BL/6J mice (Martin-Aragon et al., 1998) where liver supernatants of mice showed a decrease in GSSG/GSH ratio, which was relevant to an increase in glutathione reductase activity after esculetin treatment. Studies also report that an increase in glutathione level was preceded by stimulation of γ-glutamylcysteine synthetase (GCS), the enzyme involved in glutathione synthesis (Rodgers and Grant, 1998, Myhrstad et al., 2002, Scharf et al., 2003). Elevated total glutathione levels indicate enhanced expression and/or activity of key enzymes involved in glutathione biosynthesis, such as the catalytic subunit of glutamate-cysteine ligase and glutathione synthase (Lu, 2009). The observed increase in total intracellular glutathione levels in the present study indicates that additional glutathione is being synthesized within the cell following esculetin pre-treatment. The experimental results clearly show that esculetin treatment increases total glutathione levels, which maintains the redox state of the cell thereby preventing human hepatoma HepG2 cells from oxidative damage.

Free radicals and non-radicals derived from oxygen represent the most imperative reactive species generated in the biological system (Miller et al., 1990). The major reactive oxygen species (ROS) produced includes the superoxide and hydroxyl radical. Superoxide radical, produced by a small number of electron leakage to oxygen prematurely during the energy transduction in the mitochondria, has been reported to be involved in the pathophysiology of various diseases (Kovacic et al., 2005, Valko et al., 2004). The evaluation of generation of ROS is a good indication of oxidative stress inside the living cells. Dichlorofluorescin diacetate (DCFH) is a non-fluorescent probe that can cross the cell membrane and can get oxidised to a fluorescent compound, dichlorofluorescein. This intracellular fluorescence can be quantified and used as an index to represent the overall oxidative stress in cells (Wang and Joseph, 1999). Previously esculetin has been reported to scavenge DPPH radical, hydroxyl radical and intracellular ROS, and this property of free radical scavenging of esculetin helps in preventing cells from lipid peroxidation, protein

carbonyl and DNA damage induced by hydrogen peroxide in hamster lung fibroblast cells (Kim et al., 2008). Another study states that the protective effect of esculetin against dopamine-induced toxicity was ascribed partly to the free radical scavenging property of esculetin (Zhao et al., 2008). In the present study, esculetin treatment decreased the generation of ROS in HepG2 cells during hydrogen peroxide-induced oxidative stress, however, the generation of free radicals could not be completely prevented. These observations show that the protective effect of esculetin in HepG2 cells may be due their capacity to inhibit ROS generation and thus can delay or prevent cells from hydrogen peroxide induced oxidative damage.

Cellular antioxidant defence system plays an important role in protecting cells from oxidative damage to the cells. The enhancement of levels antioxidant and detoxifying enzymes can provide protection against carcinogens and reactive oxygen species such as superoxide anions, hydrogen peroxide and hydroxyl radicals. The induction of protective enzyme defence systems is known to play an important role in protecting cells from oxidative damage (Hayes et al., 1999). The enhanced expression of enzymes such as NQO1, glutathione S-transferases and aldo-keto reductases can provide protection against chemical stress as well as reactive oxygen species as part of an adaptive response (MacLeod et al., Jaiswal, 2000). Thus, inducers of antioxidant enzymes are considered as potential candidates for prevention from oxidative stress related diseases.

NQO1 is a good marker for the induction of an adaptive response, and is known to be induced by a variety of chemicals (Nioi and Hayes, 2004), including the polyphenol quercetin, a compound that has been reported to up-regulate NQO1 expression at both protein and mRNA levels in HepG2 cells (Tanigawa et al., 2007). External chemical signals induce NQO1 by the dissociation of NRF2 from Keap1 in the cytosol, followed by its translocation and binding to the antioxidant response element located in the nucleus (Chen and Kong, 2005). Many previous studies have shown that NQO1 can be induced via a mechanism that involves the transcription factor NRF2, via its binding to an antioxidant response element (ARE) located upstream of the NQO1 basal promoter (Nioi and Hayes, 2004, Jaiswal, 2000). NRF2 is associated

with Keap1 in the cytoplasm, and the ability of NRF2 to regulate the expression of protective genes is associated with its accumulation in the nucleus (Nguyen et al., 2009). Our data supports this hypothesis, and in this study it has been shown that NRF2 accumulates in the nucleus in HepG2 cells following esculetin treatment, suggesting that esculetin acts to regulate the expression of NQO1 through a mechanism that depends upon NRF2. In this study, it is shown that esculetin up-regulates expression of NQO1 at the mRNA and protein level, leading to the increased expression of enzyme activity. Although esculetin has been investigated previously for its chemoprotective potential (Sharma et al., 1994), this is the first time it has been shown that esculetin can induce a protective response in any cell line.

Superoxide dismutase (SOD) catalyses the dismutation of superoxide radical and is present in three different forms in mammals: CuZnSOD (SOD1: mainly in cytosol), MnSOD (SOD2: located in mitochondria) and extracellular SOD (SOD3: localised in extracellular fluid) (Rohrdanz et al., 2002). There have been no scientific studies carried out previously on the effect of esculetin on induction of SOD1. In our experiments, esculetin treatment for 8 hrs showed a significant increase in the expression of both SOD1 at protein and mRNA levels. However, no marked increase in the total SOD activity was seen after esculetin treatment and this might be due to the interference caused by the different forms of SOD. Nevertheless, in the experiments, the lack of relationship between the changes in expression at protein, mRNA levels and the specific enzyme activity indicates that the antioxidant enzymes are perhaps post-transcriptionally regulated. These findings show that the expression of SOD1 can be induced in human hepatoma HepG2 cells by esculetin, which can effectively prevent cells from further oxidative damage.

It is worth noting that previous work has shown that esculetin can induce apoptosis in cell lines (Chu et al., 2001, Yang et al., 2006), although requiring much higher concentrations (168 μM to 200 μM) than those needed to induce the protective response used in this study (25 μM). This effect is in concordance with other studies that have shown that many agents that induce protective responses at low

concentrations can also give rise to apoptotic and/or cytotoxic effects at much higher concentrations (Davies, 1999, Chen et al., 2006). These apparently contradictory effects are typical of an adaptive response, where sub-lethal concentrations of a cytotoxic compound are able to induce a beneficial protective response (Davies, 1999, Chen et al., 2006).

3.6 Conclusions

In conclusion, it is shown that esculetin a compound present in a range of fruit, vegetables and herbs is able to protect HepG2 cells against hydrogen peroxide toxicity through a mechanism that includes the induction of protective enzymes. These results indicate that a relatively low and non-toxic concentration of esculetin has powerful protective effects that do not appear to be dependent on its free radical scavenging activity alone. Esculetin used at low non-toxic concentrations therefore has the potential to provide protection against oxidative stress-related chronic diseases.

CHAPTER 4

PROTECTIVE EFFECTS OF COUMARINS ON NDEA-INDUCED HEPATOTOXICITY IN RATS

4. Protective Effects of Dietary Coumarins, Umbelliferone and Esculetin, on NDEA-induced Hepatotoxicity in Rats

4.1 Introduction

Reactive oxygen species (ROS) have been implicated in various pathological conditions including cancer, neurodegenerative diseases, cardiovascular diseases and aging (Dalle-Donne et al., 2005, Dhalla et al., 2000, Jenner, 2003, Sayre et al., 2001). ROS are produced in the cells as a by-product of normal metabolism. They can also be produced by environmental hazards such as ultraviolet light or toxic compounds, as well as by the inflammatory process. As mentioned before, elevated levels of ROS can cause potential biological damage to cell components including proteins, lipids and nucleic acids, inhibiting their normal function. ROS-induced damage to DNA causes permanent modification of the genetic material leading to mutation, carcinogenesis and aging. DNA mutation is a crucial stage in carcinogenesis and oxidative DNA lesions have been reported in various tumours, robustly supporting the implications of ROS in cancer initiation. The fact that more than 100 oxidative products of DNA have been identified in cancer cells further underscores the role of oxidative damage in carcinogenesis (Marnett, 2000, Valko et al., 2006). ROS and RNS can combine chemically to form peroxynitrite, which has also been associated with DNA damage (Hehner et al., 2000) causing nitration of the guanine base. Moreover, the role of ROS in interfering with expression of various genes and signal transduction pathways has been well established, and is thought to be instrumental in carcinogenesis (Poli et al., 2004, Valko et al., 2006).

The involvement of ROS in cancer initiation has led to investigations into the potential for using antioxidant molecules as therapeutic treatment (Halliwell, 2001, Zhou et al., 2008). Several antioxidants have been reported previously to be beneficial against carcinogenesis in several *in vitro* and *in vivo* models (Bishayee et al., 2010, Khan et al., 2008, Stoner et al., 2008). Many of these compounds are thought to act through their radical scavenging property, which requires relatively

high doses of antioxidant, but few have been effective in clinical trials (Weber and Ernst, 2006).

The aim of the work described in this chapter was to examine the protective effects of Umb and Esc against NDEA-induced oxidative damage to liver in rats, and to investigate the mechanism by which these compounds work. Liver injury was studied by measuring lipid peroxidation and glutathione levels in the liver as well as serum biomarkers. In addition, the effect of dietary consumption of Umb and Esc on the up-regulation antioxidant enzymes was also investigated.

4.2 Materials and Methods

4.2.1 NDEA

N-nitrosodiethylamine (NDEA) was procured from Sigma (Poole, UK). NDEA is a hepatocarcinogenic agent and has been shown to cause the development of tumours in several animal models (Verna et al., 1996). NDEA has been reported to cause the generation of ROS resulting in oxidative damage and cell injury (Bartsch et al., 1989). Liver is the main site of NDEA metabolism and the production of ROS may be responsible for its carcinogenic effects. Although liver has an efficient antioxidant defence system, it may be overwhelmed by the increased levels of ROS induced by NDEA. Hence, NDEA-induced liver injury model is a proficient method to study the protective effects of compounds against ROS-induced damage in liver.

4.2.2 Animals and Drug Treatment

Male Wistar 10-week old rats were used for this study. Rats were allowed to acclimatize for 2 weeks before being fed either a normal diet supplemented with 2% arachis oil or diet containing test compounds in 2% arachis oil. Arachis oil was used as a vehicle for the uniform distribution of test compounds. The test compounds in animal diet were provided in the diet for 7 days in the following amounts: BHA,

0.75% (w/w); Umbelliferone, 0.5% (w/w) and Esculetin, 0.5% (w/w). BHA (butylated hydroxyanisole); a known antioxidant was used as a positive control. During the experiment the animals were provided free access to food and water. Each rat on average consumed 30 g of the diet per day (with or without test compounds).

4.2.3 Induction of liver damage with NDEA

Following the diet and drug treatment aforementioned 11, 12 weeks old male Wistar rats were administered with 200 mg/kg NDEA, IP, before 48h of sacrifice, to initiate oxidative damage and induce toxicity in liver. After the treatment period the animals were sacrificed by cervical dislocation and the liver was removed immediately, sliced into two portions and one portion was snap-frozen in liquid nitrogen and stored at -80^{o}C until analysis. The other portion was rinsed in physiological saline and immediately fixed in 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.4, for histological and immunohistochemical studies.

No obvious visual phenotypes in the rat groups were observed after the drug treatment or NDEA intoxication.

4.2.3.1 Determination of serum alanine transaminase activity

Rats were sacrificed at the end of the treatment and blood samples were collected. Serum was then separated by spinning at 3000 rpm for 5 min at 4^{o}C and stored at -80^{o}C for further analysis. The assay for alanine transaminase activity was performed according to manufacturer's protocol (Alanine transaminase activity assay kit, Cayman Chemical Company, USA, Cat. No. 700260).

All other materials and methods for this chapter are described in Chapter 2.

4.3 Results

4.3.1 Effect of dietary administration of Umb and Esc on serum biomarkers of liver function in NDEA-induced hepatotoxicity

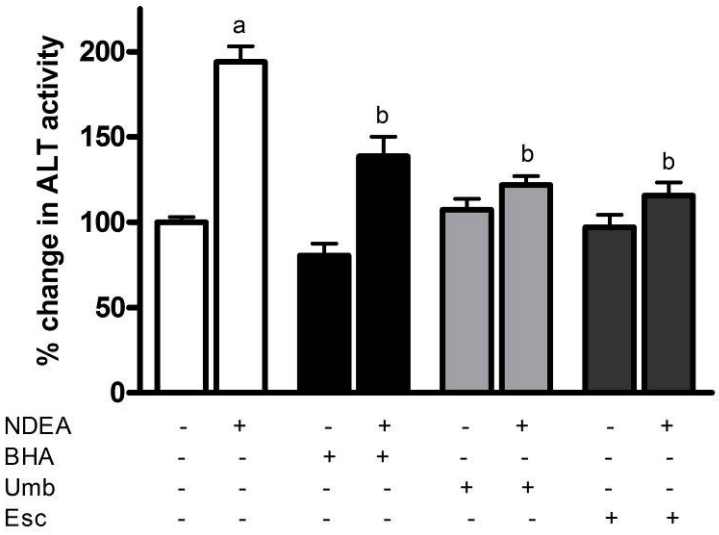
Alanine transaminase is found in abundance in liver compared to other organs such as kidney, heart and brain. The major function of ALT in liver is conversion of alanine to glucose. Serum ALT levels are generally low, but may be elevated in disease conditions or following liver injury (Pratt and Kaplan, 2000). Hence, serum ALT levels are routinely used as indicators of disease, particularly liver disease.

Rats injected with NDEA showed a severe increase in ALT levels by almost 2-fold compared to untreated control (Fig. 4.1A). Dietary supplementation of Umb and Esc inhibited the elevation of serum ALT levels by 80% and 87%, respectively compared to rats treated with NDEA alone. Diet administration of BHA, a known antioxidant, also decreased serum ALT levels by 57%.

Serum albumin levels are known to be reduced in chronic liver disease due to impaired protein synthesis in liver. NDEA-administration reduced the serum albumin levels by 22% compared to vehicle treated group (Fig. 4.1B). Umb treatment completely reversed NDEA-induced serum albumin depletion whereas Esc and BHA were only able to partially reverse the depletion.

These results show that Esc and Umb are able to reduce NDEA-induced liver damage.

A



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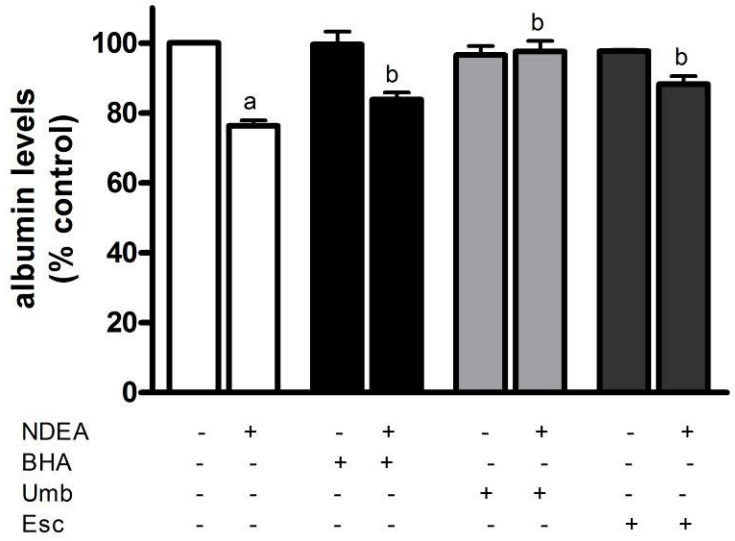


Fig. 4.1 - Umb and Esc inhibit NDEA-induced changes in serum biomarkers of hepatic injury in rat liver. Blood samples were collected after treatment; serum was isolated and stored at -80^{o}C. (A) Serum alanine transaminase levels were determined by using commercial kit as described in materials and methods. (B) Serum albumin levels were measured against albumin standard curve by colorimetric assay. Results are expressed as the mean ± SD of 3-6 rats. $P<0.05$ compared to vehicle without NDEA treatment group (a) and vehicle with NDEA treatment group (b).

4.3.2 Effect of dietary administration of Umb and Esc on NDEA-induced histological changes in rat liver

Histological examination of liver sections after haematoxylin and eosin (H&E) staining under light microscope was done to examine the effect of NDEA on the cell architecture and integrity.

Vehicle, BHA, Umb and Esc treated group (Fig 4.2A, C, E, G) showed normal appearance and histological characters of hepatic cells. The liver tissue of NDEA treated group (Fig 4.2B) showed loss of architecture, acute inflammatory cells with vacuolated cytoplasm indicating necrosis. In animals treated with BHA, Umb and Esc (Fig 4.2D, F, H), NDEA injection caused only mild necrosis and a regeneration of hepatocytes was observed.

These results indicate that animals treated with BHA and the coumarins recovered from NDEA-induced toxicity compared to vehicle treated animals.

4.3.3 Effect of Umb and Esc on NDEA-induced lipid peroxidation in rat liver

Lipid peroxidation (LPO) is a consequence of oxidative damage to cell structures and measurement of lipid peroxidation can define the level of oxidative damage to tissues.

NDEA administration showed a marked increase in LPO levels (101 nmol/mg protein) compared to vehicle administered group (26 nmol/mg protein) (Fig. 4.3). LPO levels of rats supplemented with Umb and Esc showed up to a 44% and 40% decrease respectively, compared to rats receiving vehicle alone. Treatment with the positive control BHA decreased the LPO levels by 37%.

These results show that Umb and Esc are able to protect liver against LPO, to a similar extent as BHA.

4.3.4 Effect of Umb and Esc on NDEA-induced GSH depletion in rat liver

Glutathione (GSH) is essential for the protection of cells from increased ROS levels and xenobiotics, in addition to maintaining the thiol redox status. NDEA, which induces ROS generation, is known to cause depletion of intracellular GSH. The level of intracellular glutathione was measured by an enzyme-dependent colorimetric assay.

As expected, administration of NDEA to rats sharply decreased GSH levels in liver up to 50% compared to vehicle treated control group (Fig. 4.4). However, Umb and Esc supplementation to rats completely prevented NDEA-induced GSH depletion in rat liver. A similar preventive effect was observed in rats consuming BHA diet.

These results suggest that Umb and Esc supplementation can protect against GSH depletion caused by NDEA in rat liver.

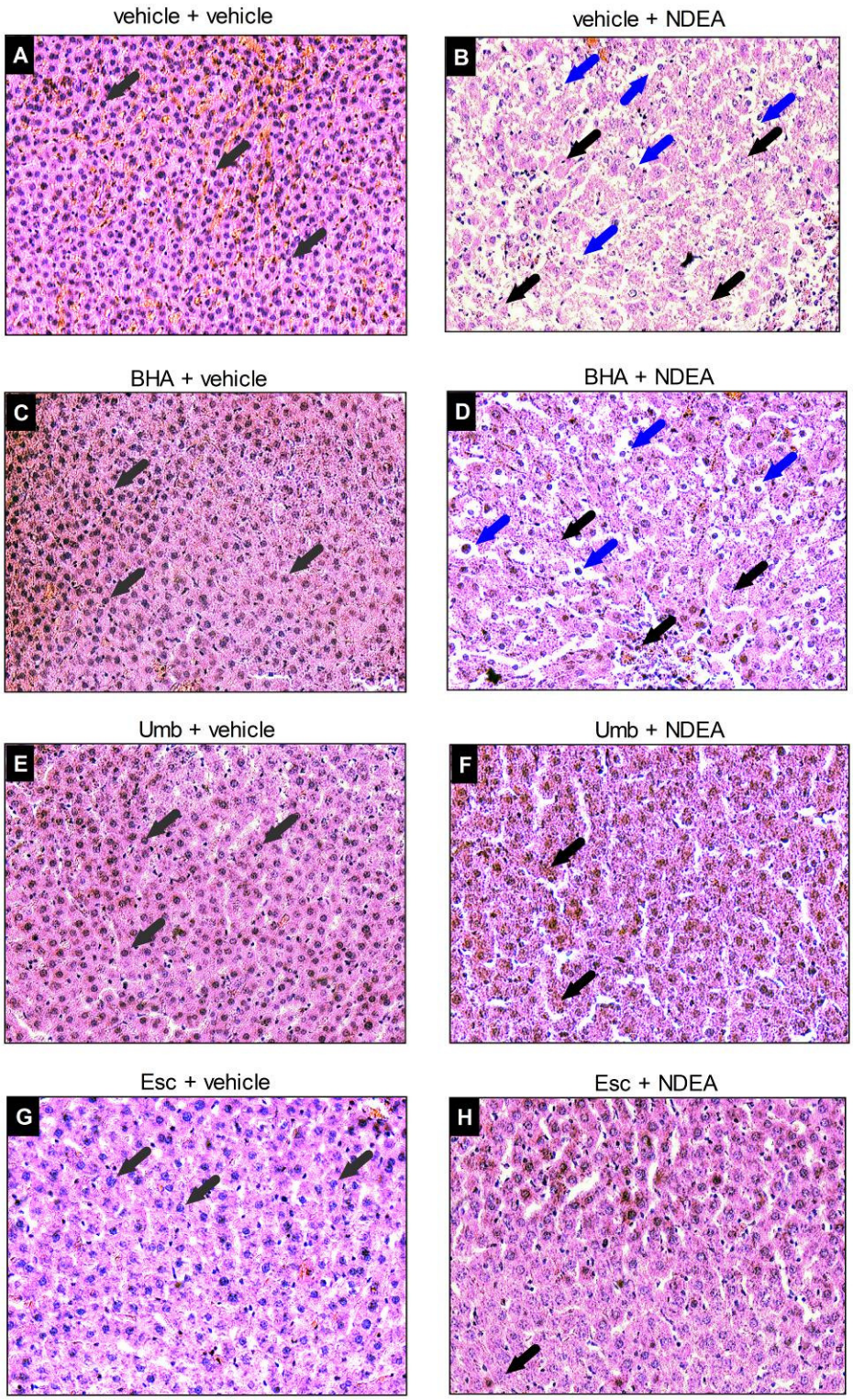


Fig. 4.2 - Histopathology of rat liver supplemented with vehicle or BHA or Umb or Esc and administered with vehicle or NDEA. Representative photomicrographs of H&E staining from 3-6 rats for each group with vehicle (A, C, E, G) or NDEA (B, D, F, H) administration. Grey arrows: normal hepatocytes; black arrows: loss of cell architecture; blue arrows: vacuolated cytoplasm.

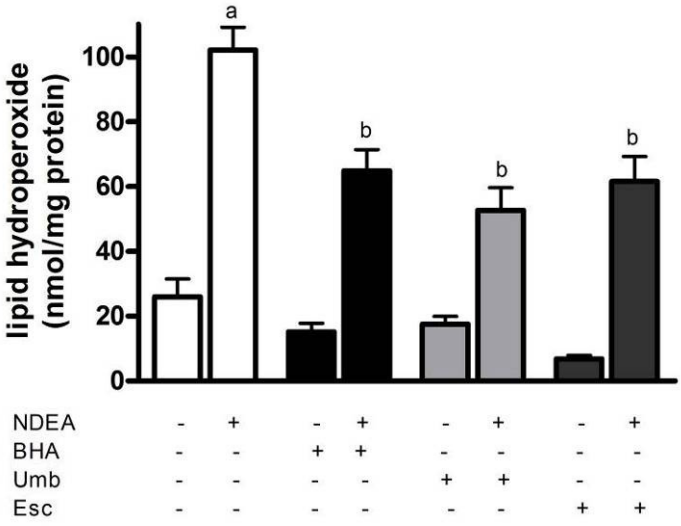


Fig. 4.3 - Umb and Esc decreased NDEA-induced hepatic lipid peroxidation in rats. Rats were sacrificed after treatment; liver was isolated, snap frozen in liquid nitrogen and stored at -80^{o}C. Hepatic lipid peroxidation was measured as lipid hydroperoxide levels in liver homogenates by using commercially available kit as mentioned in materials and methods. Results are expressed as the mean ± SD of 3-6 rats. P<0.05 compared to vehicle without NDEA treatment group (a) and vehicle with NDEA treatment group (b).

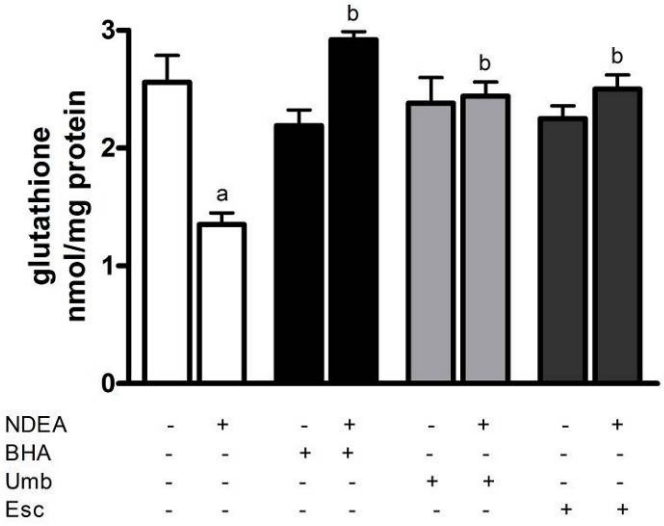


Fig. 4.4 - Umb and Esc prevent NDEA-induced glutathione depletion in rat liver. Rats were sacrificed after treatment; liver was isolated, snap frozen in liquid nitrogen and stored at -80^{o}C. Total glutathione levels were determined by enzymatic colorimetric assay as mentioned in materials and methods. Results are expressed as the mean ± SD of 3-6 rats. P<0.05 compared to vehicle without NDEA treatment group (a) and vehicle with NDEA treatment group (b).

4.3.5 Effect of Umb and Esc on expression of antioxidant enzymes in rat liver

NDEA provokes generation of ROS, which is one of the main mechanisms by which it induces hepatotoxicity in rats. Antioxidant defences play a vital role in scavenging ROS and hence up-regulation of the endogenous antioxidant system may help hepatocytes to counter NDEA-mediated toxicity. The enhanced expression of enzymes such as NAD(P)H: quinoneoxidoreductase 1 (NQO1), heme oxygenase 1 (HO1), glutathione S-transferases (GST) and aldo-keto reductases (AKR) are reported to protect against ROS as part of an adaptive response (MacLeod et al., Jaiswal, 2000) (Lin et al., 2004, Soane et al., 2010). Therefore the effect of Umb and Esc on the expression of antioxidant protective enzymes NQO1, HO1 and GSTP1 in the liver was studied.

The results in figure 4.5 show that Umb and Esc administration up-regulates the expression of several antioxidant protective enzymes including NQO1, HO1 and GSTP1. NQO1 expression was increased up to 3.6-fold and 2.7-fold respectively by Umb and Esc treatment. Umb and Esc increased HO1 expression to 2.7-fold and 3.2-fold respectively, and GSTP1 levels were up by 2.8-fold and 3.2-fold respectively in rats treated with Umb and Esc. The positive control BHA also increased the expression of NQO1 (6.2-fold), HO1 (3.3-fold) and GSTP1 (3.2-fold) significantly compared to vehicle treated control.

These results show that Umb and Esc are able to influence the cellular antioxidant defences in the liver.

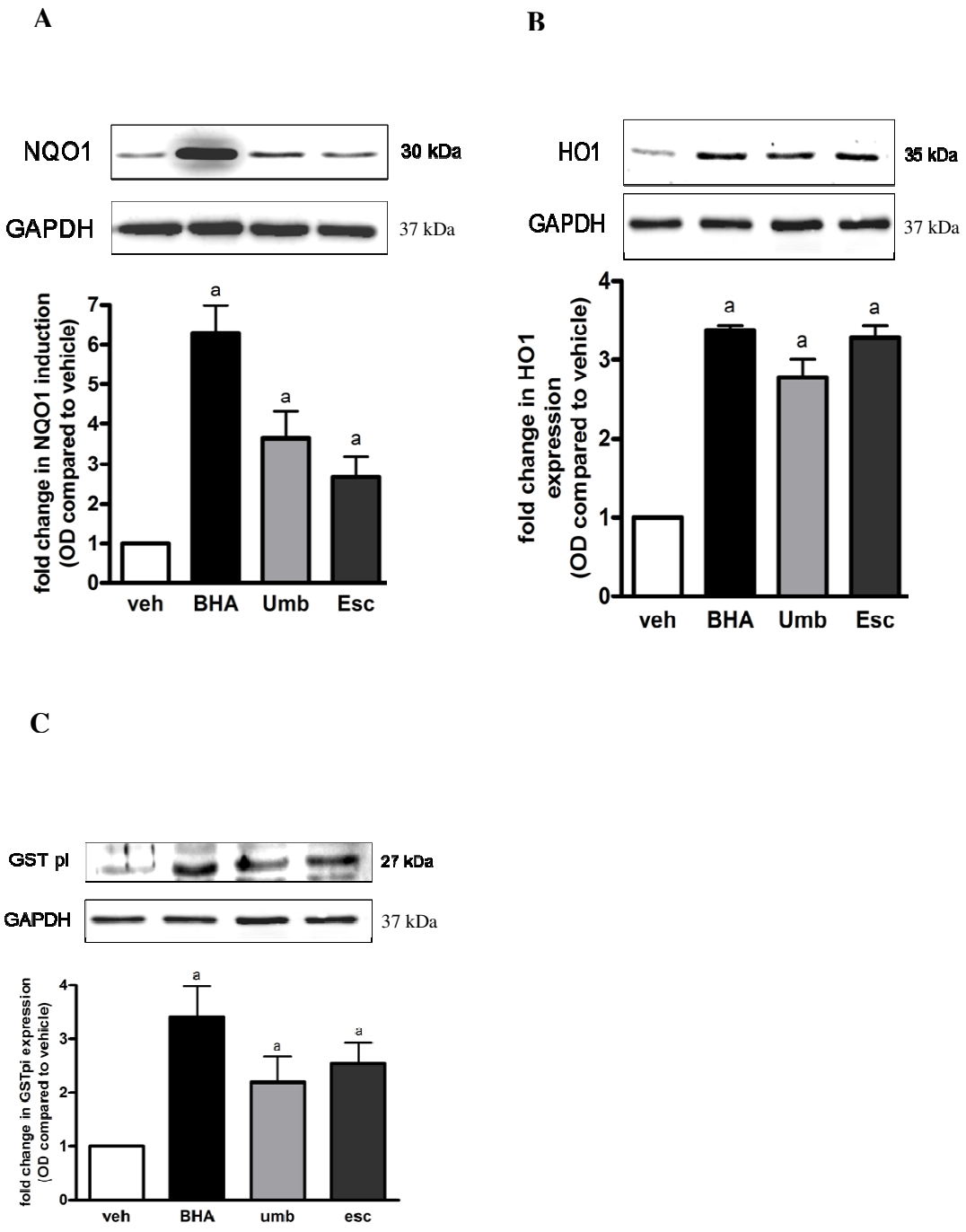


Fig. 4.5 - Effect of Umb and Esc administration on expression of (A) NQO1, (B) HO1 and (C) GSTP1 in rat liver. After drug treatments rats were euthanized, livers were individually isolated and homogenized. Liver homogenates were analyzed for NQO1, HO1 and GSTP1 levels by Western blotting. Histograms show the densitometric analysis of protein expression normalised to GAPDH by NIH ImageJ software. Results are expressed as the mean ± SD of 3-6 rats. $P<0.05$ compared to vehicle with vehicle treatment group (a).

4.4 Discussion

Oxidant-induced damage within cells is mediated by elevated levels of ROS, lipid peroxidation products and other free radicals, which are reported to be involved in initiation and progression of several pathological conditions including cancer (Valko et al., 2004, Valko et al., 2006, Valko et al., 2001) and other diseases. The ability of NDEA to generate ROS has been characterised previously and is known to disturb the antioxidant status of the cell and eventually leading to elevated ROS and cancer (Bartsch et al., 1989). It is therefore a good model compound to use to investigate the protective effects of antioxidants in liver.

Alanine transaminase (ALT) is widely distributed in many tissues but is found in great abundance in liver. Serum ALT levels are normally low, but may increase during a disease state or liver injury (Pratt and Kaplan, 2000). Serum albumin levels are high in normal conditions and are known to be reduced in chronic liver diseases due to impaired protein synthesis in liver. Hence serum ALT and albumin levels were used as biomarkers to detect hepatic liver injury. In this study, we have shown that administration of NDEA to male Wistar rats caused significant increase in serum ALT levels and a decline in serum albumin levels, which supports previous studies (Bansal et al., 2005). These effects of NDEA may be attributed to the release of ALT from the ruptured hepatocytes into the blood and impaired protein synthesis in liver, respectively. Dietary supplementation of Umb and Esc to rats for 7 days prior to administration of NDEA significantly reduced the levels of serum ALT and increased the serum albumin levels. These coumarin compounds are potent free radical scavengers (Paya et al., 1992b) and the observed protective effect may be due to their ability to reduce free radical-induced oxidative damage in the liver.

Lipid peroxidation, the process of oxidative destruction of polyunsaturated fatty acids (PUFAs) by ROS and other reactive species, plays an important role in carcinogenesis (Banakar et al., 2004). Lipid peroxidation may lead to formation of toxic compounds such as malondialdehyde (MDA), acrolein and 4-hydroxynonenal (4-HNE), which are reported to attack DNA and cause mutagenesis and

carcinogenesis (Marnett, 1999) (Marnett, 2000) (Chung et al., 2000, Hu et al., 2002). The studies performed in this chapter show that NDEA administration markedly increased lipid peroxidation, measured as lipid hydroperoxide levels, in rat liver. Lipid peroxidation levels were significantly reduced in rats on Umb and Esc diet compared to rats administered with NDEA alone. Umb, in addition to its free radical scavenging properties, has been reported to maintain redox status and prevent diabetic rats from erythrocyte lipid peroxidation (Ramesh and Pugalendi, 2005). Esc, a potent antioxidant, has been shown to protect hamster lung fibroblasts (V79-4) from lipid peroxidation, protein carbonylation and DNA damage induced by hydrogen peroxide (Kim et al., 2008). These results suggest that the coumarin compounds effectively scavenge ROS and other free radicals, preventing hepatic lipid peroxidation and thus prevent formation of mutagenic and carcinogenic lipid peroxidation products.

The steady state concentrations of ROS and other reactive species are determined by the balance between their rates of generation and elimination by antioxidant defence system. Thus the concentration of electrons (redox state) and its variation in each cell determines its functions (Schafer and Buettner, 2001). The intracellular redox status is maintained by glutathione (GSH) and thioredoxin (TRX). GSH plays an important role in cellular redox mechanisms and is therefore a representative of the redox status of the cell (Droge, 2002, Schafer and Buettner, 2001). GSH acts as antioxidant, enzyme cofactor and cysteine storage form its deficiency has been implicated in several disease states (Maher, 2005). Studies indicate that the knowledge of mechanisms of redox buffering by GSH, TRX and other small molecular weight compounds can be used in the development of targeted cancer-preventive and therapeutic drugs (Evens, 2004). The present work shows that injection of the hepatotoxin NDEA causes severe decrease in intracellular GSH levels in rat liver. Treatment with Umb and Esc completely prevented GSH depletion in rat liver compared to rats injected with NDEA alone. We have recently shown that Esc prevents GSH depletion and protects HepG2 cells from hydrogen peroxide-induced toxicity (Subramaniam and Ellis, 2011). In addition, Esc has also been reported to modulate redox status of the cell and enhance reduced glutathione levels in mouse

liver (Martin-Aragon et al., 1998). This evidence and our finding that consumption of Umb and Esc prevent GSH depletion indicate that the protective effect of these coumarin compounds involves maintenance of redox status of hepatocytes to shield them against NDEA-induced hepatotoxicity.

Enhancement of the antioxidant defence system is reported to play a important role in protecting against oxidative damage (Hayes et al., 1999). The essential components of the endogenous antioxidant defence system include phase 2 detoxifying and antioxidant enzymes. Induction of these enzymes appears to be part of a generalised mechanism triggered by the antioxidant responsive element (ARE), which include key enzymes such as NAD(P)H: oxidoreductases (Nioi and Hayes, 2004, Jaiswal, 2000), heme oxygenase 1 (HO1) (Prestera et al., 1995) and glutathione-S-transferases (GSTs) (Rushmore and Pickett, 1990). The present study shows that consumption of diet enriched with Umb and Esc up-regulates the expression of NRF2/ARE-regulated antioxidant enzymes such as NQO1, HO1 and GSTP1 in rat liver. Previous studies show that treatment with antioxidants such as curcumin, anthocyanins and melatonin increased NRF2-mediated NQO1, HO1 and GSTα expression and protected rats from dimethylnitrosamine-induced liver injury (Farombi et al., 2008, Hwang et al., Jung et al., 2009). Earlier studies also show that administration of antioxidants BHA and ethoxyquin increased the expression of GSTs in mice (Hayes et al., 2000) and rats administered with coumarin prevented them from aflotoxin B1-induced hepatocarcinogenesis (Kelly et al., 2000). Recently, we have also shown that Esc protects HepG2 cells from hydrogen peroxide-induced damage via NRF2-dependent up-regulation of NQO1 gene (Subramaniam and Ellis, 2011). These observations suggest that Umb and Esc protect rat liver from NDEA-induced hepatotoxicity through a mechanism that involves the up-regulation of NRF2/ARE-dependent antioxidant enzymes.

4.5 Conclusions

In conclusion, the results from this chapter show that umbelliferone and esculetin, compounds present in wide variety of fruits and vegetables, protects from NDEA-induced hepatotoxicity in rats. The protective effect of these compounds appears to be through several mechanisms that includes inhibition of lipid peroxidation, maintenance of intracellular redox status of cells and induction of NRF2/ARE-dependent antioxidant protective enzymes. These results support the protective effects of esculetin previously observed *in vitro* in HepG2 cells and do not appear to be solely dependent on its free radical scavenging activity. Our *in vitro* studies in Chapter 3 on HepG2 cells together with these *in vivo* studies in rats clearly suggest that these coumarins at low non-toxic concentrations have the potential to protect against oxidant-dependant liver damage.

CHAPTER 5

NEUROPROTECTIVE EFFECTS OF COUMARINS AGAINST H_2O_2 AND MPP^+-INDUCED TOXICITY IN SH-SY5Y CELL LINES AND MOUSE BRAIN

5. Neuroprotective Effects of Coumarins against H_2O_2 and MPP^+-Induced Toxicity in SH-SY5Y cell lines and Mouse Brain

5.1 Introduction

The production of reactive oxygen species (ROS) resulting from mitochondrial dysfunction, inflammation and toxicants has been associated with various neurodegenerative diseases including Parkinson's disease, Alzheimer's disease, Huntington's disease and amyotrophic lateral sclerosis (Beal, 2007, Halliwell, 2006, Hensley et al., 2006, Tansey et al., 2008, von Bernhardi, 2007). Increased production of ROS causes damage to cell membranes and results in accumulation of dysfunctional proteins, lipid peroxidation products and damaged nuclear or mitochondrial DNA (mtDNA). The mutations in mtDNA enhance normal aging, leading to further oxidative damage, causing energy failure and increased production of ROS, in a vicious cycle of events. Oxidative damage to proteins is a major mechanism underlying neurodegeneration and occurs by several pathways including carbonylation, oxidisation of critical sulfhydryl groups, nitrosylation and nitrotyrosination.

5.1.1 Parkinson's Disease (PD)

Parkinson's disease (PD) is the second most prevalent age-related neurodegenerative disorder affecting 0.3% of entire population, and more than 1% of humans over 60 years of age (de Lau and Breteler, 2006). PD is a multisystem disease which is pathologically characterized by a progressive degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNpc) resulting in the deficiency of dopamine in striatum that plays an important role in normal motor functions. In addition, PD is identified by the presence of cytoplasmic inclusions containing α-synuclein and ubiquitin called Lewy bodies in surviving dopaminergic neurons (Lang and Lozano, 1998, Forno, 1996). Parkinson's disease is clinically characterized by resting tremor,

slowness of movements, rigidity and postural instability (Ziemssen and Reichmann, 2007). Although the mechanisms behind the neurodegeneration in PD is largely unknown, there is a large body of evidence suggesting that oxidative stress, mitochondrial dysfunction, excitotoxicity and abnormal proteolytic degradation may be significant to the pathogenesis (Dawson and Dawson, 2003).

5.1.2 Role of oxidative stress, mitochondrial dysfunction and nitric oxide in PD

Reactive Oxygen Species (ROS) have been implicated in various neurodegenerative and age-related diseases including PD and Alzheimer's disease. Evidence for the involvement of oxidative stress in PD is derived from the post mortem analysis of brain tissues from PD patients that reveal increased levels of oxidized proteins, lipids and nucleic acids (Alam et al., 1997a, Alam et al., 1997b, Castellani et al., 2002, Dexter et al., 1986, Dexter et al., 1989, Dexter et al., 1994). Besides, mitochondrial complex I, which is highly susceptible to oxidants, ROS and reactive nitrogen species, had been reported to play an important role in PD pathogenesis. Mitochondrial dysfunction in PD is supported by studies suggesting that post mortem brains of idiopathic PD patients showed a defect in mitochondrial respiratory complex I with 25-30% inhibition (Hattori et al., 1991, Schapira et al., 1990, Schapira, 2006). Moreover, nitric oxide (NO) has emerged as a major endogenous modulator of neuronal function and acts as an intracellular messenger in mediating cell-to-cell interactions in brain. NO, synthesized by nitric oxide synthase, and peroxynitrite, formed by NO interaction with superoxide are known to be involved in neurodegeneration. Studies reveal that NO inhibits mitochondrial complex I by S-nitrosylation and Fe-nitrosation, and on the other hand peroxynitrite irreversibly inhibits brain mitochondrial complex I (Brown and Borutaite, 2004, Riobo et al., 2001). Thus, generation of ROS, free radical NO followed by peroxynitrite production, and mitochondrial dysfunction may be implicated in degenerative neuronal cell death in PD.

5.1.3 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced PD model

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP; Fig. 5.1) is a potent neurotoxin that causes degeneration of nigrostriatal dopaminergic neurons in primates and rodents and, therefore, induces suitable models of Parkinson's disease (Sedelis et al., 2001, Dauer and Przedborski, 2003). MPTP is highly lipophilic and readily crosses the blood brain barrier where it is converted into 1-methyl-4-phenyl-pyridinium (MPP^+; Fig. 5.1) by monoamine oxidase B (MAO-B) in glial cells. MPP^+ released in extracellular space is transported into dopaminergic neurons by dopamine transporter (DAT) (Fig 5.2). MPP^+ accumulated in dopaminergic neurons enters mitochondria and disrupts oxidative phosphorylation by inhibiting the mitochondrial complex I of the electron transport chain. The resulting ATP deficiency causes loss of plasma and mitochondrial membrane potential difference leading to neuronal cell death. Another consequence of mitochondrial complex I inhibition is the generation of reactive oxygen species (ROS), especially superoxide (Rossetti et al., 1988, Hasegawa et al., 1990, Cleeter et al., 1992). Studies suggest that such complex I inhibition of MPP^+ can generate excessive mitochondrial superoxide which can destroy the macromolecules in close proximity (Bates et al., 1994, Cleeter et al., 1992).

Several *in vivo* and *in vitro* studies suggest that induction of oxidative stress as one of the important machinery for MPTP-induced neuronal toxicity. MPP^+ has been reported to increase the production of reactive oxygen species (ROS) in neuroblastoma cells (Cassarino et al., 1997), and treatment with antioxidants were found to protect against MPP^+ toxicity in neuronal cell lines (Lai et al., 1993) and dopaminergic neurons in primary cells (Akaneya et al., 1995). In animals, MPTP was reported to induce hydroxyl radical (Chiueh et al., 1992) and increase peroxynitrite expression through nitric-oxide synthase in mouse brain (Schulz et al., 1995). MPP^+ has been reported to induce superoxide radicals by mitochondrial complex I inhibition (Hasegawa et al., 1997) and over expression of superoxide dismutase in

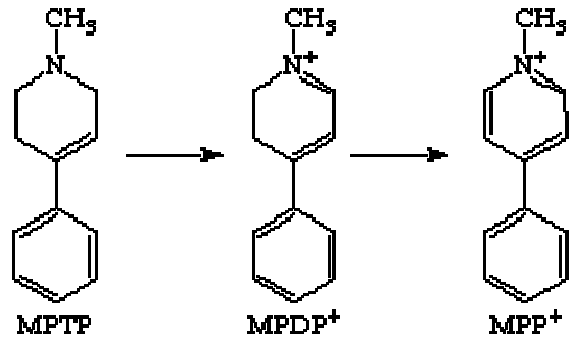


Fig. 5.1 - Structure of MPTP and its metabolites $MPDP^+$, MPP^+

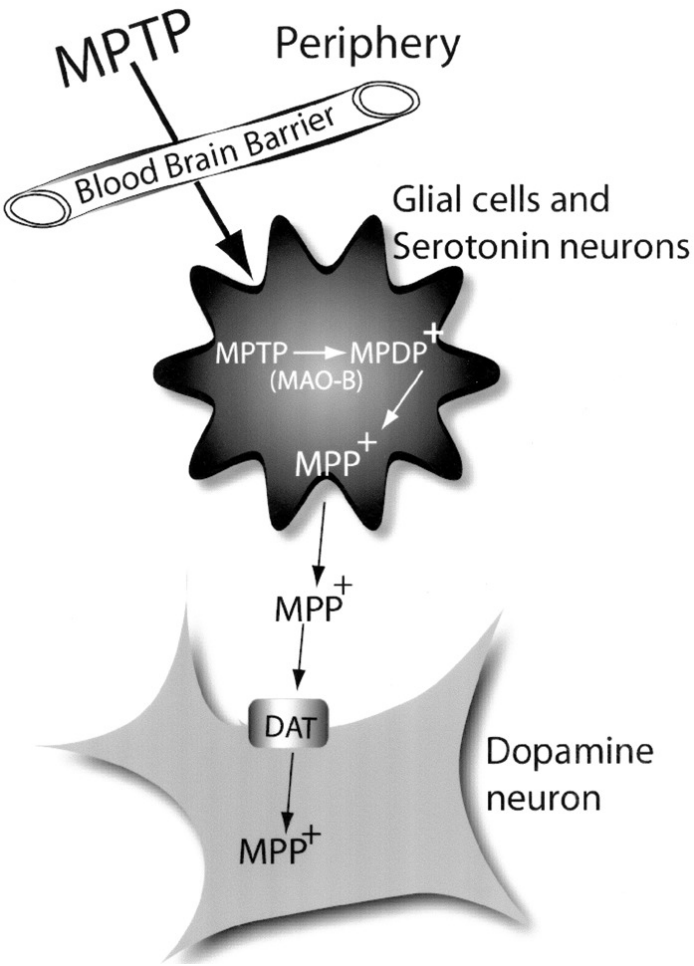


Fig. 5.2 - Schematic diagram of MPTP metabolism and uptake into dopaminergic neurons in brain (Przedborski and Vila, 2003).

mice has been found to ameliorate MPTP-induced neurotoxicity (Przedborski et al., 1992).

On the other hand, activation of microglia also contributes to the neurotoxicity of MPTP (Wu et al., 2002, Wang et al., 2005). The activation of microglia leads to the increase in production of reactive oxygen species, nitric oxide and pro-inflammatory cytokines, all of which can individually lead to neuronal toxicity (Kreutzberg, 1996). It has also been reported that MPTP causes up-regulation of nitric-oxide synthase and the mice lacking in inducible nitric-oxide synthase (iNOS) were more resistant to MPTP-induced toxicity than the wild type (Liberatore et al., 1999).

All these findings suggest that mitochondrial dysfunction, reactive oxygen species and nitric oxide are involved in MPTP-induced Parkinson's model. Several antioxidants have been reported to protect against MPTP-induced neurotoxicity including edaravone (Kawasaki et al., 2007), cytosine (Ferger et al., 1998), bromocriptine (Muralikrishnan and Mohanakumar, 1998), ginsenoside (Chen et al., 2005), deprenyl (Ebadi et al., 2002) and salicylic acid (Mohanakumar et al., 2000).

Umbelliferone (7-hydroxycoumarin; Umb) and esculetin (6,7-dihydroxycoumarin; Esc), as mentioned previously, are coumarin derivatives found in plants with proven antioxidant and free radical scavenging properties (Paya et al., 1992b). Umb has been reported to maintain redox status in diabetic rats (Ramesh and Pugalendi, 2005) and Esc has been shown to protect cells and animals from ROS and other reactive agents (Kim et al., 2008) (Kaneko et al., 2003) (Kaneko et al., 2007).

The involvement of ROS in PD has led to investigations into the potential for using antioxidant molecules as therapeutic treatment (Halliwell, 2001, Zhou et al., 2008). Several antioxidants have been reported previously to protect against MPTP-induced neurotoxicity in animal models. Many of these are thought to act through a radical scavenging activity, which requires relatively high doses of antioxidant, but few have been effective in clinical trials (Weber and Ernst, 2006).

The aim of the work carried out in this chapter was to examine the protective effects of Umb and Esc against oxidative stress-mediated toxicity induced by hydrogen peroxide and MPP^+ in SH-SY5Y neuroblastoma cells, and MPTP-induced dopaminergic neurotoxicity in the striatum and substantia nigra in C57BL/6J mouse brain. In addition, the mechanism by which these compounds work was also investigated. SH-SY5Y cell line is considered as a suitable model to study the cellular and molecular mechanisms of neurodegeneration, especially in Parkinson's disease. This cell line is reported to possess many characteristics of dopaminergic neurons. SH-SY5Y cells express biomarkers of PD such as tyrosine hydroxylase and dopamine-beta-hydroxylase, as well as the dopamine transporter (Lopes et al., 2010, Xie et al., 2010).

5.2 Materials and Methods

5.2.1 In vitro studies

SH-SY5Y cells were cultured for 48h and pre-treated with umbelliferone or esculetin or BHA or quercetin for 8h. Then cells were exposed to hydrogen peroxide or MPP^+ for 24h or 48h, respectively. Cells were then analysed for different parameters to study the neuroprotective effects of coumarins as mentioned in Chapter 2.

5.2.2 Animals and drug treatment

Male C57BL/6J 6-week old mice were used for this study. Mice were allowed to acclimatize for 2 weeks before being fed either a normal diet supplemented with 2% arachis oil or diet containing test compounds in 2% arachis oil. Arachis oil was used as a vehicle for the uniform distribution of test compounds. The test compounds were provided in the animal diet for 7 days in the following amounts: BHA, 0.75% (w/w); Umbelliferone, 0.5% (w/w) and Esculetin, 0.5% (w/w). During the experiment the animals were provided free access to food and water. Each mouse on average consumed 5 g of the diet per day (with or without test compounds).

5.2.3 Induction of MPTP-mediated dopaminergic neurotoxicity

Following the diet and drug treatment, seven- to eight-week old male C57BL/6J mice were administered with 30 mg/kg MPTP, IP, once a day, for five consecutive days to induce dopaminergic neurotoxicity in brain. Mice were euthanized by cervical dislocation and the brains were quickly removed and dissected on an ice-cold glass plate. The striatum and midbrain of three-six mice in each group were individually isolated, frozen on dry ice and stored at -80^{o}C until further analysis. Similarly brains of three-six mice in each group after the treatments were rinsed in physiological saline and immediately fixed in PFA for immunohistochemical studies.

Mouse groups intoxicated with MPTP showed restricted motor activity compared to their respective control groups. However, no tests for motor activity were performed due to the Home Office project license restrictions.

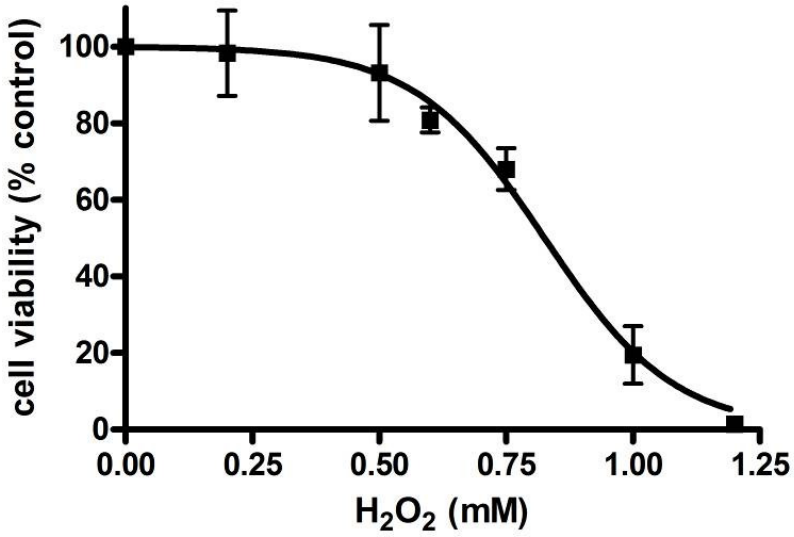
All other methods in this chapter were performed as described in chapter 2.

5.3 Results

5.3.1 Cytotoxic effect of hydrogen peroxide and MPP^{+} on SH-SY5Y cell lines

The cytotoxic effects of hydrogen peroxide and MPP^{+} on SH-SY5Y cell lines were determined using the MTT assay, which measures cell viability. SH-SY5Y cells were plated and incubated for 48 hrs in 96-well plates followed by exposure to hydrogen peroxide (0.1 - 1.2mM) for 24 hrs or MPP^{+} (25 - 10 μM) for 48 hrs. As shown in figure 5.1, hydrogen peroxide exhibited a dose-dependent toxicity on SH-SY5Y cells with an IC_{50} value of 0.79±0.19mM (Fig. 5.3A). The concentrations of the neurotoxin MPP^{+} used showed severe toxicity on SH-SY5Y cells, with an IC_{50} value of approximately 25μM (Fig. 5.3B).

A



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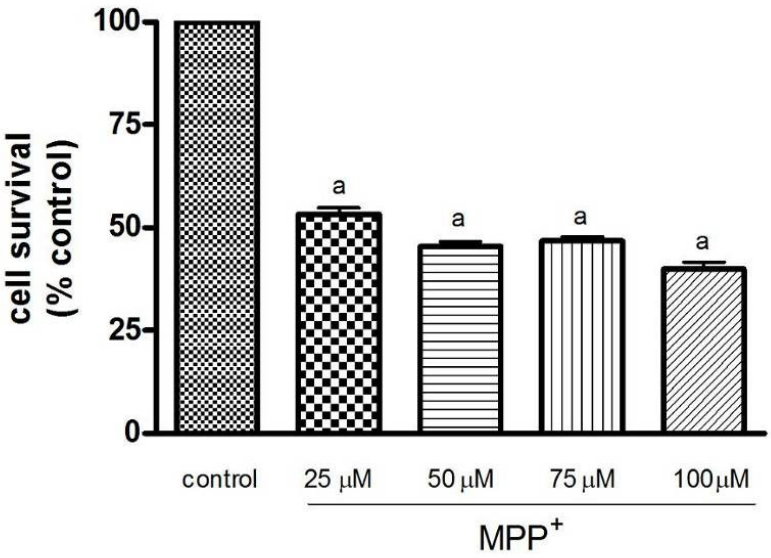


Fig. 5.3 - Cytotoxic effect of H_2O_2 and MPP^+ on SH-SY5Y cells. Cells were treated with increasing concentrations of (A) H_2O_2 (0.1 – 1.2 mM) for 24 hrs and (B) MPP^+ (25 - 100 μM) for 48 hrs. The level of toxicity was measured by MTT assay. Samples represent a mean ± SD of samples in triplicate (n=3) and cell viability was calculated as % untreated control cells. $P < 0.05$ compared to untreated control (a).

5.3.2 Neuroprotective effect of coumarins on hydrogen peroxide and MPP^+-induced neurotoxicity in SH-SY5Y cell lines

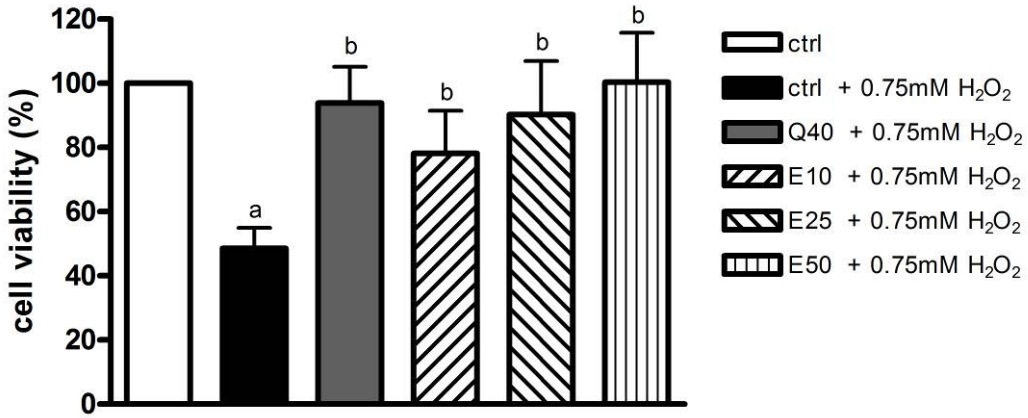
The neuroprotective effect of esculetin (10-50 μM) and umbelliferone (25 μM) were determined as cell viability using the MTT assay. Quercetin (40 μM) and BHA (50 μM) are known for their protective effects against oxidant-induced toxicity and were used as positive controls. SH-SY5Y cells were pre-treated with the coumarins for 8 hrs followed by exposure to the oxidant (0.75 mM H_2O_2) and neurotoxin (50 μM MPP^+) for 24 hrs and 48 hrs respectively.

Pre-treatment with esculetin (25-50 μM) significantly increased cell viability compared to untreated control after exposure to H_2O_2 in a dose-dependent manner. Esculetin (50 μM) showed an equivalent protective effect compared to positive control quercetin (40 μM) (Fig 5.4A).

Umbelliferone (25 μM) and esculetin (25 μM) pre-treatment showed no increase in cell viability on MPP^+-induced toxicity on SH-SY5Y cell lines. Similarly the positive control BHA (50 μM) could not elevate the cell viability in MPP^+-induced neurotoxicity (Fig 5.4B).

These results suggest that Umb and Esc protect SH-SY5Y cells from hydrogen peroxide induced decrease in cell viability, however the coumarins did not prevent from MPP^+-induced cell death.

A



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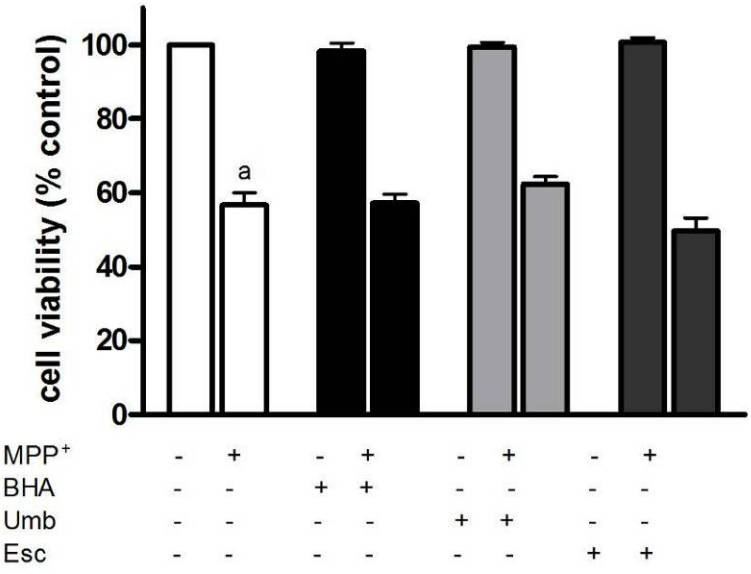


Fig. 5.4 - Effect of coumarins on H_2O_2 and MPP^+-induced neurotoxicity in SH-SY5Y cells. Cells were pre-treated with esculetin or umbelliferone or quercetin or BHA for 8 hrs and further exposed to (A) 0.75 mM H_2O_2 for 24 hrs or (B) 50 μM MPP^+ for 48 hrs. The level of toxicity was measured by MTT assay. Samples represent a mean ± SD of samples in triplicate and cell viability was calculated as % untreated control cells. $P < 0.05$ compared to untreated control (a) and H_2O_2-treated control (b).

5.3.3 Effect of coumarins on glutathione depletion during hydrogen peroxide and MPP^+-induced neurotoxicity in SH-SY5Y cell lines

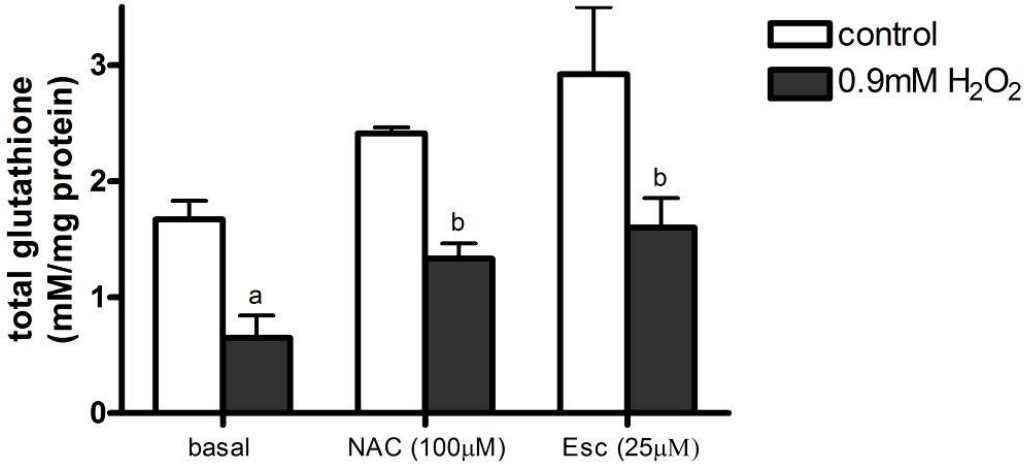
Total intracellular glutathione level is an indicator of the redox status of the cell (Droge, 2002, Schafer and Buettner, 2001). GSH levels during hydrogen peroxide and MPP^+-induced neurotoxicity were measured by an enzyme-dependent spectrophotometric assay. NAC has been shown to increase glutathione levels (Dodd et al., 2008) and BHA is a potent antioxidant; these compounds were used as positive controls for this experiment.

Treatment of cells with 0.9mM hydrogen peroxide toxicity for 24 hrs decreased total GSH levels up to 60% (Fig 5.5A). However pre-treatment of cells with esculetin (25μM) completely prevented GSH depletion and maintained the levels of GSH during H_2O_2-induced toxicity. Esculetin had an equivalent effect compared to NAC (100 μM), a well-documented antioxidant (Dodd et al., 2008).

The treatment of SH-SY5Y cells with the neurotoxin MPP^+ (50 μM) depleted total intracellular GSH levels by 85% after 48 hrs exposure. Pre-treatment with umbelliferone (25 μM) and esculetin (25 μM) for 8 hrs totally prevented GSH depletion. The positive control BHA (50 μM) also showed a similar effect (Fig 5.5B).

These results indicate that Umb and Esc prevent SH-SY5Y cells from hydrogen peroxide and MPP^+-induced depletion in total intracellular glutathione levels.

A



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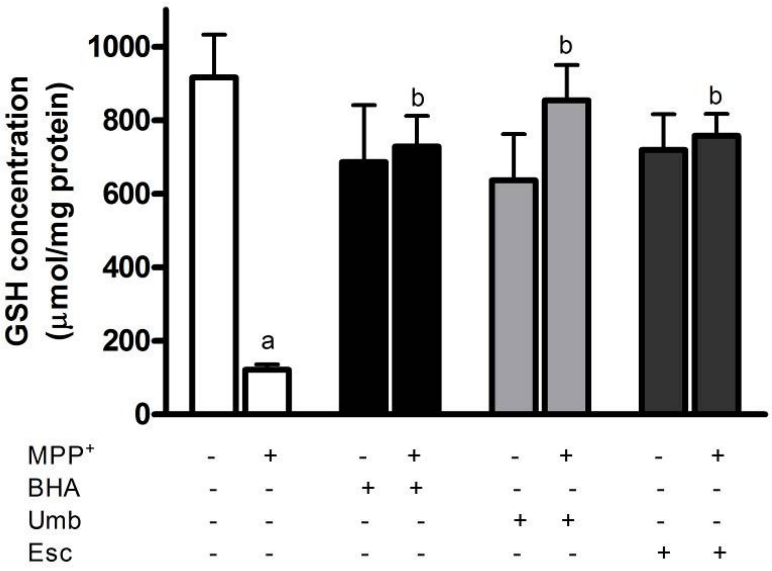


Fig. 5.5 - Coumarins prevent GSH depletion during H_2O_2 and MPP^+-induced toxicity. Cells were pre-treated with umbelliferone (25 μM) or esculetin (25 μM) or NAC (100 μM) or BHA (50 μM) for 8 hrs. Then cells were exposed to (A) 0.9 H_2O_2 for 24 hrs or (B) 50 μM MPP^+ for 48 hrs and the glutathione levels were measured by a colorimetric reaction with DTNB. Samples represent a mean ± SD of samples in triplicate and total glutathione was calculated per milligram of protein. $P < 0.05$ compared to untreated control (a) and 0.9 mM H_2O_2-treated or MPP^+-treated (b).

5.3.4 Effect of coumarins on ROS generation during hydrogen peroxide and MPP^+-induced neurotoxicity in SH-SY5Y cell lines

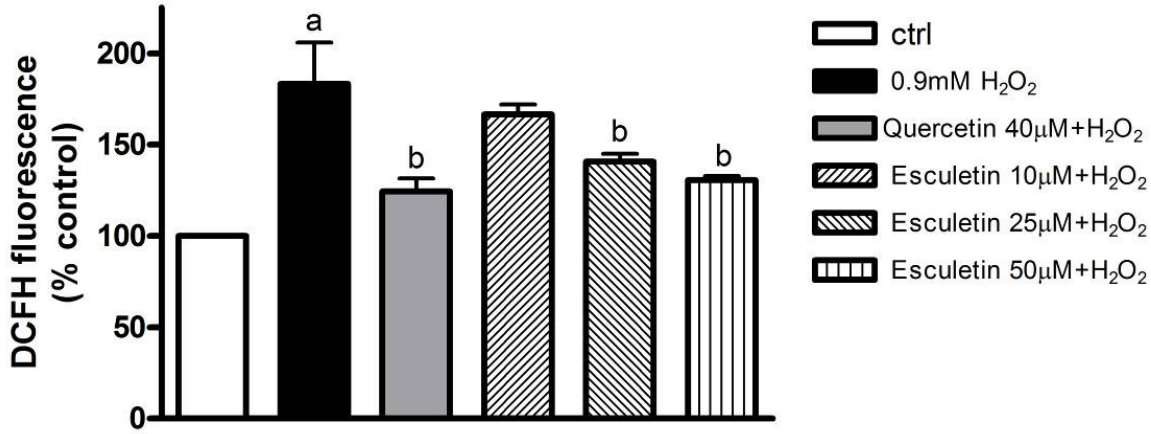
Generation of ROS in excess of the antioxidant capabilities of the cell can lead to damage to cellular components including proteins, lipids and nucleic acids. To examine the ability of hydrogen peroxide and MPP^+ to generate ROS, ROS levels were determined by using a fluorescent probe, dichlorofluorescein diacetate (Wang and Joseph, 1999).

Exposure of the neuroblastoma cells to 0.9mM H_2O_2 drastically increased intracellular ROS by 1.8-fold. Pre-treatment of SHSY-5Y cells with esculetin (25-50μM) significantly decreased the elevated ROS levels by 50% and 58% respectively, compared to vehicle treated control. Similarly, the positive control quercetin (40μM), a potent scavenger of ROS (Tanigawa et al., 2007), showed a marked reduction (70%) in H_2O_2-induced ROS levels (Fig. 5.6A).

The neurotoxin MPP^+ markedly increased the ROS levels by 1.7-fold in SH-SY5Y cells compared to control treatment. The elevation in ROS was significantly reduced by pre-treatment with umbelliferone (25μM) as well as the positive control BHA (50μM) by 55% and 45% respectively. However, esculetin (25μM) did not provide any decrease in ROS levels during MPP^+-induced neurotoxicity in SHSY-5Y cells (Fig. 5.6B).

Overall these results show that Umb and Esc reduce the amount of ROS induced by hydrogen peroxide and MPP^+ in SH-SY5Y cells.

A



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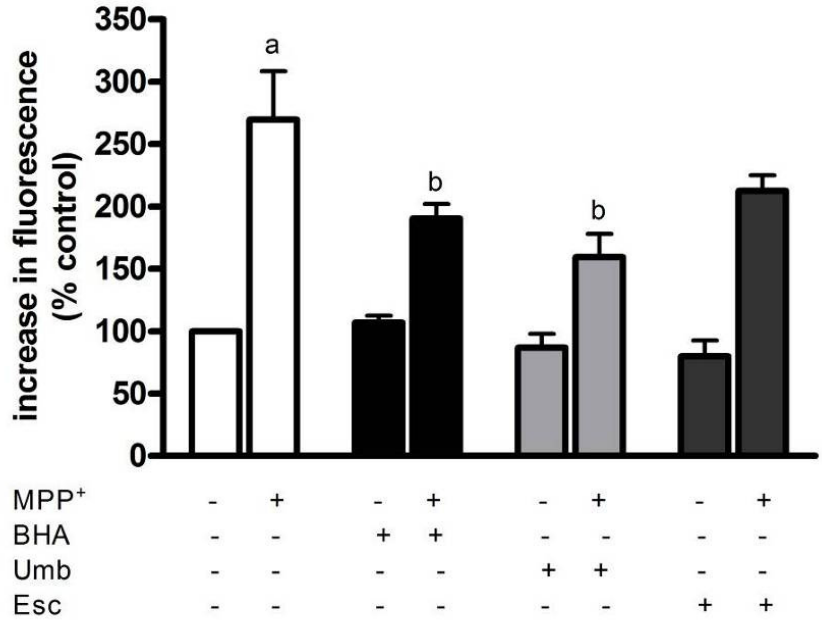


Fig. 5.6 - Coumarins decrease ROS generation during H_2O_2 and MPP^+-induced oxidative stress. Cells were pre-treated with esculetin or quercetin or BHA for 8 hrs and incubated with 100μM DCFH for 30 min. The cells were washed and then exposed to (A) 0.9 H_2O_2 or (B) 50μM MPP^+ for 30 min and the level ROS generation was measured as fluorescence in a plate reader. Samples represent a mean ± SD of samples in triplicate and fluorescence was calculated as% of untreated cells. $P < 0.05$ compared to untreated control (a) and 0.9mM H_2O_2 or 50μM MPP^+ treated control (b).

5.3.5 Coumarins inhibit MPP^+-induced increase in pro-apoptotic Bax expression in SH-SY5Y cell lines

The members of B-cell lymphoma 2 protein (Bcl-2) family including pro-apoptotic Bax (Vila et al., 2001) and anti-apoptotic Bcl-XL (Dietz et al., 2008) are reported to be involved in MPTP induced neuronal apoptosis. To evaluate the link between elevated ROS and apoptosis, the effect of Umb and Esc on MPP^+-induced apoptosis was measured.

SH-SY5Y cells were treated with coumarins followed by MPP^+ (50μM) and Bax expression levels were measured by Western blotting using specific antibodies to Bax. Treatment with MPP^+ (50μM) markedly increased expression of Bax by approximately 3-fold. Umbelliferone (25μM) and esculetin (25μM) pre-treatment for 8 hrs completely inhibited MPP^+-induced elevation in pro-apoptotic Bax expression. BHA (50μM), the positive control, also inhibited the neurotoxin-induced expression of Bax (Fig 5.7).

These results suggest that Umb and Esc reduce MPP^+-induced increase in Bax levels in SH-SY5Y cells.

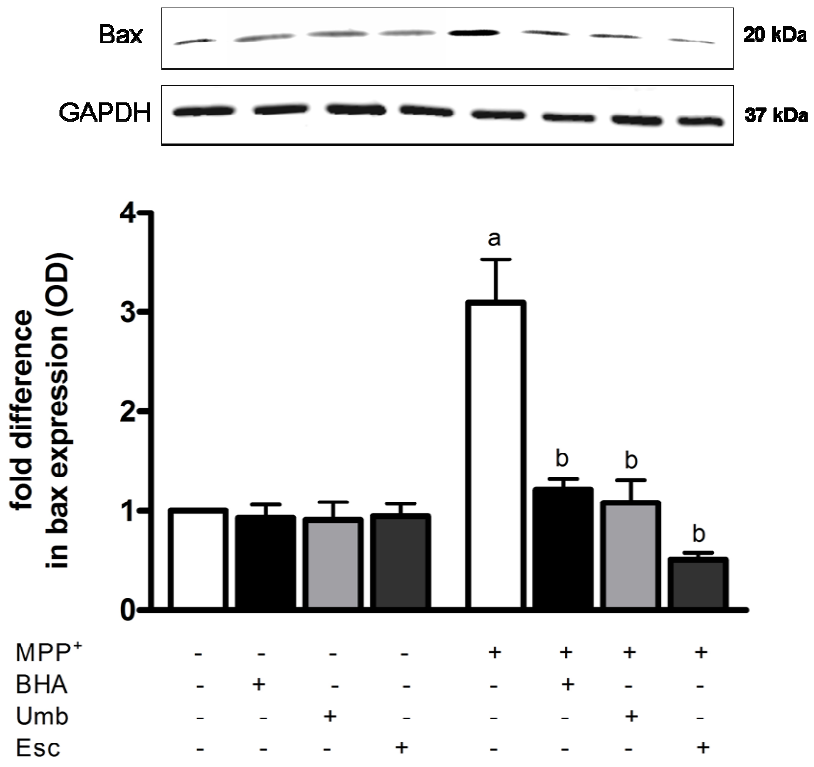


Fig. 5.7 - Coumarins inhibit MPP$^+$-induced increase in Bax expression. Cells were pre-treated with umbelliferone (25 µM) or esculetin (25 µM) or BHA (50 µM) for 8 hrs and cells lysates were analysed by immunoblotting using specific antibodies for Bax or GAPDH as loading control. The blots represent experiments performed in triplicate. Histograms show the densitometric analysis of Bax expression normalised to GAPDH (B) by NIH ImageJ software. $P < 0.05$ compared to vehicle treated control (a) and MPP$^+$-treated control (b).

5.3.6 Coumarins induce antioxidant protective enzyme SOD1 in SH-SY5Y cell lines

Studies suggest that mitochondrial complex I inhibition of MPP^+ can generate excessive mitochondrial superoxide which can destroy the macromolecules in close proximity (Bates et al., 1994, Cleeter et al., 1992). Superoxide dismutase (SOD) catalyses the dismutation of superoxide radical and is present in three different forms in mammals: CuZnSOD (SOD1: mainly in cytosol), MnSOD (SOD2: located in mitochondria) and extracellular SOD (SOD3: localised in extracellular fluid) (Rohrdanz et al., 2002). In this study we determined the levels of SOD1 in SH-SY5Y cell lines by Western blotting.

Results show that esculetin up-regulates the expression of SOD1 by 1.8-fold compared to the vehicle treated control. The positive control quercetin also increased SOD1 levels but to a lesser extent (1.3-fold) (Fig 5.8).

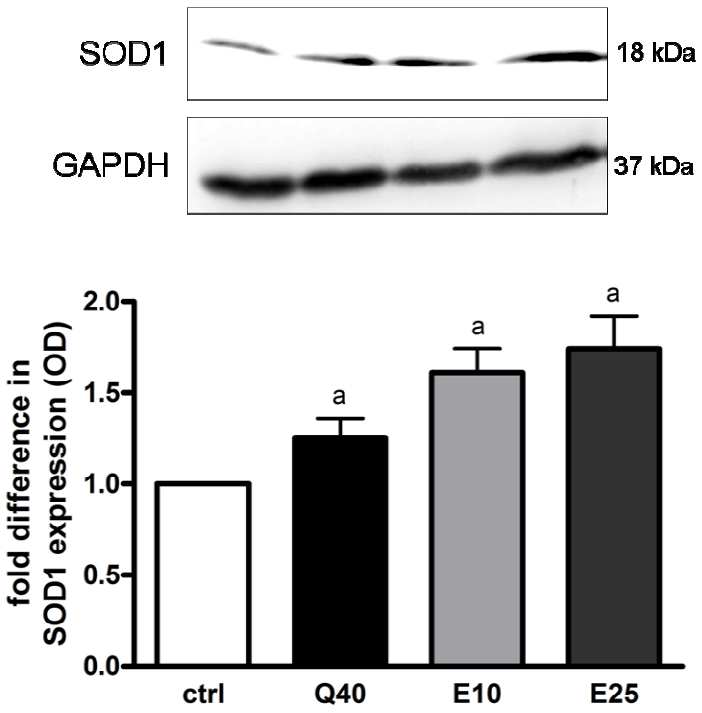


Fig. 5.8 - Induction of antioxidant enzyme SOD1 by esculetin in SH-SY5Y cells. Cells were pre-treated with esculetin (10-25 µM) or quercetin (40 µM) for 8 hrs and the cell lysates were analysed by immunoblotting using SOD1 or GAPDH as loading control. The blots represent experiments performed in triplicate. Histogram shows the densitometric analysis of SOD1 expression normalised to GAPDH by NIH ImageJ software. $P < 0.05$ compared to untreated control (a).

5.3.7 Effect of Umbelliferone and Esculetin on MPTP-induced decrease in tyrosine hydroxylase and neurotoxicity in mouse brain

MPP^+ has a high affinity for plasma membrane dopamine transporter (DAT) and enters the dopaminergic neurons by active transport. The enzyme tyrosine hydroxylase (TH) mediates the catalytic conversion of tyrosine to DOPA, which is the precursor for dopamine. Once inside dopaminergic neurons, MPP^+ causes neurotoxicity by multiple mechanisms leading to decrease in dopamine and tyrosine hydroxylase levels.

To evaluate the dopaminergic neurotoxicity caused by MPTP in mouse brain, the levels of tyrosine hydroxylase (TH) were monitored by immunohistochemistry. Mice treated with MPTP for five days showed a severe decrease in TH levels in the striatum (97%) (Fig. 5.9) and substantia nigra (75%) (Fig. 5.10). Treatment with the coumarins failed to alleviate MPTP-induced reduction in TH levels in the striatum. In contrast, BHA increased the TH levels to 37% in striatum (Fig. 5.9). In SNpc, Umb and Esc equally attenuated MPTP-induced decrease in TH levels by 25%, superior to that of the known antioxidant BHA (55%) (Fig. 5.10).

MPTP-induced neurotoxicity was further evaluated by determining Nissl-positive neurons by staining the mouse brain with Nissl's crystal violet stain. Treatment with MPTP markedly reduced the number of Nissl-positive neurons in the striatum (10%) and SNpc (22%). Dietary administration of Umb and Esc significantly ameliorated MPTP-induced neuronal cell loss up to 60 % and 78% respectively in the striatum. A similar neuroprotective effect (Umb 62%, Esc 72%) was observed in SNpc. These effects were comparable to that of BHA in the striatum (65%) and SNpc (67%) (Fig. 5.11, 5.12).

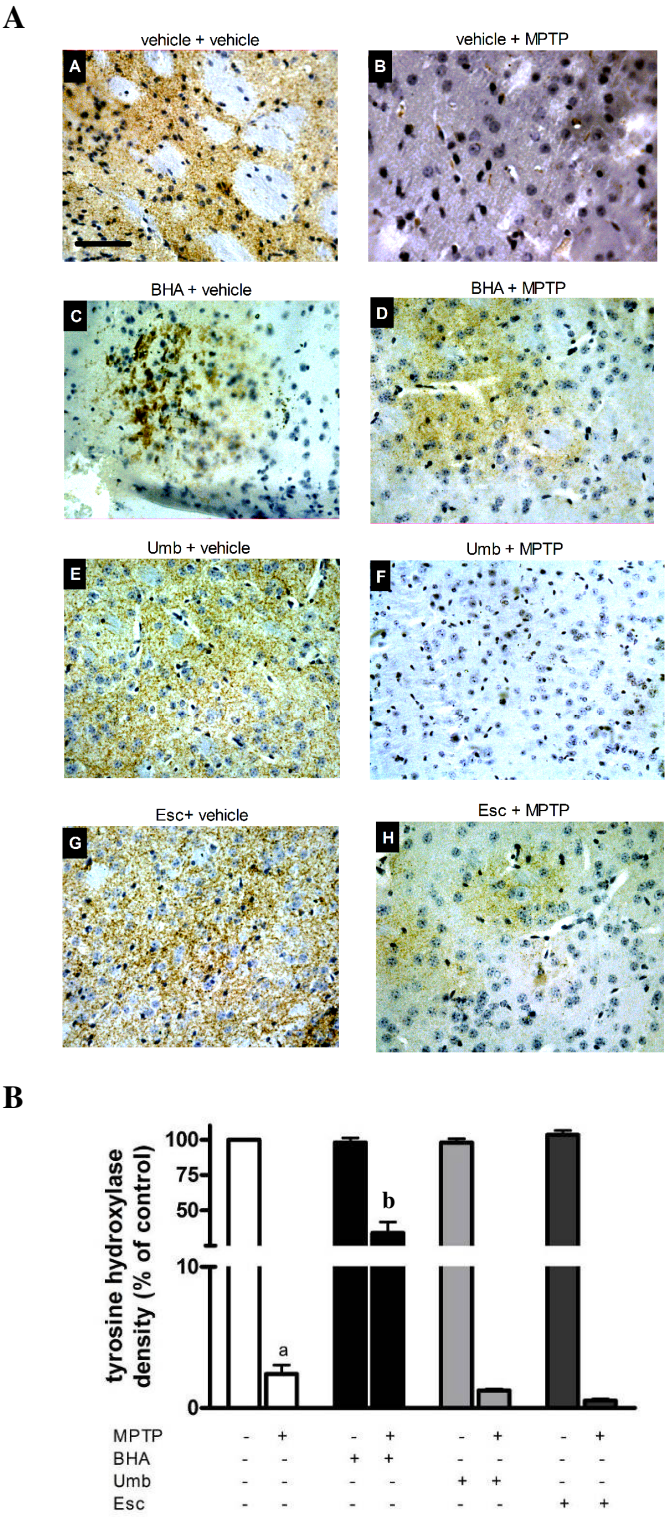


Fig. 5.9 - Effect of Umb and Esc on MPTP-induced decrease in immunoreactivity for tyrosine hydroxylase (TH) in striatum. (A) Representative photomicrographs of TH staining from 3-6 mice for each group with vehicle (A, C, E, G) or MPTP (B, D, F, H) administration. Scale bar 200µM. (B) Densitometric analysis using NIH ImageJ software by measuring the mean threshold of TH immunostaining from 3 images. Results are expressed as the mean ± SD of 3-6 mice. $P<0.05$ compared to vehicle with vehicle treatment group (a) and vehicle with MPTP treatment group (b).

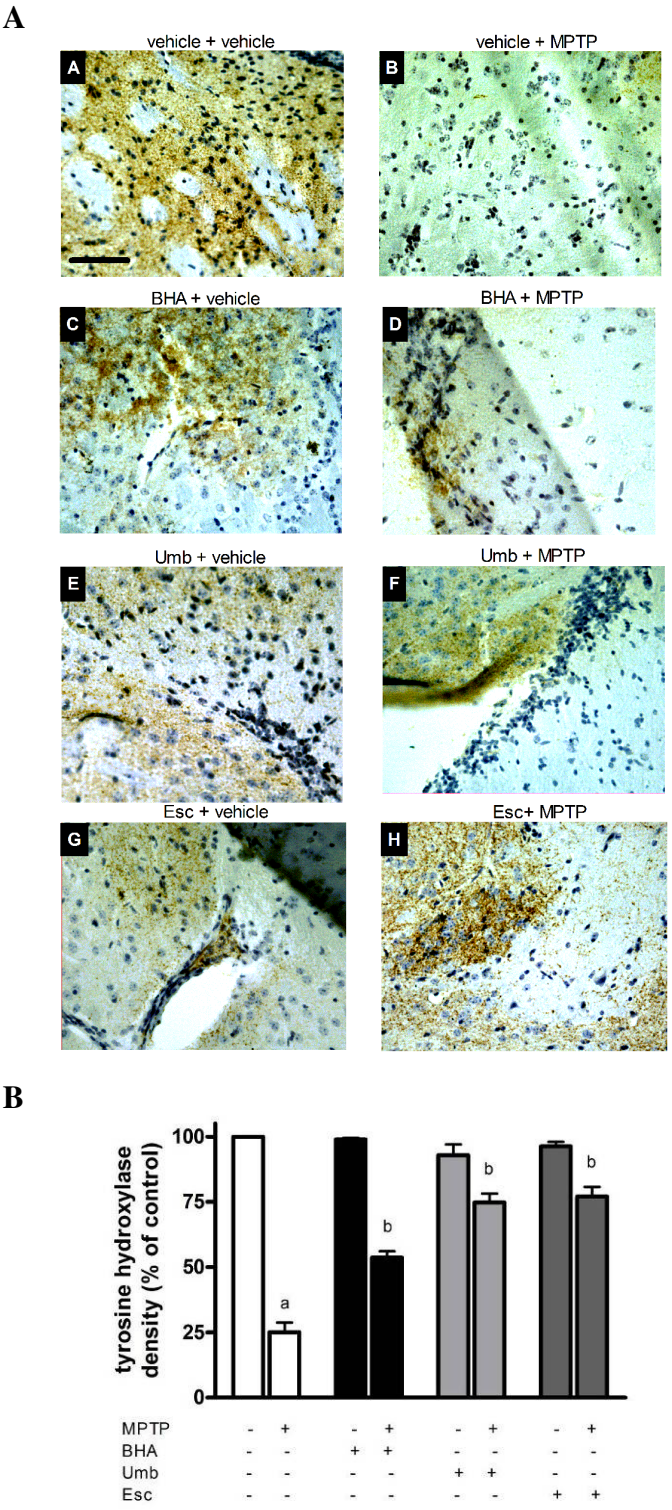


Fig. 5.10 - Effect of Umb and Esc on MPTP-induced decrease in immunoreactivity for tyrosine hydroxylase (TH) in SNpc. (A) Representative photomicrographs of TH staining from 3-6 mice for each group with vehicle (A, C, E, G) or MPTP (B, D, F, H) administration. Scale bar 200µM. (B) Densitometric analysis using NIH ImageJ software by measuring the mean threshold of TH immunostaining from 3 images. Results are expressed as the mean ± SD of 3-6 mice. $P<0.05$ compared to vehicle with vehicle treatment group (a) and vehicle with MPTP treatment group (b).

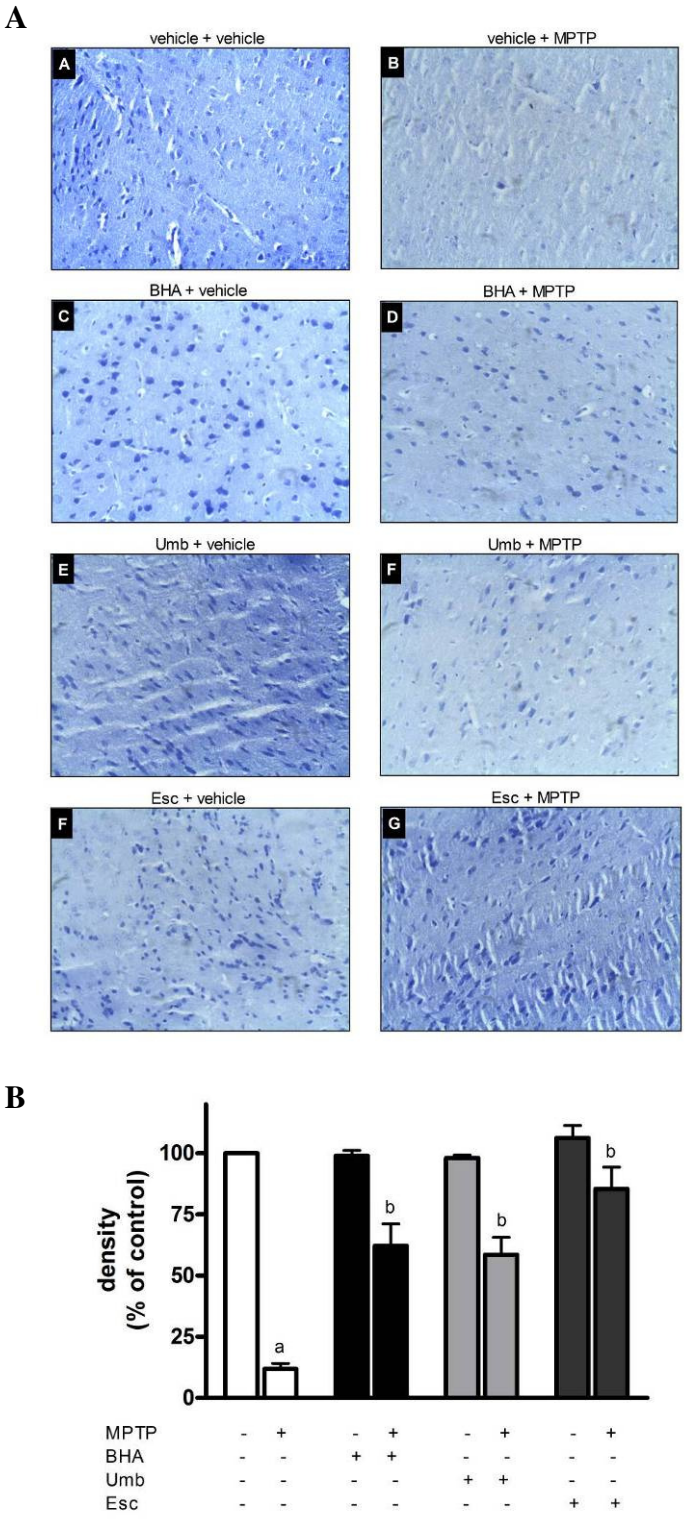


Fig. 5.11 - Effect of Umb and Esc on MPTP-induced decrease in Nissl-positive neurons in striatum. (A) Representative photomicrographs of Nissl's staining from 3-6 mice for each group with vehicle (A, C, E, G) or MPTP (B, D, F, H) administration (20×). (B) Densitometric analysis using NIH ImageJ software by measuring the mean threshold of Nissl's staining from 3 images. Results are expressed as the mean ± SD of 3-6 mice. $P<0.05$ compared to vehicle with vehicle treatment group (a) and vehicle with MPTP treatment group (b).

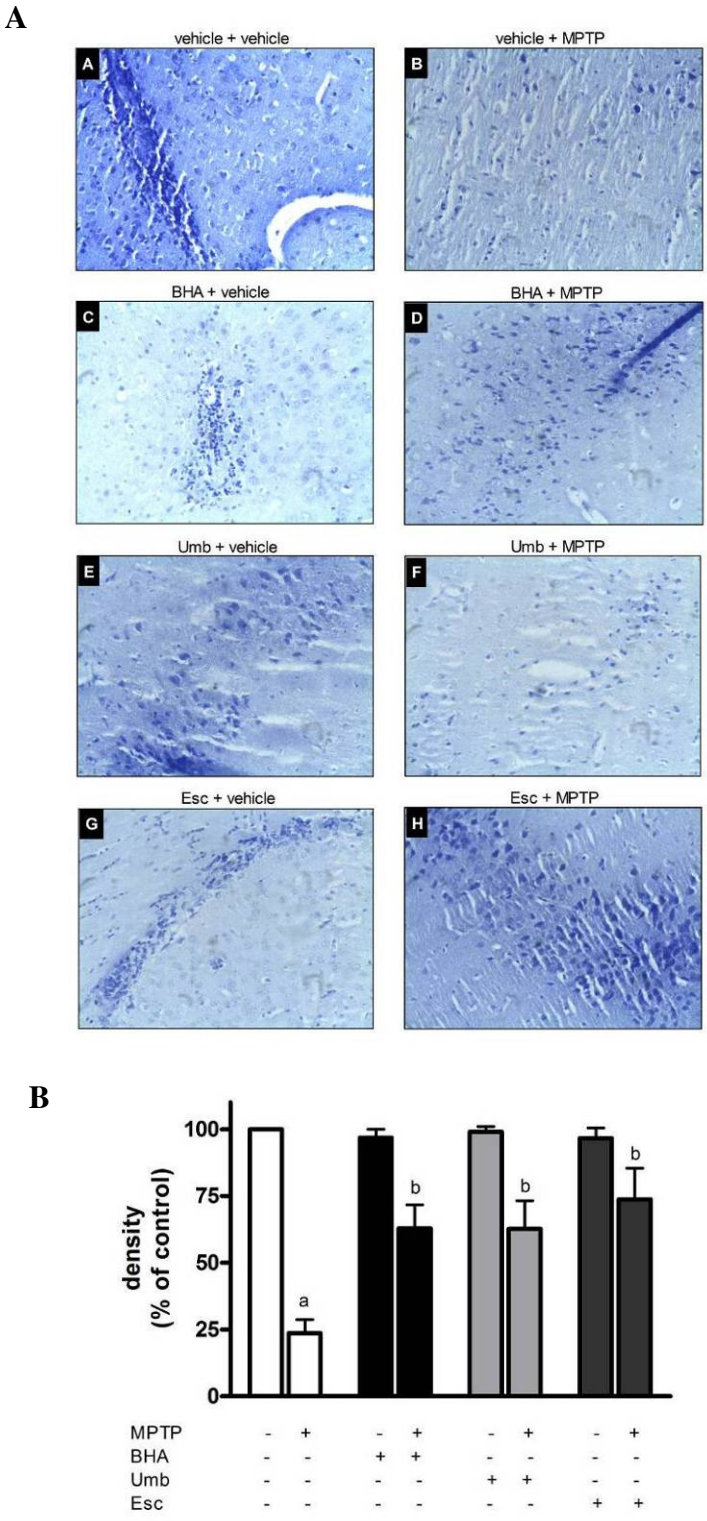


Fig. 5.12 - Effect of Umb and Esc on MPTP-induced decrease in Nissl-positive neurons in SNpc. (A) Representative photomicrographs of Nissl's staining from 3-6 mice for each group with vehicle (A, C, E, G) or MPTP (B, D, F, H) administration (20×). (B) Densitometric analysis using NIH ImageJ software by measuring the mean threshold of Nissl's staining from 3 images. Results are expressed as the mean ± SD of 3-6 mice. $P<0.05$ compared to vehicle with vehicle treatment group (a) and vehicle with MPTP treatment group (b).

5.3.8 Effect of Umbelliferone and Esculetin on MPTP-induced peroxynitrite production

Nitric oxide produced by nitric oxide synthase has been implicated to play a major role in altering neuronal functions leading to its toxicity. Nitric oxide combines with superoxide and forms peroxynitrite, which is detrimental to neuronal cells.

The levels of 3-nitrotyrosine (3-NT) was measured as an index of peroxynitrite production in mouse brain by immunohistochemistry. MPTP treated mice showed higher concentrations of 3-NT in both striatum and SNpc compared to vehicle treated control group. Umb and Esc treatment showed a significant reduction in MPTP induced elevated levels of 3-NT by 42% and 35% in striatum, 58% and 80% in SNpc, respectively. However, BHA failed to decrease MPTP-induced increase in 3-NT in striatum and showed 20% reduction in SNpc (Fig. 5.13, 5.14).

These results suggest that Umb and Esc reduce MPTP-induced elevation of peroxynitrite production in mouse SNpc and striatum.

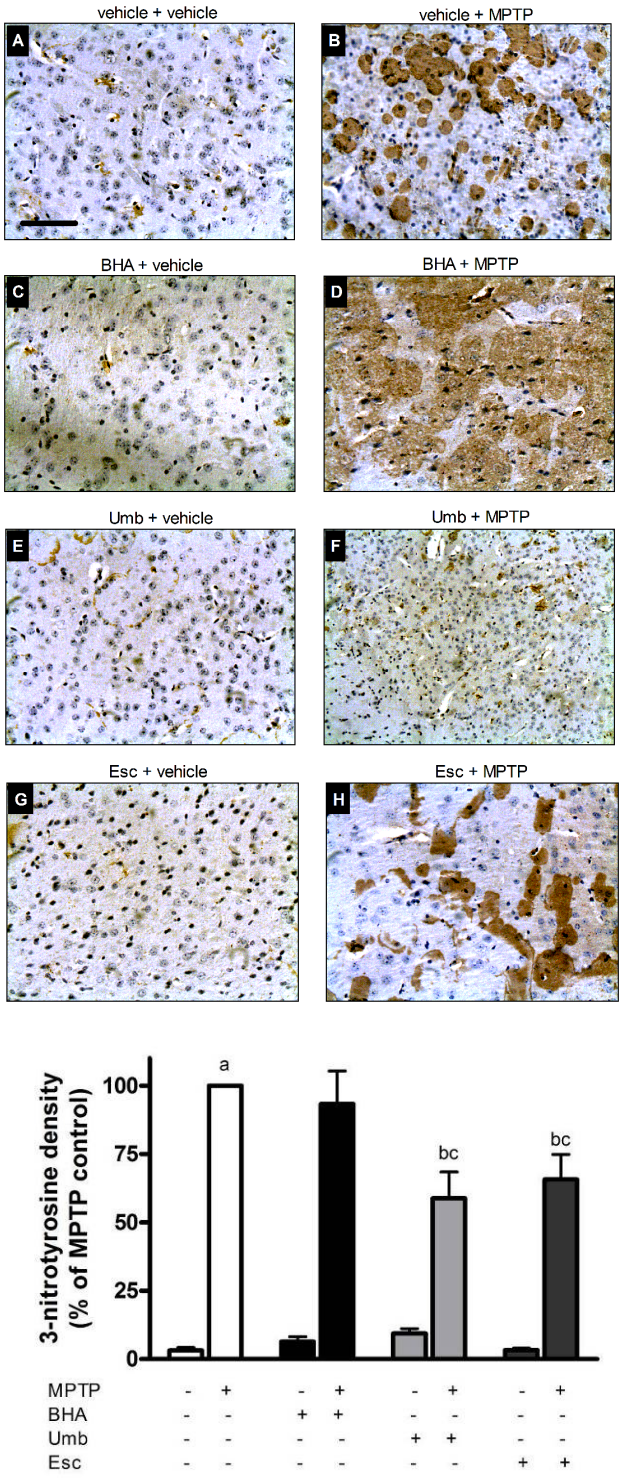


Fig. 5.13 - Effect of Umb and Esc on MPTP-induced peroxynitrite production in striatum. (A) Representative photomicrographs of 3-nitrotyrosine (3-NT) staining from 3-6 mice for each group without (A, C, E, G) or with (B, D, F, H) MPTP administration. Scale bar 200µM. (B) Densitometric analysis using NIH ImageJ software by measuring the mean threshold of 3-NT immunostaining from 3 images. Results are expressed as the mean ± SD of 3-6 mice. $P<0.05$ compared to vehicle without MPTP treatment group (a), vehicle with MPTP treatment group (b) and BHA with MPTP group (c).

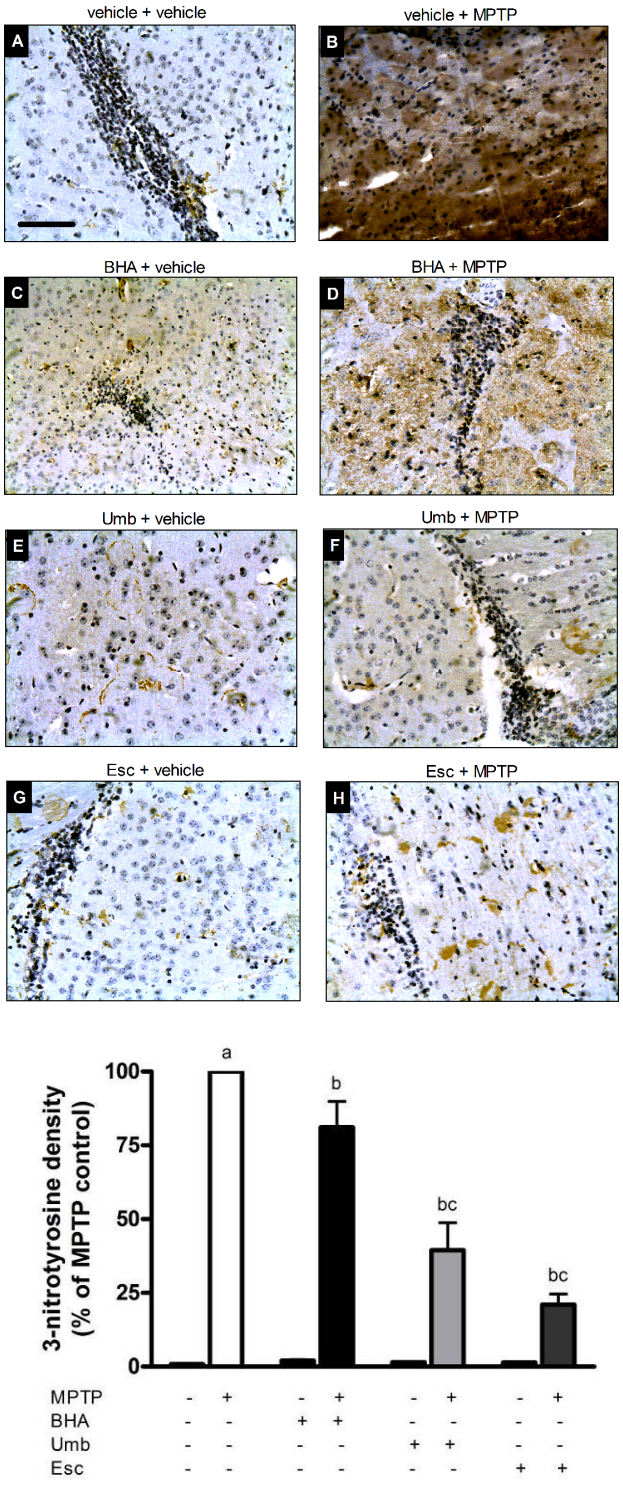


Fig. 5.14 - Effect of Umb and Esc on MPTP-induced peroxynitrite production in SNpc. (A) Representative photomicrographs of 3-nitrotyrosine (3-NT) staining from 3-6 mice for each group without (A, C, E, G) or with (B, D, F, H) MPTP administration. Scale bar 200µM. (B) Densitometric analysis using NIH ImageJ software by measuring the mean threshold of 3-NT immunostaining from 3 images. Results are expressed as the mean ± SD of 3-6 mice. $P<0.05$ compared to vehicle without MPTP treatment group (a), vehicle with MPTP treatment group (b) and BHA with MPTP group (c).

5.3.9 Effect of Umbelliferone and Esculetin on MPTP-induced decrease in glutathione in brain

Glutathione (GSH) is essential for the protection of cells from oxidative stress and xenobiotics, in addition to maintaining the thiol redox status. MPTP, which induces mitochondrial dysfunction leading to increase in oxidative stress, causes depletion of intracellular GSH. The aim of this experiment was to test whether Umb and Esc could prevent GSH depletion following MPTP treatment.

To examine the concentrations of total GSH in the nigrostriatal regions of mice, enzymatic GSH assay was carried out. Mice injected with MPTP showed a significant decrease in GSH levels by approximately 30% in striatum and 50% in midbrain compared to the vehicle treated animals (Fig 5.15). Treatment of mice with Umb and Esc completely prevented MPTP-induced striatal GSH depletion. In addition, in the midbrain Umb totally prevented GSH depletion while Esc reduced the loss by 50%. However, BHA failed to prevent the GSH decrease slump in both striatum and midbrain.

These results show that Umb and Esc act to reduce MPTP-induced GSH depletion in mouse striatum and midbrain.

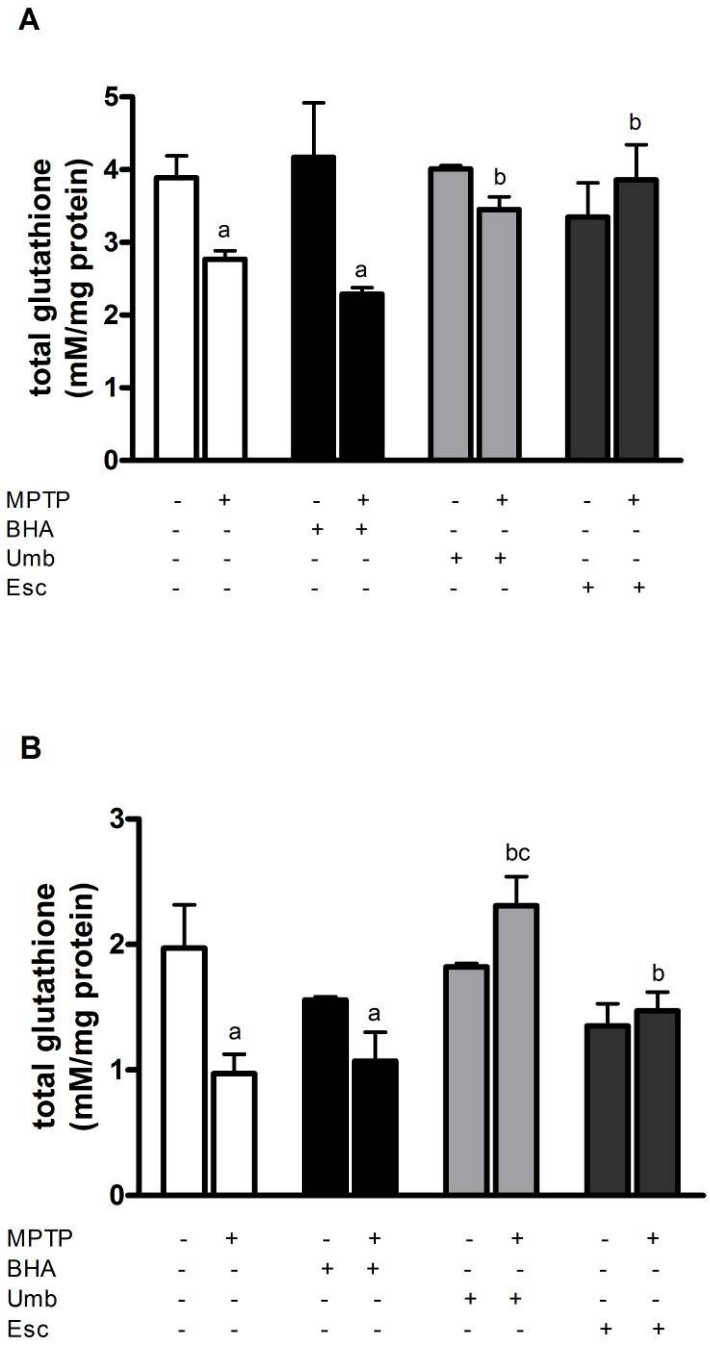


Fig. 5.15 - Effect of Umb and Esc on MPTP-induced decrease in glutathione levels in striatum (A) and midbrain (B). Mice were fed with normal diet or diet containing BHA (0.75% w/w) or Umb (0.5% w/w) or Esc (0.5%) for 7 days. Vehicle or MPTP (30 mg/kg), IP, was administered for five consecutive days to induce dopaminergic neurotoxicity. Mice were sacrificed and brains were isolated after the treatments and total glutathione was determined by enzymatic assay. Results are expressed as the mean ± SD of 3-6 mice. $P<0.05$ compared to vehicle without MPTP treatment group (a), vehicle with MPTP treatment group (b) and BHA with MPTP treatment group (c).

5.3.10 Effect of Umbelliferone and Esculetin on MPTP-induced apoptosis in the brain

MPTP has been reported to induce apoptosis in nigrostriatal neurons (Vila et al., 2001). The ability of Umb and Esc to prevent MPTP-induced neuronal apoptosis was investigated by determining the induction of Caspase 3 and Bax, and release of Cytochrome C from mitochondria into the cytosol.

MPTP treatment of mice showed up-regulation of Caspase 3 expression in striatum (2.9 fold) and midbrain (2.4 fold) (Fig. 5.16A, 5.17). An increase in Caspase 3 activity (1.3 fold) was observed in the striatum (Fig. 5.16B). Esc administration completely alleviated the elevated levels of Caspase 3 expression in striatum and midbrain. Umb showed significant decrease in Caspase 3 levels in the midbrain, however failed to attenuate MPTP-induced elevated Caspase 3 levels in striatum. BHA markedly reduced Caspase 3 levels in both striatum and midbrain. Treatment with Umb and Esc showed reduced Caspase 3 activities in striatum but no significant difference was observed in midbrain. The antioxidant BHA administration showed no significant decrease in Caspase 3 activity.

Mice administered with MPTP showed marked increase in pro-apoptotic Bax protein in both striatum (2.4 fold) and midbrain (7.1 fold) (Fig. 5.18). MPTP induced expression of Bax protein was completely attenuated by dietary administration of Umb and Esc in both striatum and midbrain. BHA treatment was 50% less effective than the coumarins in reducing Bax levels in the striatum, besides BHA failed to decrease Bax levels in midbrain. MPTP treated mice showed significantly high levels of Cytochrome C in the cytosolic fractions of striatum (19-fold) (Fig. 5.19). The release of Cytochrome C into cytosol from mitochondria was completely inhibited by dietary administration Umb, Esc and BHA. In contrast, midbrain cytosolic fractions did not show Cytochrome C release after MPTP treatment (data not shown).

These results show that the administration of Umb and Esc reduce MPTP-induced increase in pro-apoptotic Bax and Caspase 3 expression in mouse SNpc and striatum. The coumarins also completely reduced cytosolic Cytochrome C in the striatum.

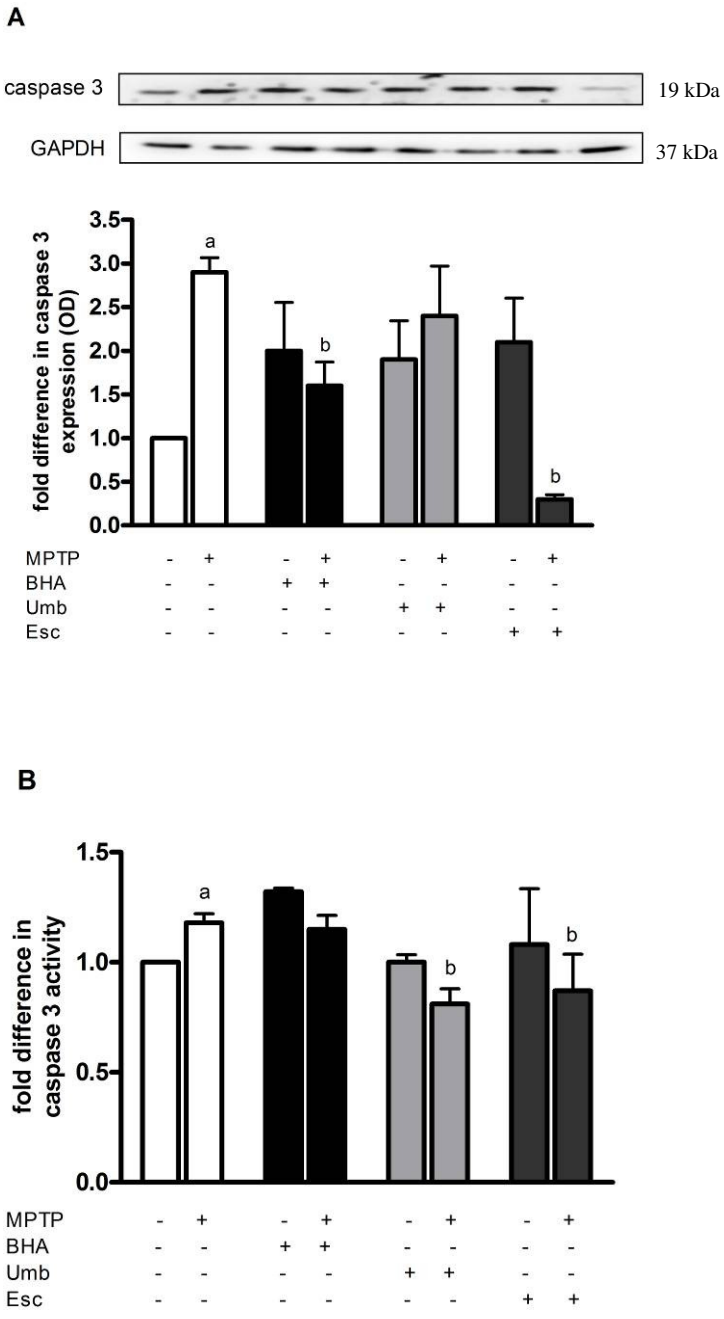


Fig. 5.16 - Effect of Umb and Esc on MPTP-induced increase in Caspase 3 levels (A) and caspase activity (B) in striatum. Mice were fed with normal diet or diet containing BHA (0.75% w/w) or Umb (0.5% w/w) or Esc (0.5%) for 7 days. Vehicle or MPTP (30 mg/kg), IP, was administered for five consecutive days to induce dopaminergic neurotoxicity. Mice were sacrificed and brains were isolated after the treatments. Brain homogenates were analyzed for Caspase 3 expression by Western blotting and activity was measured as described. Results are expressed as the mean ± SD of 3-6 mice. $P<0.05$ compared to vehicle without MPTP treatment group (a) and vehicle with MPTP treatment group (b).

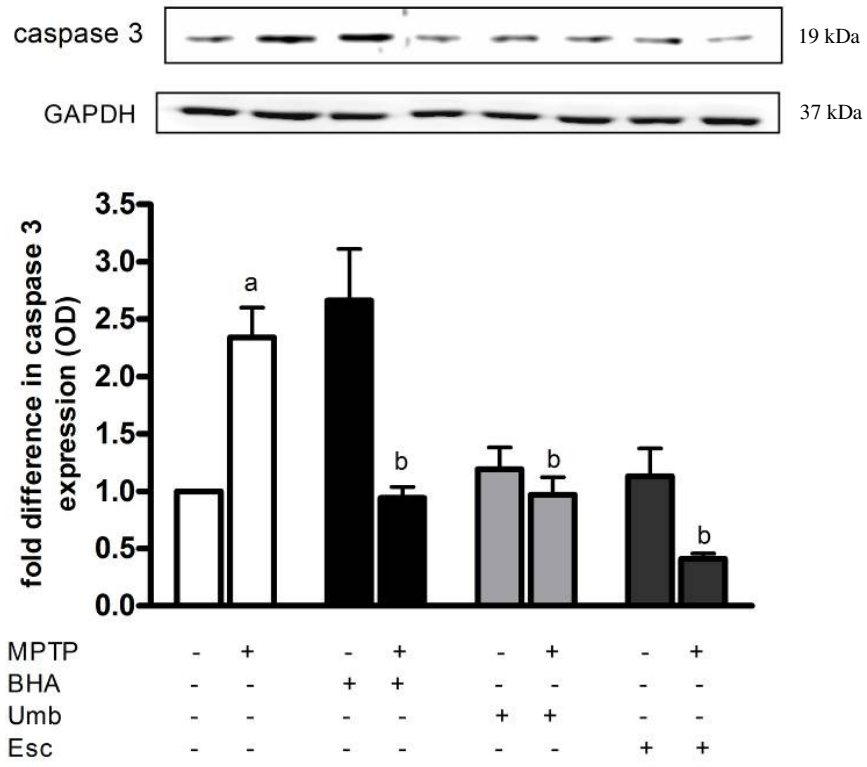


Fig. 5.17 - Effect of Umb and Esc on MPTP-induced increase in Caspase 3 levels in midbrain. Mice were fed with normal diet or diet containing BHA (0.75% w/w) or Umb (0.5% w/w) or Esc (0.5%) for 7 days. Vehicle or MPTP (30 mg/kg), IP, was administered for five consecutive days to induce dopaminergic neurotoxicity. Mice were sacrificed and brains were isolated after the treatments. Brain homogenates were analyzed for Caspase 3 expression by Western blotting. Histogram shows the densitometric analysis of Caspase 3 expression normalised to GAPDH by NIH ImageJ software. Results are expressed as the mean ± SD of 3-6 mice. $P<0.05$ compared to vehicle without MPTP treatment group (a) and vehicle with MPTP treatment group (b).

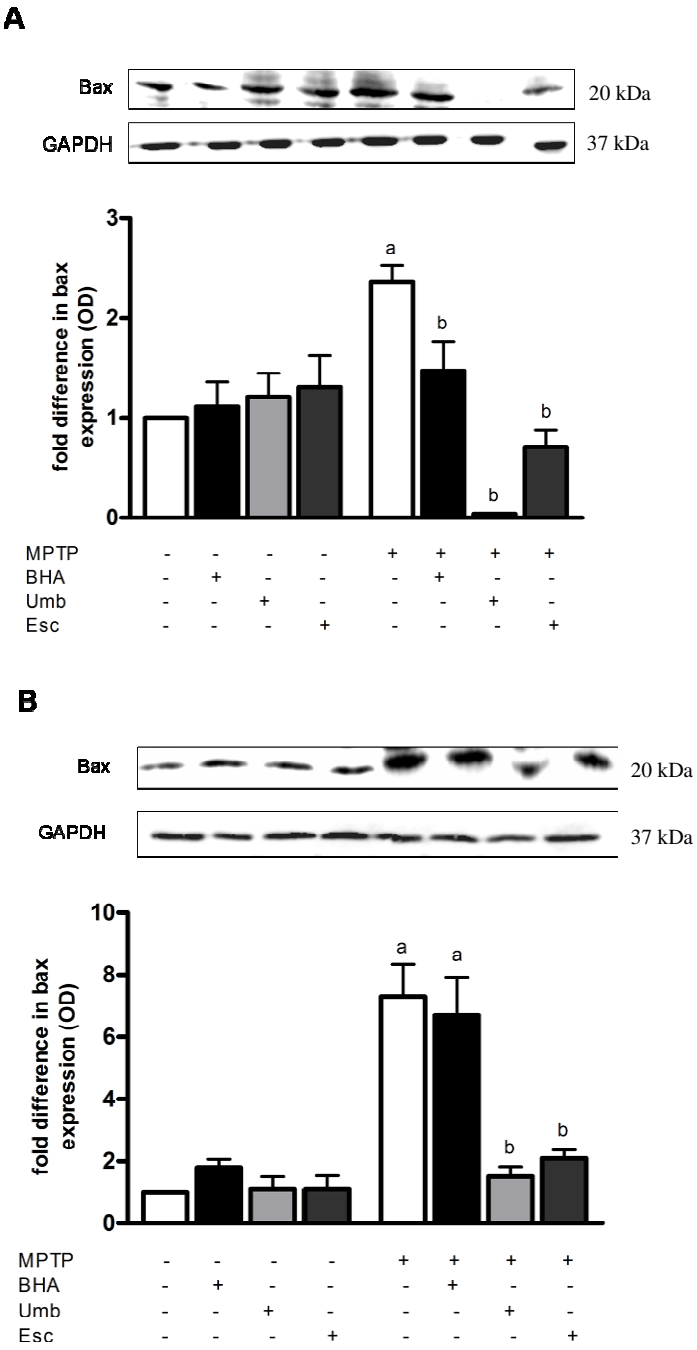


Fig. 5.18 - Effect of Umb and Esc on MPTP-induced increase in Bax levels in striatum (A) and midbrain (B). Mice were fed with normal diet or diet containing BHA (0.75% w/w) or Umb (0.5% w/w) or Esc (0.5%) for 7 days. Vehicle or MPTP (30 mg/kg), IP, was administered for five consecutive days to induce dopaminergic neurotoxicity. Mice were sacrificed and brains were isolated after the treatments. Brain homogenates were analyzed for Bax expression by Western blotting. Histograms show the densitometric analysis of Bax expression normalised to GAPDH by NIH ImageJ software. Results are expressed as the mean ± SD of 3-6 mice. $P<0.05$ compared to vehicle without MPTP treatment group (a) and vehicle with MPTP treatment group (b).

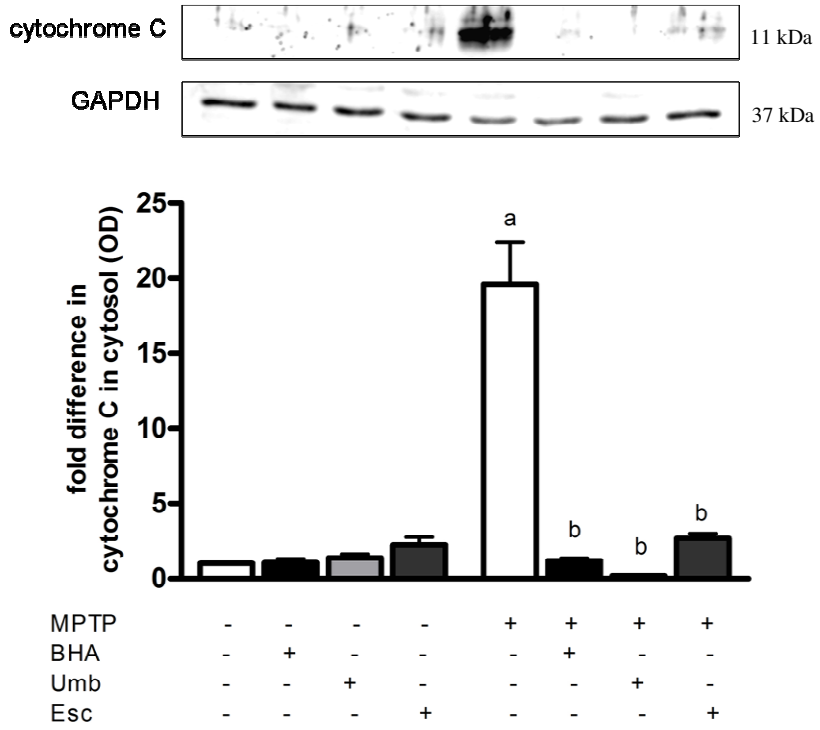


Fig. 5.19 - Effect of Umb and Esc on MPTP-induced release of Cytochrome C into cytosol in striatum. Mice were fed with normal diet or diet containing BHA (0.75% w/w) or Umb (0.5% w/w) or Esc (0.5%) for 7 days. Vehicle or MPTP (30 mg/kg), IP, was administered for five consecutive days to induce dopaminergic neurotoxicity. Mice were sacrificed and brains were isolated after the treatments. Cytosolic fractions of brain homogenates were analyzed for Cytochrome C by Western blotting. No significant release was observed in midbrain (data not shown). Histogram shows the densitometric analysis of Cytochrome C expression normalised to GAPDH by NIH ImageJ software. Results are expressed as the mean ± SD of 3-6 mice. $P<0.05$ compared to vehicle without MPTP treatment group (a) and vehicle with MPTP treatment group (b).

5.3.11 Effect of Umbelliferone and Esculetin on MPTP-induced phosphorylation of c-Jun NH_2 terminal kinase (JNK)

The JNK signalling cascade has been implicated in MPTP-induced apoptotic neuronal cell death (Saporito et al., 2000). In this study, we examined the activation of JNK pathway by measuring phospho-JNK (p-JNK) levels in mouse brain.

Mice administered with MPTP increased phosphorylation of JNK in midbrain (2.5-fold) and striatum (21-fold) (Fig. 5.20). Dietary administration of Umb and Esc significantly inhibited the phosphorylation of JNK in striatum by 49% and 56% respectively, compared to MPTP treated control group; however BHA showed no decrease in the p-JNK levels. Further, in the midbrain Umb treatment reduced p-JNK levels up to 55%. In contrast, Esc and BHA administration to mice failed to decrease the elevated MPTP-mediated p-JNK levels.

These results indicate MPTP-induced activation of JNK is inhibited by Umb and Esc in SNpc, and Umb in midbrain of mice.

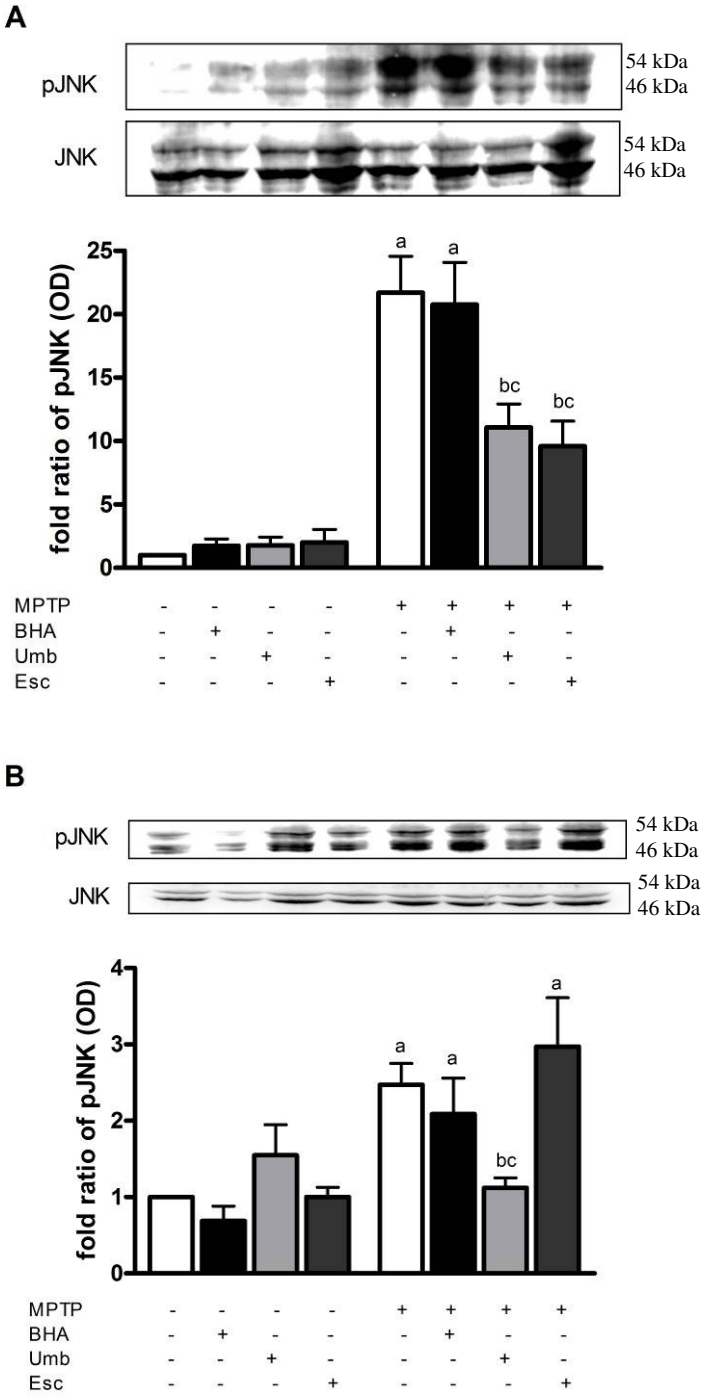


Fig. 5.20 - Effect of Umb and Esc on MPTP-induced phosphorylation of JNK in striatum (A) and midbrain (B). Mice were fed with normal diet or diet containing BHA (0.75% w/w) or Umb (0.5% w/w) or Esc (0.5%) for 7 days. Vehicle or MPTP (30 mg/kg), IP, was administered for five consecutive days to induce dopaminergic neurotoxicity. Mice were sacrificed and brains were isolated after the treatments. Brain homogenates were analyzed for pJNK and total JNK by Western blotting. Histograms show the densitometric analysis of pJNK normalised to total JNK by NIH ImageJ software. Results are expressed as the mean ± SD of 3-6 mice. $P<0.05$ compared to vehicle without MPTP treatment group (a), vehicle with MPTP treatment group (b) and BHA with MPTP treatment group (c).

5.3.12 Effect of Umbelliferone and Esculetin on induction and activity of antioxidant and protective enzymes

MPTP induces mitochondrial dysfunction leading to increase in generation of ROS. Phase II detoxifying and antioxidant enzymes may play an important role in countering elevated ROS by boosting the endogenous antioxidant system. In the present study, the potential of the coumarins, Umb and Esc, to induce the expression and activity of antioxidant enzymes NAD(P)H: quinone oxidoreductase 1 (NQO1) and glutathione S-transferase 3 (GSTP1) in mouse brain was investigated.

Dietary administration of Umb and Esc markedly induced the expression of NQO1 up to 2.2-fold and 2.7-fold, respectively, in midbrain but not in striatum (Fig. 5.21A, 5.22A). BHA increased NQO1 expression by 1.5-fold in midbrain but not in striatum. Similarly, mice consuming Umb (1.5-fold) and Esc (1.7-fold) diet showed a significant increase in GSTP1 expression in midbrain, although no such induction was observed in the striatum. BHA showed 1.4-fold induction of GSTP1 in midbrain but not in striatum (Fig. 5.21B, 5.23A).

Mice administered with MPTP showed an increase in expression of NQO1 (3.2-fold) and GSTP1 (1.4-fold) expression in the midbrain and not in striatum, compared to vehicle treated controls. Further, Umb and Esc treated mice maintained the elevated NQO1 and GSTP1 levels in midbrain after MPTP administration (Fig. 5.22A, 5.23A).

However, no significant changes in the enzymatic activities of NQO1 and GST were observed after Umb, Esc or BHA treatments (Fig. 5.22B, 5.23B).

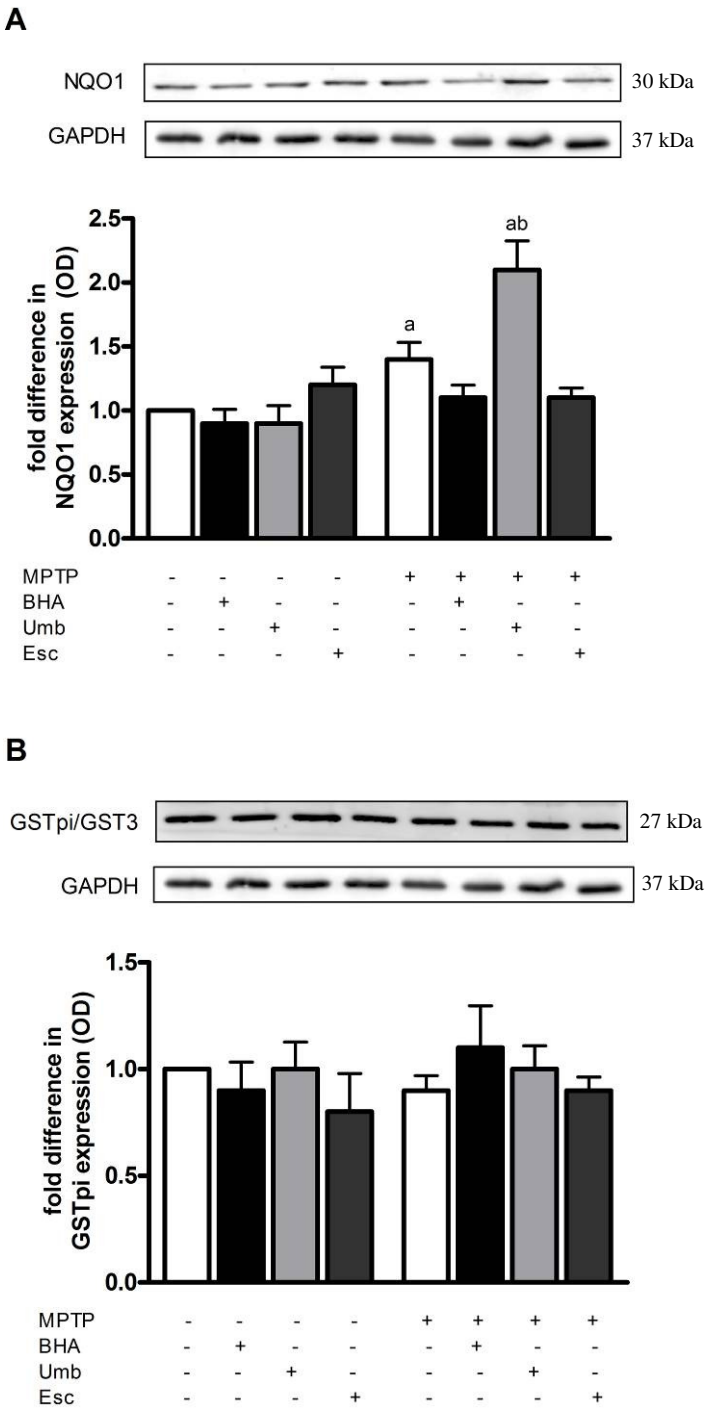


Fig. 5.21 - Effect of Umb and Esc on NQO1 (A) and GSTP1 (B) expression in striatum. Mice were fed with normal diet or diet containing BHA (0.75% w/w) or Umb (0.5% w/w) or Esc (0.5%) for 7 days. Vehicle or MPTP (30 mg/kg), IP, was administered for five consecutive days to induce dopaminergic neurotoxicity. Mice were sacrificed and brains were isolated after the treatments. Brain homogenates were analyzed for NQO1 and GSTP1 expression by Western blotting. Histograms show the densitometric analysis of protein expression normalised to GAPDH by NIH ImageJ software. Results are expressed as the mean ± SD of 3-6 mice. $P<0.05$ compared to vehicle without MPTP treatment group (a) and vehicle with MPTP treatment group (b).

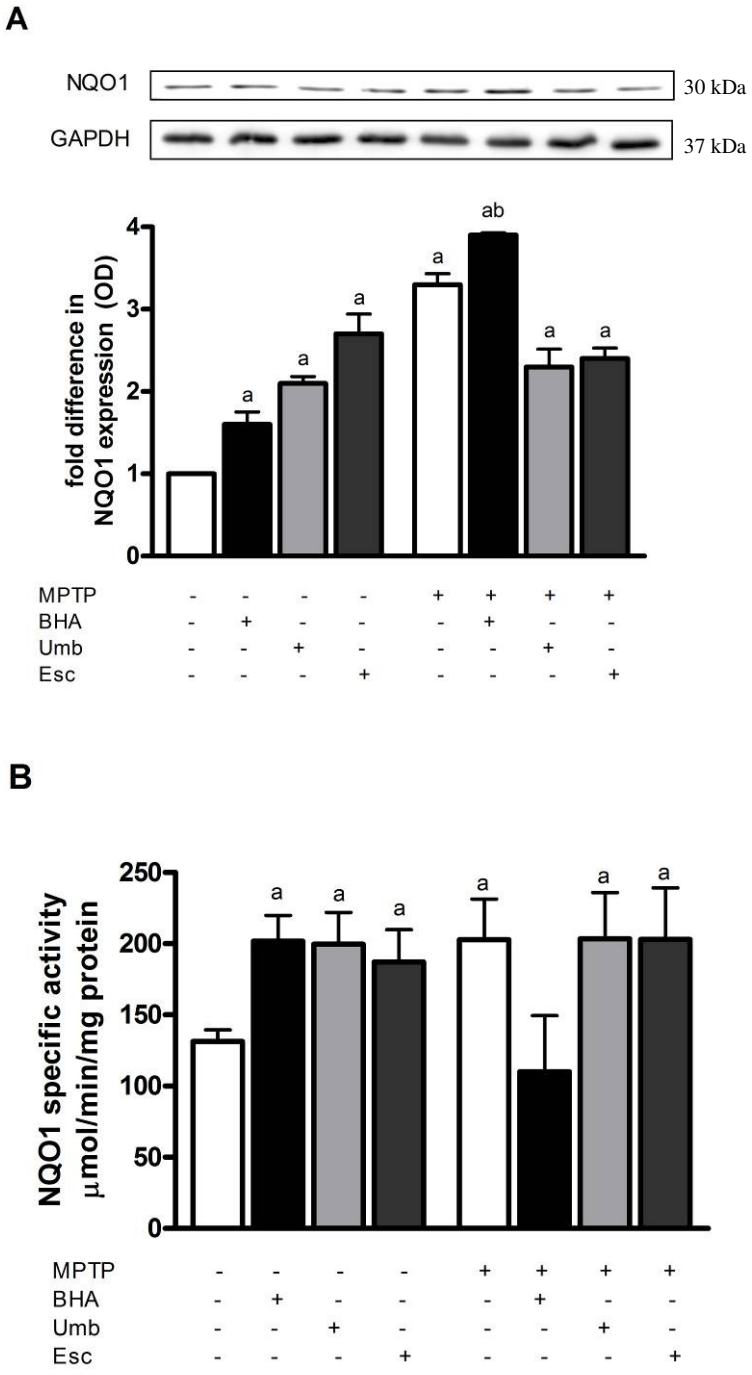


Fig. 5.22 - Effect of Umb and Esc on NQO1 expression (A) and activity (B) in midbrain. Mice were fed with normal diet or diet containing BHA (0.75% w/w) or Umb (0.5% w/w) or Esc (0.5%) for 7 days. Vehicle or MPTP (30 mg/kg), IP, was administered for five consecutive days to induce dopaminergic neurotoxicity. Mice were sacrificed and brains were isolated after the treatments. Brain homogenates were analyzed for NQO1 expression by Western blotting and activity by assay described. Histogram (A) shows the densitometric analysis of NQO1 expression normalised to GAPDH by NIH ImageJ software. Results are expressed as the mean ± SD of 3-6 mice. $P<0.05$ compared to vehicle without MPTP treatment group (a) and vehicle with MPTP treatment group (b).

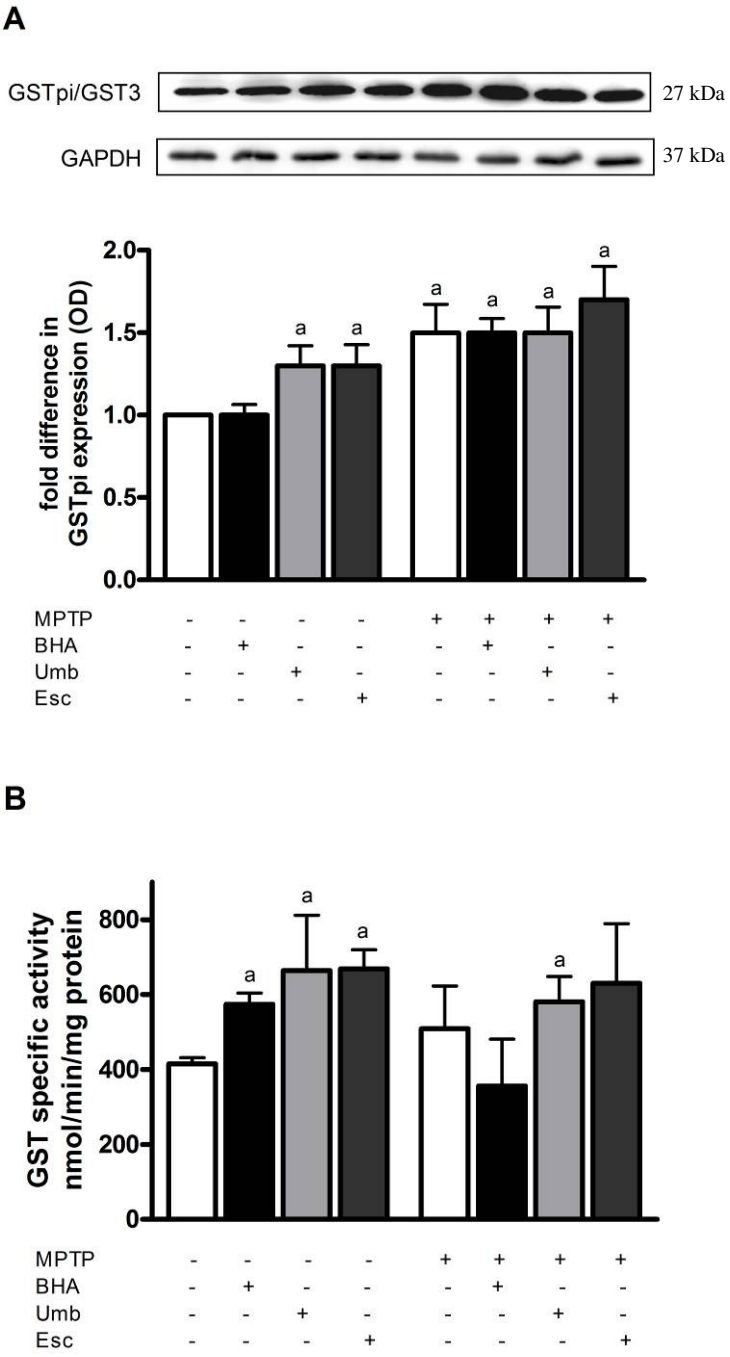


Fig. 5.23 - Effect of Umb and Esc on GSTP1 expression (A) and GST activity (B) in midbrain. Mice were fed with normal diet or diet containing BHA (0.75% w/w) or Umb (0.5% w/w) or Esc (0.5%) for 7 days. Vehicle or MPTP (30 mg/kg), IP, was administered for five consecutive days to induce dopaminergic neurotoxicity. Mice were sacrificed and brains were isolated after the treatments. Brain homogenates were analyzed for GSTP1 expression by Western blotting and GST activity by assay described. Histogram (A) shows the densitometric analysis of GSTP1 expression normalised to GAPDH by NIH ImageJ software. Results are expressed as the mean ± SD of 3-6 mice. $P<0.05$ compared to vehicle without MPTP treatment group (a).

5.4 Discussion

Oxidative stress has been implicated in initiating or aggravating various age-related disease conditions. In aging, oxidative stress increases due to steady depletion of the antioxidant defence mechanisms in the cell and consequently, aging and associated oxidative stress are major risk factors in several neurodegenerative diseases. Several studies suggest that mitochondrial dysfunction and resulting ROS/RNS generation, inflammation or toxic substances are the major factors in the oxidative stress-induced neuronal cell death that underlies various neurodegenerative disorders, including neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, Huntington's disease and amyotrophic lateral sclerosis. (Beal, 2007, Halliwell, 2006, Hensley et al., 2006, Tansey et al., 2008, von Bernhardi, 2007). In particular, previous studies show that mitochondrial dysfunction and the resulting ROS/RNS generation are major factors in the neuronal cell death that underlies Parkinson's disease (Thomas, 2009, Halliwell, 2006). Hence the strategies to mitigate oxidative stress are the principal focus of current therapeutic programs to alleviate the symptoms or postpone the progress of the disease.

In the work described in this chapter, the effects of esculetin on hydrogen peroxide-induced oxidative stress on SH-SY5Y neuroblastoma cells were studied. Hydrogen peroxide is known to cause damage to cell components, lipids in particular leading to lipid peroxidation, which subsequently produces ROS causing further damage to DNA, proteins and lipids (Esterbauer, 1993). The protective effects of umbelliferone and esculetin on MPP^+-induced neurotoxicity on SHSY-5Y cells were also investigated. MPP^+, the active metabolite of MPTP, is reported to cause increase in ROS (Rossetti et al., 1988) resulting in the neurodegeneration of dopaminergic neurons, reproducing many of the pathological and clinical features of Parkinson's disease (Bove et al., 2005).

In this chapter, results show that the coumarin, esculetin, has the potential to protect against hydrogen peroxide-induced oxidative stress in SH-SY5Y cell lines, and maintains cell viability as determined by MTT assay. Esculetin and umbelliferone

have been previously reported to scavenge a wide range of free radicals and possess potent antioxidant property (Lin et al., 2000, Paya et al., 1992a). The free radical scavenging activity of esculetin resulted in protection of the hamster lung fibroblasts (V79-4) from lipid peroxidation, protein carbonyl, and DNA damage induced by hydrogen peroxide (Kim et al., 2008). Esculetin has also been shown to protect SH-SY5Y neuroblastoma cells against dopamine-induced toxicity (Zhao et al., 2008). In contrast, the coumarins, umbelliferone and esculetin, did not prevent cell death when exposed to the neurotoxin MPP^+. These observations indicate that esculetin protected SH-SY5Y neuroblastoma cells from oxidative insult induced by hydrogen peroxide but failed to alleviate MPP^+-induced neurotoxicity in SH-SY5Y cells. It is worth noting that MPP^+-mediated toxicity to neuronal cells is not only due to elevated ROS levels but also through various mechanisms including depletion of ATP levels leading to the loss of plasma and mitochondrial membrane potential difference, resulting in neuronal cell death (Przedborski and Vila, 2003). Hence, the failure of Umb and Esc to protect SH-SY5Y against MPP^+ toxicity may be attributed to their inability to prevent neuronal cell death mediated through mechanisms other that increase in ROS levels.

In addition, dopaminergic neurotoxicity in mice was induced through MPTP; a neurotoxin which incites symptoms identical to PD. The study showed that MPTP treatment significantly decreased tyrosine hydroxylase levels in striatum and substantia nigra (SNpc) indicating that the neurotoxin causes dopaminergic neuronal cell death in mouse brain. MPTP reduced tyrosine hydroxylase levels to higher extent in striatum than SNpc suggesting that MPTP causes superior degenerative loss of striatal dopaminergic neurons compared to SNpc. Incidentally, Kurosaki and co-workers reported that MPTP induces degeneration of dopaminergic neurons in striatum faster than SNpc (Kurosaki et al., 2004). These results suggest that MPTP causes loss of nigrostriatal dopaminergic neurons, but the progression of striatal damage worsens by time in comparison to nigral neurons.

Dietary supplementation of umbelliferone and esculetin attenuated MPTP-induced decrease in tyrosine hydroxylase levels in SNpc but not striatum implying that the

coumarins prevented the loss of nigral neurons but failed to inhibit the loss of striatal neurons. These findings indicate that MPTP-induced damage in the striatum was progressive and relatively higher compared to the SNpc, and eventually the coumarins were unable to prevent neuronal cell death in the striatum. The ability of Umb and Esc to scavenge MPTP-mediated generation of ROS (Hoult and Paya, 1996, Lin et al., 2000), besides their capacity to cross the blood brain barrier (Sundt and Anderson, 1980, Barber et al., 2009) may contribute to the neuroprotective effect of these coumarins against dopaminergic neurodegeneration in mouse brain.

Glutathione (GSH) plays an important role in protecting against oxidative stress and maintaining the cellular thiol redox state in central nervous system (Dringen, 2000). GSH acts as antioxidant, enzyme cofactor, cysteine storage form and neuromodulator in CNS and its deficiency has been implicated in neurodegenerative diseases. Aging is a critical factor in GSH homeostasis and several lines of evidence suggests depletion of GSH with aging in brain (Maher, 2005). PD is characterised by decrease in concentrations of GSH in substantia nigra (Sian et al., 1994) and the severity of GSH depletion is parallel to that of its clinical severity (Sechi et al., 1996). The redox environment of the cell is maintained by glutathione and thioredoxin (Droge, 2002, Schafer and Buettner, 2001). A number of physiological functions are controlled by redox-responsive signalling pathways which include ROS-induced apoptosis, production of NO, regulation of immune response and cell adhesion, and other mechanisms (Droge, 2002). In this chapter, results show that the coumarins prevented glutathione depletion during hydrogen peroxide and MPP^+-induced oxidative stress in SH-SY5Y cell lines. In addition, the study on mice MPTP-model also reveals that MPTP causes marked decrease in GSH levels in the striatum and midbrain including SNpc. Dietary administration of Umb and Esc completely attenuated GSH depletion in striatum. Similarly, Umb treatment totally inhibited GSH reduction in midbrain though Esc significantly reduced the deficit. Previous studies show that elevated total intracellular glutathione levels indicate enhanced expression and/or activity of key enzymes involved in glutathione biosynthesis, such as the catalytic subunit of glutamate-cysteine ligase and glutathione synthase (Lu, 2009). These results suggest that the increase in total glutathione levels by the

coumarins in SH-SY5Y cells and mouse brain maintains the redox state of the cell thereby preventing them from further oxidative damage. In addition, it appears that Umb and Esc increase GSH biosynthesis through activation of key enzymes and thus enhance the endogenous antioxidant defence system.

Reactive oxygen species represent the most imperative reactive species generated in the biological system (Miller et al., 1990). Superoxide radical, one of the major ROS, is produced by electron leakage to oxygen prematurely during the energy transduction in the mitochondria and has been reported to be involved in the pathophysiology of various diseases (Kovacic et al., 2005, Valko et al., 2004). Evaluation of generation of ROS is a good indication of the intensity of oxidative stress inside the living cells. Previous evidence has suggested that umbelliferone and esculetin scavenge ROS, and thus help in preventing cells from oxidative damage such as lipid peroxidation, protein carbonyl and DNA damage (Kim et al., 2008). In the present study, umbelliferone and esculetin treatment decreased the generation of ROS in SH-SY5Y cells during hydrogen peroxide and MPP^+-induced oxidative stress. These results indicate that these coumarins can delay or prevent cells from oxidative injury by lowering ROS in SH-SY5Y cells.

Nitric oxide (NO), produced by nitric oxide synthase (NOS), is involved in variety of physiological and pathological mechanisms including cardiovascular and nervous systems, as well as cellular toxicity (Dawson et al., 1991, Dawson and Snyder, 1994). NO can combine with superoxide to form peroxynitrite, which in the presence of hydroxyl radicals can lead to nitration of proteins, primarily tyrosine residues (Beckman and Koppenol, 1996). Studies show that induction of iNOS (inducible NOS) in glial cell contributes to the toxicity of dopaminergic neurons in MPTP models of PD (Liberatore et al., 1999) and that inhibition of nNOS (neuronal NOS) prevents Parkinsonism in animal models (Hantraye et al., 1996, Dehmer et al., 2000). In the present study it was observed that MPTP treatment increased 3-nitrotyrosine, a marker for peroxynitrite production, in striatum and SNpc significantly. This increase in 3-nitrotyrosine was attenuated by Umb and Esc administration suggesting that these coumarins inhibit peroxynitrite production, hence protect nigrostriatal

dopaminergic neurons from peroxynitrite-induced toxicity. Our studies also reveal that Umb and Esc induce antioxidant enzymes such as NAD(P)H: quinone oxidoreductase 1 (NQO1) and glutathione S-transferase 3 (GSTP1) in mouse brain. The reported antioxidant properties of these coumarins (Hoult and Paya, 1996, Lin et al., 2000) and their ability to induce antioxidant enzymes might contribute to scavenge mitochondrial superoxide and thus, reducing MPTP-mediated peroxynitrite production.

Mounting evidence suggests that cell death-associated molecular mechanisms may participate in neuronal cell death in degenerative diseases (Rudin and Thompson, 1997, Pettmann and Henderson, 1998). MPP^+ is reported to inhibit mitochondrial respiratory pathway resulting in ATP depletion and ROS generation, which trigger cell death pathways within the intoxicated neurons (Jackson-Lewis et al., 1995, Tatton and Kish, 1997). The members of B-cell lymphoma 2 protein (Bcl-2) family including pro-apoptotic Bax (Vila et al., 2001) and anti-apoptotic Bcl-XL (Dietz et al., 2008) are reported to be involved in MPTP induced neuronal apoptosis. The core function of the Bcl-2 family seems to be the release of pro-apoptotic Cytochrome-c from mitochondria (Hengartner, 2000), increasing caspase-3 like activity in the cytosol resulting in apoptotic cell death (Du et al., 1997, Dodel et al., 1998). In the present work, treatment of SHSY-5Y cells with MPP^+ increased the expression of pro-apoptotic Bax and pre-treatment with umbelliferone and esculetin attenuated the elevation in Bax levels. In addition, the study shows that in mouse brain MPTP up-regulates the expression of pro-apoptotic Bax, increases cytosolic Cytochrome-c release and induces caspase-3 expression. Interestingly, treatment with Umb and Esc alleviated the elevation of Bax levels; inhibited Cytochrome-c release and decreased caspase-3 induction in mouse brain. Previously, antioxidant properties such as puerarin and (-)-deprenyl have been shown to prevent neurotoxicity by inhibiting apoptosis in neuronal cell lines and animals models by decreasing Bax expression (Zhu et al., 2010, Magyar and Szende, 2004). These observations indicate that these coumarins may be involved in inhibiting MPP^+-mediated increase in pro-apoptotic Bax expression and thus prevent apoptosis in SH-SY5Y cell lines and mouse brain.

Another pathway of apoptotic cell death in neurodegeneration involves the activation of c-Jun n-terminal kinase (JNK), a component of stress-activated protein kinase (SAPK) pathway (Ham et al., 1995, Xia et al., 1995, Eilers et al., 1998). MPTP is reported to be involved in phosphorylation of JNK in mice leading to dopaminergic degeneration (Saporito et al., 1999, Maroney et al., 1999, Saporito et al., 2000). Downstream targets for JNK that have been identified include death domain proteins p53, Bcl-2/Bcl-XL, Bax, Fas-ligand, Tau and caspase-1 (Seimiya et al., 1997, Herdegen et al., 1998, Zhang et al., 1998). In the present study it is shown that MPTP increases phosphorylation of JNK and the treatments with Umb and Esc reduced the p-JNK levels in mouse brain. In previous studies, similar reduction in the activation of JNK was observed in mice administered with antioxidant compounds including NAC and ginsenoside (Chen et al., 2005). These observations indicate that Umb and Esc protect dopaminergic cell death by inhibiting phosphorylation of JNK in MPTP mice models. It is possible that the regulation of Bcl-2 family members by these coumarins might be linked to the inhibition of JNK phosphorylation due to fact that JNK downstream targets include Bcl-2/Bcl-XL and Bax.

Previous studies suggest that MPP^+ can generate excessive mitochondrial superoxide by mitochondrial complex I inhibition which can destroy the cellular macromolecules in close proximity (Bates et al., 1994, Cleeter et al., 1992). Superoxide dismutase (SOD) catalyses the dismutation of superoxide radical and is present in three different forms in mammals: CuZnSOD (SOD1: mainly in cytosol), MnSOD (SOD2: located in mitochondria) and extracellular SOD (SOD3: localised in extracellular fluid) (Rohrdanz et al., 2002). In this chapter, it is shown that esculetin enhanced the expression of superoxide dismutase 1 (SOD1) in SH-SY5Y cells. These results suggest that esculetin induces the expression of the antioxidant protective enzyme SOD1, which can effectively prevent cell components from further oxidative damage. Although esculetin has been investigated previously for its chemoprotective potential (Sharma et al., 1994), this is the first time it has been shown that esculetin can induce expression of SOD1 in a neuronal cell line.

The induction of antioxidant enzyme defence systems is known to play an important role in protecting cells from oxidative damage (Hayes et al., 1999). The enhanced expression of enzymes such as NQO1, glutathione S-transferases and aldo-keto reductases can provide protection against chemical stress as well as reactive oxygen species as part of an adaptive response (MacLeod et al., Jaiswal, 2000). As mentioned before, NQO1 is an enzyme that catalyzes the reduction of quinones to hydroquinones, thereby preventing the one electron reduction of quinones that would otherwise produce reactive oxygen species within the cell (Vasiliou et al., 2006). Recently, we have shown that Esc protects HepG2 cells from hydrogen peroxide-induced oxidative stress by NRF2-dependent up-regulation of NQO1 gene (Subramaniam and Ellis, 2011). Alternatively, GSTP1 is an isoenzyme belonging to the GST family, which catalyzes the nucleophilic attack by reduced glutathione on non-polar compounds that contain an electrophilic carbon, nitrogen or sulphur atom resulting in detoxification. In this study, it is shown that Umb and Esc administration up-regulates expression of NQO1 and GSTP1. These results indicate that the induction of these protective enzymes by the coumarins reduce oxidative stress caused by the MPTP-induced mitochondrial dysfunction. In addition, up-regulation of NQO1 and GSTP1 by coumarins might also decrease the elevated peroxynitrite production and maintain cellular redox homeostasis in MPTP mouse model.

5.5 Conclusions

In conclusion, it is shown that the coumarin compounds, umbelliferone and esculetin, present in a range of fruit, vegetables and herbs can reduce hydrogen peroxide and neurotoxin MPP^+-induced toxicity in SH-SY5Y cells and C57BL/6J mouse striatum and SNpc. The neuroprotective effects of Umb and Esc were through several mechanisms including inhibition of ROS generation, reduction of peroxynitrite production and glutathione depletion, reduction of apoptotis biomarkers and induction of the protective antioxidant enzymes NQO1 and GSTP1 (Fig 7.2). In addition, the coumarins decrease MPTP-induced apoptosis by regulating Bcl-2 family members and blocking activation of JNK in mouse brain. These results also

indicate that these coumarin derivatives possess neuroprotective properties that do not appear to be solely dependent on its free radical scavenging activity. Umbelliferone and Esculetin at low non-toxic concentrations therefore have the potential to provide protection against oxidative stress-related neurodegenerative diseases.

CHAPTER 6

UP-REGULATION OF NRF2/ARE-DEPENDANT PROTECTIVE ENZYMES BY DIETARY SUPPLEMENTATION OF COUMARINS IN MOUSE AND RAT

6. Up-regulation of NRF2/ARE-dependant Protective Enzymes by Dietary Supplementation of Coumarin Compounds in Mouse and Rat: Possible Role in Protection against Chronic Diseases with an Underlying Oxidative Stress

6.1 Introduction

Agents that are produced within the biological system, which delay, prevent or remove oxidative damage are referred to as endogenous antioxidants. They include usually enzymes, metal sequestering agents and low molecular mass compounds produced *in vivo*. Enzymes catalytically remove xenobiotics, oxidants and toxins in the biological system. These include superoxide dismutase (SOD) (Fridovich, 1995), catalase (Chance et al., 1979), glutathione peroxidise family (Chance et al., 1979, Brigelius-Flohe, 1999), NAD(P)H: oxidoreductases (Nioi and Hayes, 2004, Jaiswal, 2000), heme oxygenases (HOs) (Prestera et al., 1995), glutathione S-transferases (GSTs) (Rushmore and Pickett, 1990), aldo-keto reductase family (AKRs) (Ellis et al., 2003), γ-glutamylcysteine synthase (γ-GCS) (Wild and Mulcahy, 1999), other peroxidases such as Cytochrome C peroxidase (CCP) and NADH oxidase (Halliwell B., 2007).

Although some of these enzymes are constitutive, the enhanced expression of inducible enzymes such as NQO1, glutathione S-transferases and aldo-keto reductases can provide protection against chemical stress as well as reactive oxygen species as part of an adaptive response. NAD(P)H: quinone oxidoreductase (NQO1) leads to detoxification of quinones and its derivatives leading to protection of cells against oxidative stress and redox cycling. NQO1 gene expression is induced along with other detoxifying enzyme genes in response to antioxidants and other agents, and prevents against oxidative damage (Nioi and Hayes, 2004, Jaiswal, 2000). Heme oxygenase (HO) mediates the catalytic conversion of heme to biliverdin, which is enzymatically reduced to bilirubin (Prestera et al., 1995). The aldo-keto reductase (AKR) superfamily consists of enzymes that are induced by antioxidants and is

implicated in detoxification or bioactivation of wide variety of carbonyl-bearing compounds (MacLeod et al., Jaiswal, 2000). Alternatively, glutathione S-transferase family catalyzes the nucleophilic attack by reduced glutathione on non-polar compounds that contain an electrophilic carbon, nitrogen or sulphur atom resulting in detoxification (Hu et al., 2006, Nair et al., 2006). Several members of GST-family are reported to be induced by antioxidants for protection against oxidative injury (Hayes et al., 2000).

6.2 Aim of the chapter

The work described in previous chapters has shown that coumarin compounds induce the expression of antioxidant and detoxifying enzymes via the NRF2-ARE pathway. The aim of the work describe in this chapter is to investigate the effect of dietary consumption of the simple coumarins on the up-regulation and activity of these antioxidant protective enzymes in key organs such as liver, brain and kidney *in vivo* in mouse and rat.

6.3 Materials and Methods

6.3.1 Animals and Drug Treatment

Male Wistar 10-week old rats and male C57BL/6J 6-week old mice were used for this study. Animals were allowed to acclimatize for 2 weeks before being fed either a normal diet (powdered RM1 diet, Special Diet Services, UK) supplemented with 2% arachis oil or diet containing test compounds with 2% arachis oil. Arachis oil was used as a vehicle for the uniform distribution of test compounds. The test compounds were provided in the diet for 7 days in the following amounts: BHA, 0.75% (w/w); Umbelliferone, 0.5% (w/w); Esculetin, 0.5% (w/w); Fraxetin, 0.5% (w/w); and Scopoletin, 0.5% (w/w). During the experiment the animals were provided free access to food and water.

6.3.2 Tissue Harvesting

After the treatment period the animals were sacrificed by cervical dislocation and organs such as brain, liver and kidney were removed immediately, snap-frozen in liquid nitrogen and stored at -80^{o}C until analysis.

All other materials and methods for this chapter are described in chapter 2.

6.4 Results

6.4.1 Coumarins induce antioxidant protective enzymes in rat liver

The induction of antioxidant protective enzymes levels in rat and mouse by coumarins was investigated by immunoblotting using specific antibodies. After dietary administration of coumarins compounds for 7-days, the liver was removed, homogenised and extracts separated by SDS-PAGE before blotting to nitrocellulose membranes as described in Chapter 2. Results in figure 6.1A show that in rats the expression of NQO1 is significantly up-regulated in liver compared to vehicle treated control. All the compounds tested, Umb (3.7-fold), Esc (2.8-fold), Fra (3.9-fold) and Sco (5-fold), induced NQO1 to a similar extent to the known chemopreventive agent, BHA (6.2-fold).

In addition, rats consuming a diet supplemented with coumarin compounds, increased hemeoxygenase 1 expression by 2.8-fold (Umb), 3.2-fold (Esc), 3.5-fold (Fra) and 2.4-fold (Sco). This level of induction was similar to that observed for BHA (3.4-fold) in rat liver (Fig. 6.1B).

In addition, the dietary consumption of coumarins induced expression of protective enzymes of the AKR family including AKR1A3, AKR7A1 and AKR7A4. AKR1A3 was induced by 1.6-fold (Umb), 1.3-fold (Esc) and 1.9-fold (Fra) in the liver of rats. Sco failed to induce AKR1A3 whereas BHA treatment showed a higher induction (3.2-fold) (Fig. 6.1C). Only Sco (1.6-fold) showed a significant increase in AKR7A1

expression while BHA exhibited a 2-fold induction (Fig. 6.1D). AKR7A4 levels were significantly increased by Umb (3-fold), Esc (2.3-fold) and Sco (3.1-fold) slightly to a lesser extent compared to BHA (5-fold) (Fig. 6.1E).

6.4.2 Effect of coumarin compounds on NQO1, GST, AKR and GRed enzyme activity in rat liver

The effect of dietary intake of coumarins compounds on activity of antioxidant protective enzymes in rat liver was also investigated using enzyme assays as described in chapter 2. Generally consumption of coumarins compounds Umb, Fra and Sco increased the activity of NQO1 to 39, 43 and 41 µmol/min/mg protein, respectively compared to vehicle treated control (22 µmol/min/mg protein). However, BHA treatment (83 µmol/min/mg protein) showed a much higher activity (Fig 6.2A).

Moreover, the GST activity in the liver of rats receiving Esc, Fra and Sco increased to 220, 230 and 320 nmol/min/mg protein, respectively compared to vehicle treatment group (100 nmol/min/mg protein). BHA treatment group showed an increase up to 300 nmol/min/mg protein, although Umb receiving rats showed no significant difference in GST activity over 7 days (Fig 6.2B).

AKR activity in the liver of rats treated with coumarin compounds, Umb (42 nmol/min/mg protein), Esc (31 nmol/min/mg protein), Fra (23 nmol/min/mg protein) and Sco (29 nmol/min/mg protein) increased significantly compared to vehicle treatment group (6 nmol/min/mg protein) (Fig 6.2C).

Further, GRed activity in the liver of rats treated with the test compounds significantly increased compared to vehicle treated control group. The coumarins Umb, Esc, Fra and Sco showed an activity of 3.7, 7.0, 3.8 and 6.0 µmol/min/mg protein respectively with the control group exhibiting 1.1 µmol/min/mg protein

activity. In contrast, GRed activity was markedly decreased after BHA treatment (Fig 6.2D).

6.4.3 Coumarins increase GSH levels in rat liver

To investigate the effect of coumarins in modulation GSH levels, total GSH levels in rat liver were determined by enzymatic recycling assay using DTNB as described in the methods chapter.

Dietary administration of Umb and Sco significantly increased glutathione levels in rat liver by 1.5-fold and 1.6-fold to 36 and 40 nmol/µg protein, respectively compared to vehicle treated control (24 nmol/µg protein). BHA, a known chemoprotective agent, also showed elevated levels of GSH (42 nmol/µg protein). Surprisingly, Esc and Fra showed no increase in GSH levels (Fig. 6.3)

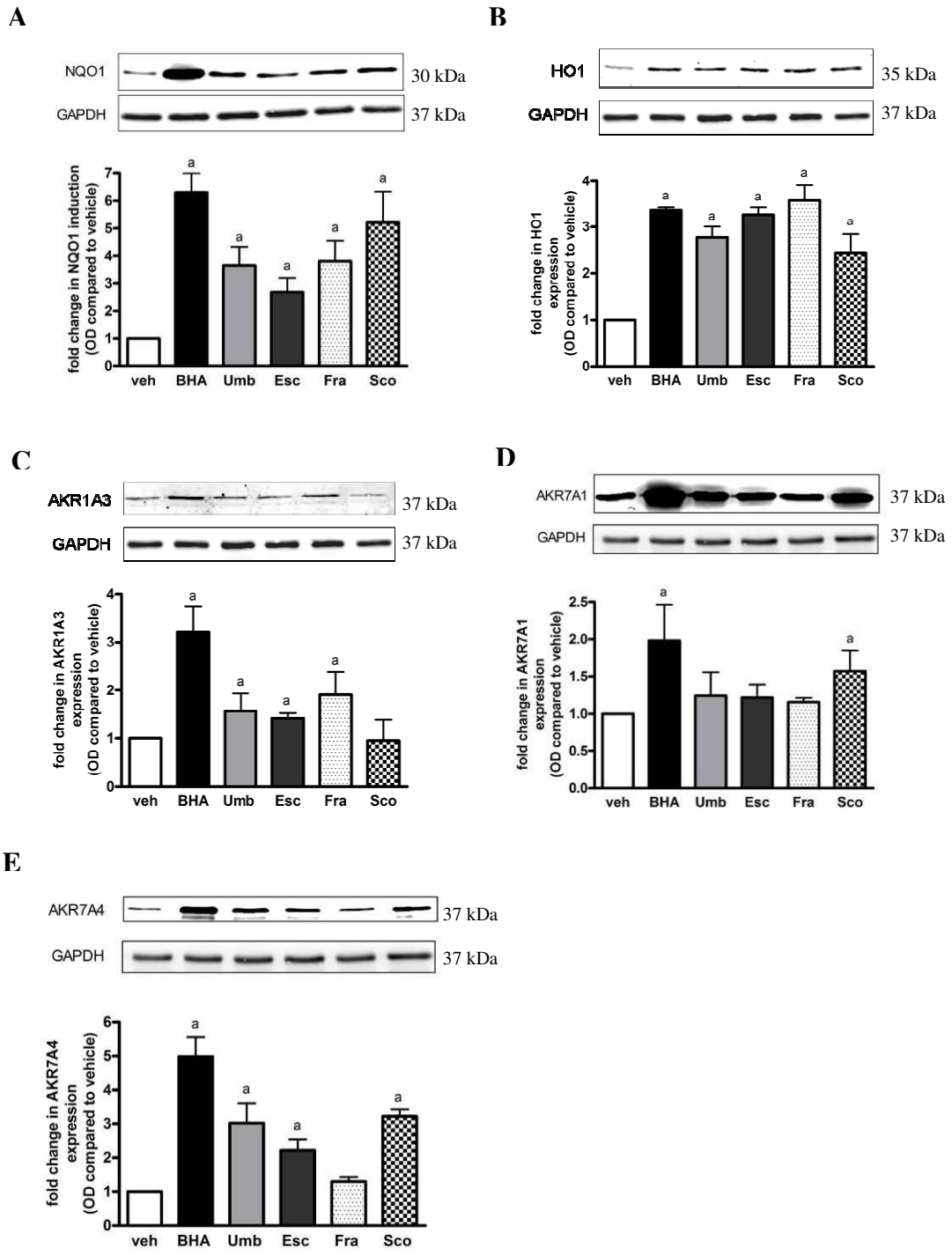


Fig. 6.1 - Coumarin compounds up-regulate the expression of antioxidant protective enzymes in rat liver. Rats were fed with vehicle (2% v/w arachis oil) or BHA (0.75%) or Umb (0.5%) or Esc (0.5%) or Fra (0.5%) or Sco (0.5%) in vehicle for 7 days. Animals were euthanized and livers were immediately frozen on dry ice and stored at -80^{o}C. Liver homogenates were analysed for NQO1 (A), HO1 (B), AKR1A3 (C), AKR7A1 (D) and AKR7A4 (D) by Western blotting. Histograms show the densitometric analysis of protein expression normalised to GAPDH by NIH ImageJ software. Results are expressed as the mean ± SD of 3-6 rats. $P<0.05$ compared to vehicle treatment group (a).

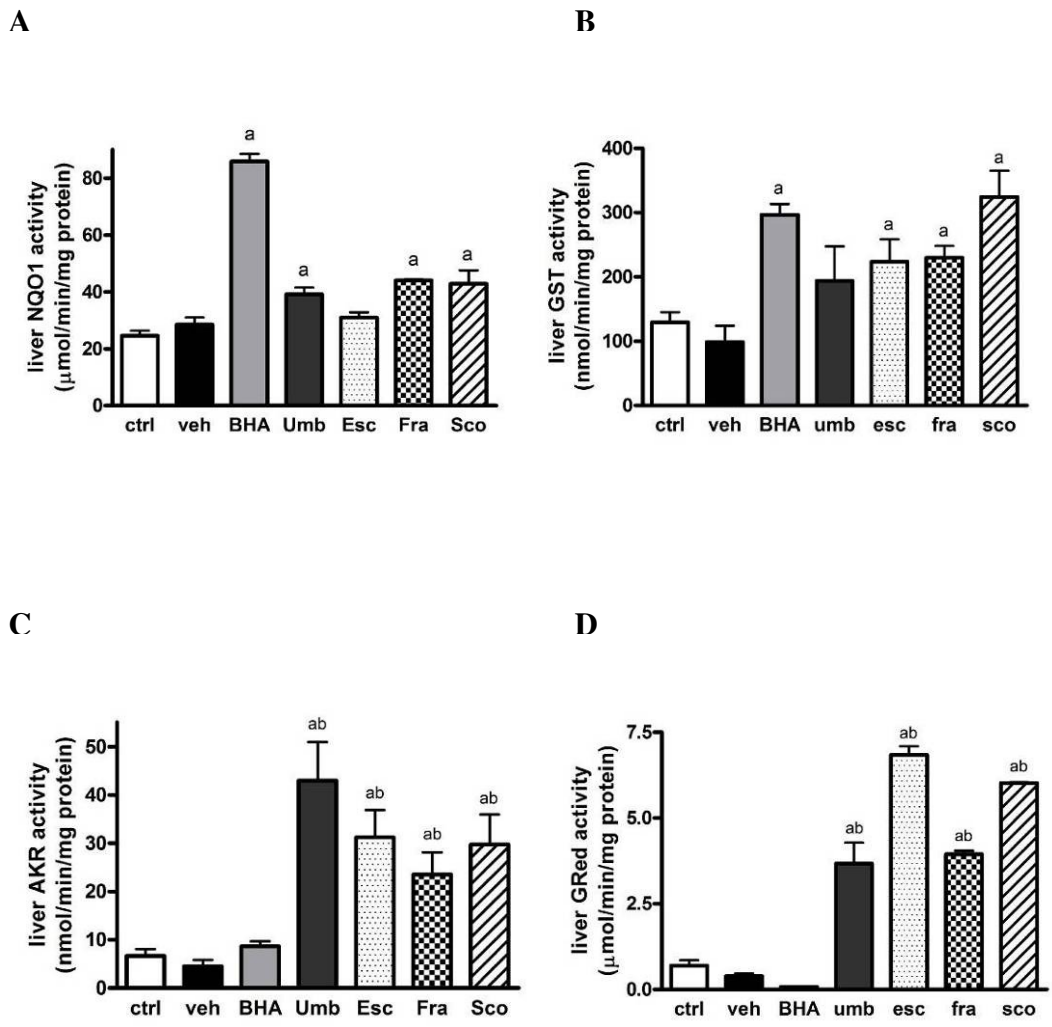


Fig. 6.2 - Coumarin compounds increase the activity of antioxidant protective enzymes in rat liver. Rats were fed with vehicle (2% v/w arachis oil) or BHA (0.75%) or Umb (0.5%) or Esc (0.5%) or Fra (0.5%) or Sco (0.5%) in vehicle for 7 days. Animals were euthanized and livers were immediately frozen on dry ice and stored at -80^{o}C. Liver homogenates were analysed for enzyme activity of NQO1 (A), GST (B), AKR (C) and GRed (D) by methods mentioned in chapter 2. Results are expressed as the mean ± SD of 3-6 mice. $P<0.05$ compared to vehicle treatment group (a) and BHA treatment group (b).

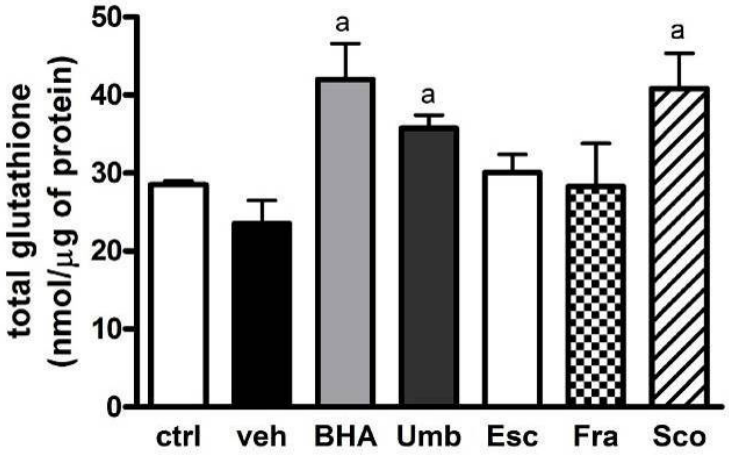


Fig. 6.3 - Coumarin compounds increase GSH levels in rat liver. Rats were fed with vehicle (2% v/w arachis oil) or BHA (0.75%) or Umb (0.5%) or Esc (0.5%) or Fra (0.5%) or Sco (0.5%) in vehicle for 7 days. Animals were euthanized and livers were immediately frozen on dry ice and stored at -80^{o}C. Liver homogenates were analysed for GSH levels as described in chapter 2. Results are expressed as the mean ± SD of 3-6 rats. $P<0.05$ compared to vehicle treatment group (a).

6.4.4 Coumarins induce antioxidant protective enzymes in rat brain

The induction of antioxidant protective enzymes by dietary administration of coumarin compounds in rat brain was examined by immunoblotting. The coumarins enhanced the levels of NQO1 in rat brain significantly. Compared to vehicle treated control group the rats administered with Umb, Esc and Fra showed 2.4-fold, 3.6-fold, and 3.7-fold increase in NQO1 expression respectively. The chemoprotective compound BHA showed a 2.7-fold induction, but Sco failed to show any significant increase in NQO1 levels in rat brain (Fig. 6.4A).

The levels of HO1 in rat brain was significantly increased by consumption of Umb, Esc, Fra and Sco for 7-days by 5-fold, 7-fold, 7-fold and 5-fold respectively, compared to vehicle treated control rats. However BHA failed to increase HO1 levels in rat brain (Fig. 6.4B).

Similarly, GSTY'8c/GSTA3 expression in rat brain was enhanced by Umb, Esc, Fra and Sco consumption for 7-days by 2.4-fold, 2.3-fold, 2.3-fold and 2-fold respectively. This is in contrast to BHA which did not show an increase in GSTY'8c/GSTA3 levels in rat brain (Fig. 6.4C).

In addition, the induction of enzymes belonging to the AKR family in rat brain was investigated. Umb (1.5-fold), Esc (1.7-fold) and Fra (1.6) treatment of rats showed an increase in AKR1A3 levels compared to vehicle treated control group. BHA exhibited a 1.3-fold induction. AKR7A1 expression was increased by Esc (1.4-fold), Sco (1.5-fold) and BHA (1.7-fold) administration. Further the levels of AKR7A4, another member of the AKR family, were increased significantly only by Esc (1.3-fold). In comparison, BHA showed 1.7-fold elevation in AKR7A4 levels in rat brain (Fig. 6.4D, 6.4E, 6.4F).

6.4.5 Effect of coumarin compounds on GST activity in rat brain

Consumption of coumarin compounds Umb, Esc and Fra by rats for 7-days showed a significant increase brain GST activity to 1.3, 2.3, 0.7 μmol/min/mg protein respectively compared to vehicle treated control rats (0.4 μmol/min/mg protein). However, BHA administration to rats did not show any elevation in brain GST activity (Fig. 6.5).

6.4.6 Coumarins induce antioxidant protective enzymes in rat kidneys

The kidney is another organ that is involved in detoxication and maintenance of homeostasis. The levels of NQO1, GST and AKR enzymes were measured in rat kidney after dietary consumption of coumarin compounds for 7 days.

NQO1 expression in rat kidney was significantly increased by dietary administration of Umb (2.5-fold), Esc (3.7-fold), Fra (3.8-fold) and Sco (3.2-fold) compared to vehicle treated control group. All the tested coumarin compounds elevated NQO1 levels to a greater extent than the known chemoprotective compound BHA (1.5-fold) (Fig. 6.6A).

In addition, Esc and Fra treatment of rats slightly elevated the levels of GSTY'8c/GSTA3 enzyme to 1.3-fold and 1.2-fold respectively. BHA showed an increase of GSTY'8c/GSTA3 expression to 1.4 fold (Fig. 6.6B).

Further, AKR7A4 expression in rat kidneys were increased by Umb (1.5-fold), Esc (1.7-fold) and Sco (2.1-fold) treatment in comparison to vehicle treated control. The coumarins showed a similar capacity as BHA (1.6-fold) to induce AKR7A4 expression in rat kidneys (Fig. 6.6C).

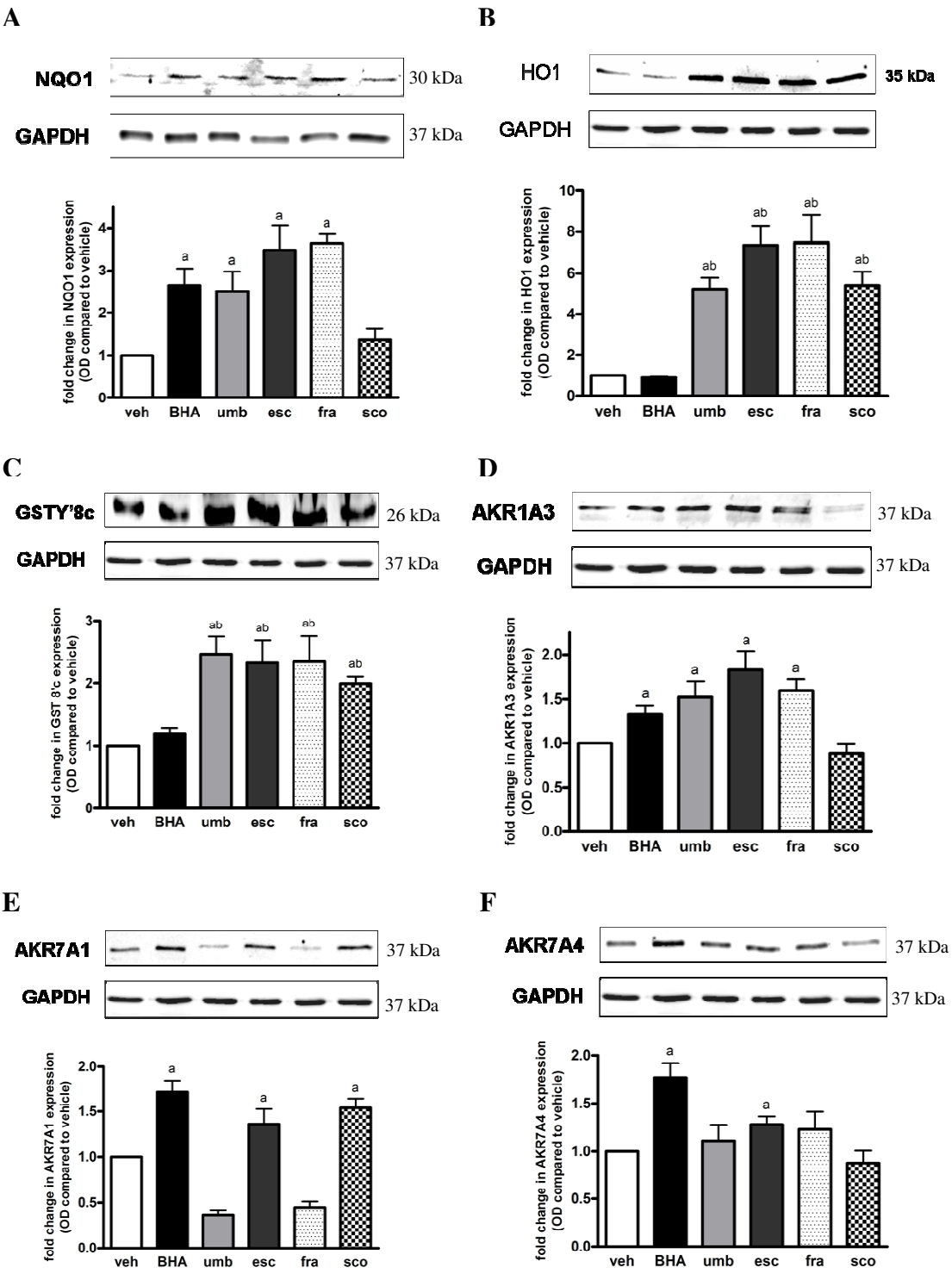


Fig. 6.4 - Coumarin compounds up-regulate the expression of antioxidant protective enzymes in rat brain. Rats were fed with vehicle (2% v/w arachis oil) or BHA (0.75%) or Umb (0.5%) or Esc (0.5%) or Fra (0.5%) or Sco (0.5%) in vehicle for 7 days. Animals were euthanized and brains were immediately frozen on dry ice and stored at -80°C. Brain homogenates were analysed for NQO1 (A), HO1 (B), GSTY'8C/GSTA3 (C), AKR1A3 (D), AKR7A1 (E) and AKR7A4 (F) by Western blotting. Histograms show the densitometric analysis of protein expression normalised to GAPDH by NIH ImageJ software. Results are expressed as the mean ± SD of 3-6 rats. P<0.05 compared to vehicle treatment group (a) and BHA treatment group (b).

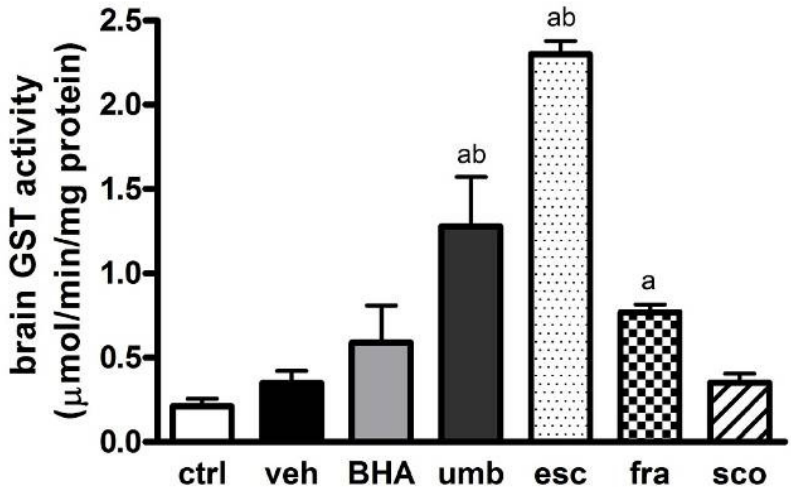


Fig. 6.5 - Coumarin compounds increase the activity of antioxidant protective enzymes in rat brain. Rats were fed with vehicle (2% v/w arachis oil) or BHA (0.75%) or Umb (0.5%) or Esc (0.5%) or Fra (0.5%) or Sco (0.5%) in vehicle for 7 days. Animals were euthanized and brains were immediately frozen on dry ice and stored at -80^{o}C. Brain homogenates were analysed for GST activity as mentioned in chapter 2. Results are expressed as the mean ± SD of 3-6 mice. $P<0.05$ compared to vehicle treatment group (a) and BHA treatment group (b).

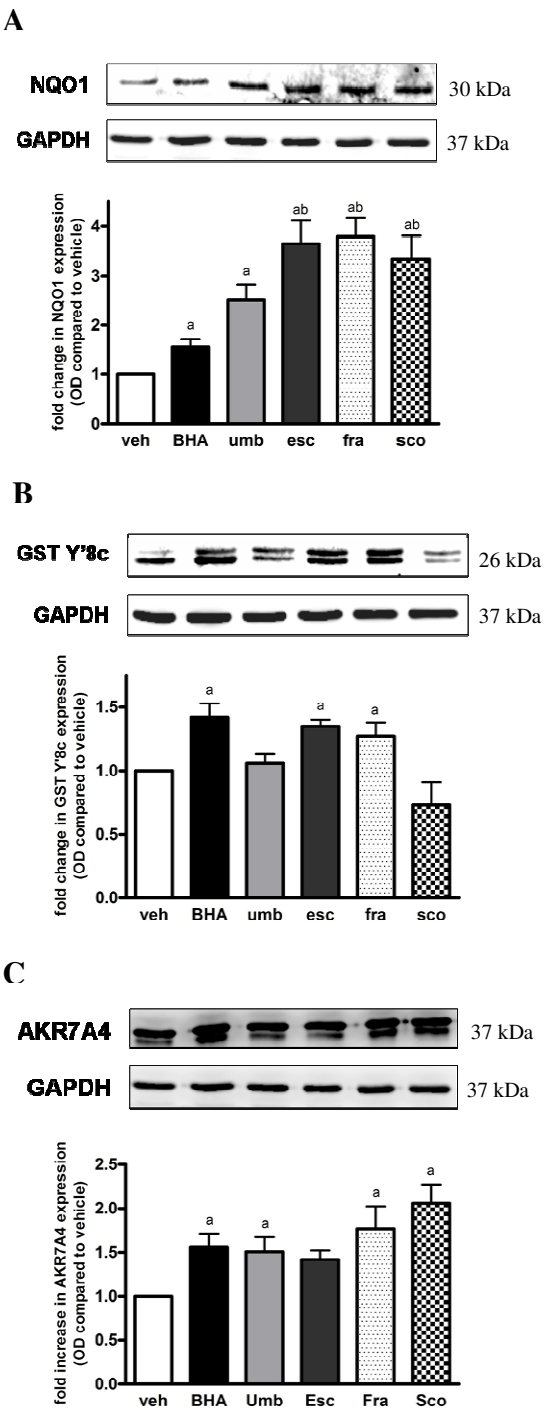


Fig. 6.6 - Coumarin compounds up-regulate the expression of antioxidant protective enzymes in rat kidneys. Rats were fed with vehicle (2% v/w arachis oil) or BHA (0.75%) or Umb (0.5%) or Esc (0.5%) or Fra (0.5%) or Sco (0.5%) in vehicle for 7 days. Animals were euthanized and kidneys were immediately frozen on dry ice and stored at -80^{o}C. Kidney homogenates were analysed for NQO1 (A), GSTY'8C/GSTA3 (B), and AKR7A4 (C) by Western blotting. Histograms show the densitometric analysis of protein expression normalised to GAPDH by NIH ImageJ software. Results are expressed as the mean ± SD of 3-6 rats. $P<0.05$ compared to vehicle treatment group (a).

6.4.7 Coumarins induce antioxidant protective enzymes in mouse midbrain

The level of the antioxidant protective enzymes NQO1 and GSTP1 was determined in mouse midbrain by Western blotting.

Dietary consumption of Umb and Esc for 7 days significantly increased the expression of NQO1 in mouse midbrain compared to vehicle treated control group. Umb and Esc treatment elevated NQO1 levels by 2.2-fold and 2.7-fold respectively, superior to BHA (1.7-fold) (Fig. 6.7A).

Similarly, mice consuming Umb and Esc supplemented diet enhanced GSTP1 expression to 1.3-fold and 1.3 fold respectively, compared to vehicle treated control. However, BHA administration failed to increase GSTP1 levels in mouse midbrain (Fig. 6.7B).

6.4.8 Effect of coumarin compounds on NQO1 and GST activity in mouse midbrain

The activity of NQO1 and GST enzymes in mouse midbrain was measured after treatment with Umb and Esc for 7 days.

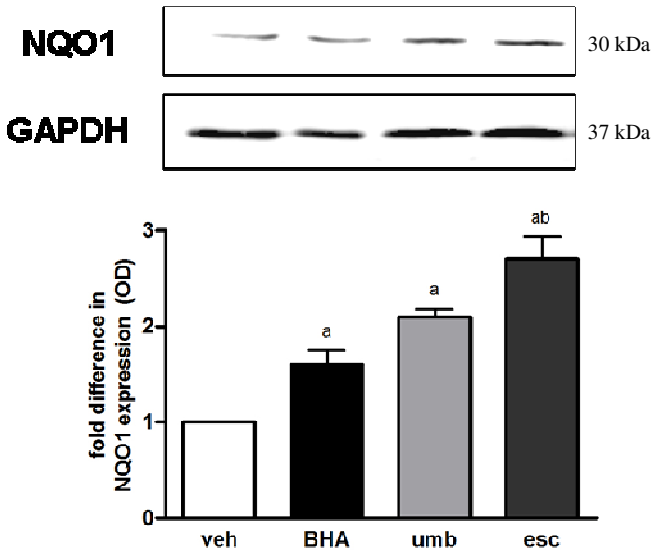
The results show that Umb and Esc consumption increased the activity of NQO1 to 195 and 180 μmol/min/mg protein, respectively compared to vehicle treated control (130 μmol/min/mg protein). Both the coumarin compounds showed a relatively similar effect to BHA (200 μmol/min/mg protein) (Fig. 6.8A).

In addition, GST activity in mouse midbrain was significantly enhanced by Umb and Esc to 640 and 650 nmol/min/mg protein, respectively, in comparison to vehicle control (410 nmol/min/mg protein). BHA showed similar effects to that of the coumarins (595 nmol/min/mg protein) (Fig. 6.8B).

6.4.9 Effect of coumarin compounds on GSH levels in mouse midbrain

GSH levels in mouse midbrain were measured after treatment with Umb and Esc for 7 days. However, no significant difference in GSH levels was observed after the coumarin consumption. BHA treatment also showed no increase in GSH concentration compared to vehicle treated control group (Fig. 6.9).

A



B

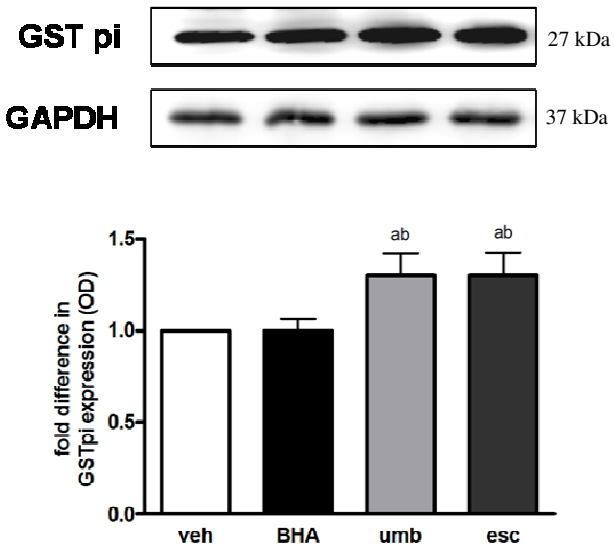
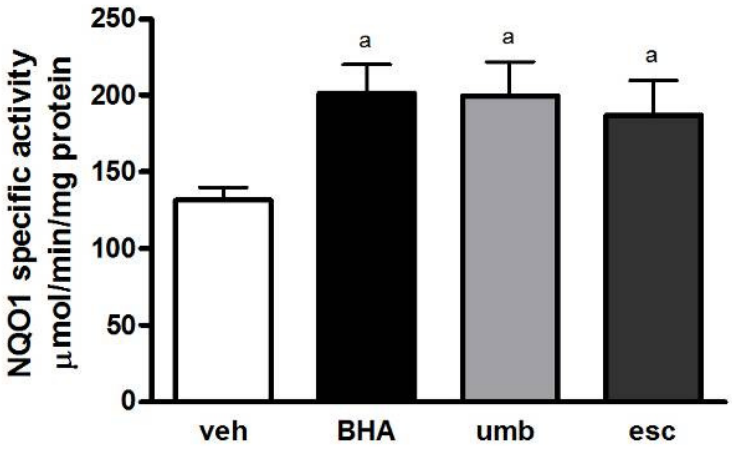


Fig. 6.7 - Coumarin compounds up-regulate the expression of antioxidant protective enzymes in mouse midbrain. Mice were fed with vehicle (2% v/w arachis oil) or BHA (0.75%) or Umb (0.5%) or Esc (0.5%) in vehicle for 7 days. Animals were euthanized; midbrains isolated and were immediately frozen on dry ice and stored at -80^{o}C. Midbrain homogenates were analysed for NQO1 (A) and GSTP1 (B) by Western blotting. Histograms show the densitometric analysis of protein expression normalised to GAPDH by NIH ImageJ software. Results are expressed as the mean ± SD of 3-6 rats. $P<0.05$ compared to vehicle treatment group (a).

A



B

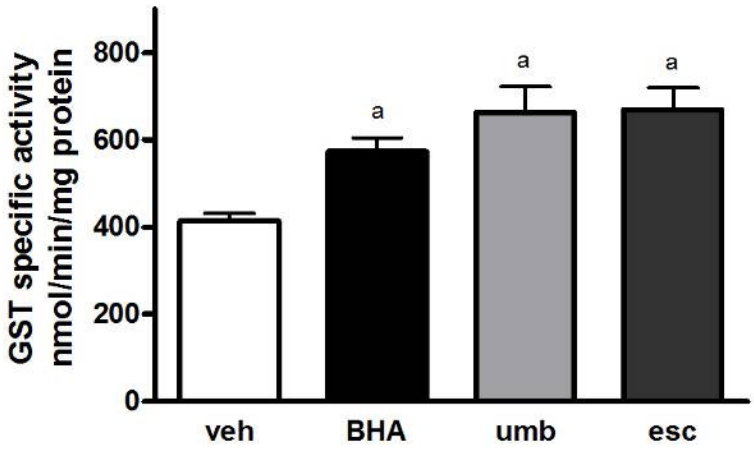


Fig. 6.8 - Coumarin compounds increase the activity of antioxidant protective enzymes in mouse midbrain. Mice were fed with vehicle (2% v/w arachis oil) or BHA (0.75%) or Umb (0.5%) or Esc (0.5%) in vehicle for 7 days. Animals were euthanized; midbrains isolated and were immediately frozen on dry ice and stored at -80^{o}C. Midbrain homogenates were analysed for enzyme activity of NQO1 (A) and GST (B) by methods mentioned in chapter 2. Results are expressed as the mean ± SD of 3-6 mice. $P<0.05$ compared to vehicle treatment group (a) and BHA treatment group (b).

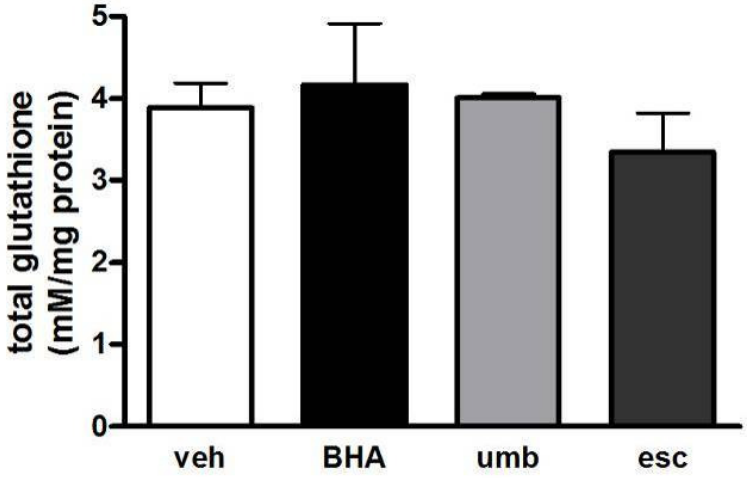


Fig. 6.9 - Effect of coumarin compounds on GSH levels in mouse midbrain. Mice were fed with vehicle (2% v/w arachis oil) or BHA (0.75%) or Umb (0.5%) or Esc (0.5%) in vehicle for 7 days. Animals were euthanized; midbrains isolated and were immediately frozen on dry ice and stored at -80^{o}C. Homogenates were analysed for GSH levels by enzymatic assay. Results are expressed as the mean ± SD of 3-6 rats.

6.5 Discussion

The focus of the current study was to evaluate the effects of dietary consumption of coumarin compounds on the antioxidant defence system in organs and tissues of rats and mice. The results presented in this chapter demonstrate that dietary consumption of coumarin compounds can modulate antioxidant protective enzyme expression and activities in several vital tissues including liver, brain and kidneys. These results also confirmed that dietary consumption of coumarin compounds elevated the intracellular GSH levels, thus enhancing the antioxidant defences.

Induction of antioxidant enzymes can protect from electrophile and reactive oxygen species toxicities by wide variety of mechanisms. These mechanisms include conjugation with endogenous ligands, modification of reactive molecules that can damage cellular components and generation or augmentation of cellular antioxidants (Talalay, 2000). Induction of antioxidant enzymes appears to be part of a generalised mechanism of protection against electrophiles and reactive oxygen species, thereby ameliorating the risk of damage to cellular components. Antioxidant responsive element (ARE) or electrophile responsive element (EpRE) is involved in triggering the transcription of a battery of genes encoding detoxification enzymes (Rushmore et al., 1991, Rushmore and Pickett, 1990). The genes that are driven by ARE and induced co-ordinately include NAD(P)H: oxidoreductases (Nioi and Hayes, 2004, Jaiswal, 2000), heme oxygenase 1 (HO1) (Prestera et al., 1995), glutathione-S-transferases (GSTs) (Rushmore and Pickett, 1990), aldo-keto reductases (AKRs) (Ellis et al., 2003), γ-glutamylcysteine synthase (γ-GCS) (Wild and Mulcahy, 1999).

NQO1 gene is a part of cellular defence mechanism that is responsible for the induction of several other protective genes in response to electrophilic and/or oxidative stress. As mentioned before, NQO1 catalyses two-electron reduction of quinones, which do not result in the formation of free radicals and highly reactive oxygen species, thus involved in chemoprotection preventing cells from oxidative damage and xenobiotics (Joseph and Jaiswal, 1994, Lind et al., 1982). Studies show that NQO1 (-/-) mice exhibit increased toxicity when exposed to the oxidant,

menadione compared to the wild type NQO1 (+/+) mice (Long II and Jaiswal, unpublished). Our studies revealed that administration of coumarin compounds in diet for seven days significantly increased the expression and activity of NAD(P)H: oxidoreductase 1 (NQO1) in various tissues including liver, brain and kidneys. Previous studies show that treatment with anthocyanins, melatonin, increased the expression of NRF2 and NQO1, and protected from dimethylnitrosamine-induced liver injury in rats (Hwang et al., Jung et al., 2009). Studies also reveal that NRF2-mediated expression of NQO1 can protect rats from oxidative stress-induced traumatic brain injury (Hong et al.). These results suggest that dietary intake of coumarin compounds induce NQO1 expression in several tissues and hence may protect cells from oxidative stress and xenobiotics.

Heme oxygenase (HO) is a protective enzyme that is responsible for the catalytic conversion of heme to biliverdin, which is enzymatically reduced to bilirubin. The present study shows that the dietary consumption of coumarin compounds induce HO1 in rat liver and brain. Previous studies show that treatment with curcumin, anthocyanins and melatonin increased NRF2-meidated HO1 expression and protected rats from dimethylnitrosamine-induced liver injury (Farombi et al., 2008, Hwang et al., Jung et al., 2009). The NRF2-mediated expression of HO1 has been also previously shown to protect rats from oxidative stress-induced traumatic brain injury (Hong et al.). These observations indicate that the coumarin compounds induce the expression of HO1 and might act as chemoprotective agents preventing tissues from oxidants and other toxic substances.

Glutathione S-transferases are enzymes that are involved the catalysis of nucleophilic attack by reduced glutathione on non-polar compounds containing an electrophile resulting in detoxification (Hu et al., 2006, Nair et al., 2006). In addition, GSTs also detoxify products of biological oxidation. In the current study it is shown that coumarin compounds up-regulate the expression of various enzymes of the GST family in various tissues such as brain, liver and kidneys. Previously, treatment of rats with anthocyanins protected from dimethylnitrosamine-induced liver injury by the up-regulation of NRF2 and GSTα (Hwang et al.). Earlier studies also show that

administration of BHA and ethoxyquin increased the expression of GSTs in mice (Hayes et al., 2000) and rats administered with coumarin prevented them from aflotoxin B1-induced hepatocarcinogenesis (Kelly et al., 2000). These observations indicate that consumption of coumarin rich diet might generally prevent these tissues against oxidative damage and related pathological conditions.

Aldo-keto reductases (AKRs) are enzymes that catalyse compounds via NAD(P)(H)-dependent reduction or oxidation reactions (Jin and Penning, 2007). AKR metabolism of xenobiotics often prepares substrate for phase 2 conjugation by sulfotransferases or UDP-glucuronosyl transferases, ultimately leading to excretion of the product. In the present study, the dietary consumption of coumarin compounds induced the expression of AKR1A3, AKR7A1 and AKR7A4 in various rodent tissues including liver, brain and kidney. Previous evidence reveals the role of aldo-keto reductases in protecting cells from aldehydes and ketones. The human AKR1A1 has been shown to be involved in the reduction of the reactive dicarbonyl, methylglyoxal (Vander Jagt et al., 1992). In addition, overexpression of AKR1A3 increased protection from deoxyglucosone and methylglyoxal induced cytotoxicity (Suzuki et al., 1998). AKR1A enzymes are also known to play a vital role in the detoxification of reactive aldehydes generated through lipid peroxidation reactions. AKR1A1 is capable of reducing highly reactive carbonyl compounds including acrolein and 4-hydroxynonenal (4-HNE) (O'Connor et al., 1999) and hence could prevent cells from these lipid peroxidation products. Another member of the AKR family, AKR7A1 was shown capable of reducing aflotoxin dialdehyde and other aldehydes and ketones (Ellis et al., 1993, Ellis and Hayes, 1995). AKR7A1 overexpression in V79 cells was shown to offer protection against acrolein induced toxicity (Gardner et al., 2004). This evidence suggests that AKRs may play an important role in the reduction of toxic aldehydes and ketones, and resulting reactive oxygen species in the biological system. Hence, dietary supplementation of the tested coumarin compounds may offer protection against toxicity induced by lipid peroxidation products such as acrolein and 4-HNE.

6.6 Conclusions

In conclusion, the results suggest that dietary supplementation of coumarin compounds, umbelliferone, esculetin, fraxetin and scopoletin, present in fruits, vegetables and herbs induces the expression of antioxidant and protective enzymes such as NQO1, HO1, GSTs and AKRs in several tissues of rats and mice. The induction of antioxidant enzymes is considered as an effective and sufficient strategy in protection against deleterious effects of electrophiles and reactive oxygen species. Hence, dietary supplementation of these coumarin compounds at non-toxic concentrations has the potential to protect against toxic effects of electrophiles and reactive oxygen species.

CHAPTER 7

SUMMARY AND GENERAL DISCUSSION

7. Summary and General Discussion

Oxidative stress has been implicated in various age-related diseases such as cancer, neurodegenerative diseases, cardiovascular diseases and others (Halliwell B., 2007, Valko et al., 2007). Several epidemiological studies have suggested that consumption of fruits and vegetables rich in phytochemicals is a simple but realistic strategy to prevent these diseases (Block et al., 1992, Cantuti-Castelvetri et al., 2000, Reddy et al., 2003, Mathew et al., 2004). However, earlier clinical studies involving antioxidants such as vitamin E, vitamin C, N-acetyl cysteine and glutathione did not show any significant beneficial effects on patients (Casetta et al., 2005). Recently, researchers have focused on novel antioxidant strategies to target particular organelles such as mitochondria that are known to generate huge amounts of ROS. Studies are being carried out on mitochondria-targeting antioxidant peptides, vitamin E and ubiquinone to enhance the benefits of antioxidant therapies (Kelso et al., 2001, Smith et al., 1999, Zhao et al., 2004). In addition to that newer compounds with the potential to enhance the endogenous antioxidant defence systems including antioxidant transcriptional regulators to stimulate NRF2 and PGC-1α are also being investigated (Calkins et al., 2009, Clark and Simon, 2009). The huge resources of natural products and diet-derived plant chemicals are being explored for compounds primarily with the capacity to enhance endogenous antioxidant defences in addition to their intrinsic antioxidant properties (Linseman, 2009, Vincent et al., 2009, Tanigawa et al., 2007). The interest to explore the huge resources of diet derived compounds has gained importance as many age-related diseases are constantly being associated with cellular damage caused by reactive oxygen species and other free radicals.

Plant-derived coumarin compounds are present in a wide range of fruits and vegetables, with a low toxicity profile and ready availability in variety of daily food. Umbelliferone (7-hydroxycoumarin; Umb) and esculetin (6,7-dihydroxycoumarin; Esc) are coumarin derivatives found in plants with proven antioxidant and free radical scavenging properties. Previous studies described in Chapter 1 show that

these coumarin derivatives can effectively scavenge free radicals and inhibit lipid peroxidation, which make them potential candidates as therapeutic agents against oxidative stress-induced diseases. Despite these observations, little is known about the protective effects and mechanism of protection elicited by these coumarin derivatives on oxidative stress-induced damage. Therefore in the present study the chemopreventive properties of dietary coumarin compounds against oxidant-induced hepatotoxicity and neurodegeneration have been investigated using *in vitro* and *in vivo* biological models.

7.1 Summary

7.1.1 Coumarins protect against ROS-induced hepatotoxicity

In Chapter 3, the protective effects of esculetin in human hepatoma HepG2 cells against ROS induced by hydrogen peroxide and the mechanism of protection were investigated. Cell survival, cell integrity, intracellular glutathione levels, generation of reactive oxygen species and expression of antioxidant enzymes were used as markers to measure cellular oxidative stress and response to ROS. The protective effect was compared to a well-characterised chemoprotective compound quercetin. Pre-treatment of HepG2 cells with sub-lethal (10-50 μM) esculetin for 8h prevented cell death and maintained cell integrity following 24h hydrogen peroxide exposure. An increase in the generation of ROS following hydrogen peroxide treatment was significantly attenuated by 8h pre-treatment with esculetin. In addition, esculetin ameliorated the decrease in intracellular glutathione caused by hydrogen peroxide exposure. Moreover, treatment with esculetin for 8h increased the expression of NAD(P)H: quinone oxidoreductase (NQO1) and superoxide dismutase 1 (SOD1) at both protein and mRNA levels significantly. Esculetin treatment also increased nuclear accumulation of NRF2 by 8-fold indicating that increased NQO1 expression is NRF2-mediated. These results indicate that esculetin protects human hepatoma HepG2 cells from hydrogen peroxide induced oxidative injury and this protective is provided through a combination of the ROS scavenging effect, maintenance of redox status and NRF2-mediated induction of NQO1 gene.

To further widen the understanding of this protective effect of esculetin against oxidative damage, the aim of the study in Chapter 4 was to investigate the effect of esculetin on ROS-induced hepatotoxicity using an *in vivo* rat model. Male Wistar strain rats were fed with the coumarins, umbelliferone (0.5% w/w) and esculetin (0.5% w/w), in diet for 7 days. Following the coumarin treatment the rats were administered with NDEA to induce oxidative damage in liver. The rats were sacrificed after 48h of NDEA administration and the hepatic injury was analysed by measuring lipid peroxidation and glutathione levels in the liver, serum biomarkers as well as histopathological studies. The protective effects of coumarins were compared with a positive control BHA, a potent antioxidant and an antioxidant enzyme inducer (Kelly et al., 2000). Results showed that NDEA administration caused severe liver damage with elevated lipid peroxidation levels, depleted glutathione levels, variation in serum biomarkers and changes in histological characters of hepatocytes. NDEA administration showed a marked increase in LPO levels (101 nmol/mg protein) compared to vehicle administered control group (26 nmol/mg protein) but rats supplemented with Umb and Esc showed up to a 44% and 40% decrease, respectively in LPO levels. Rats administered with NDEA showed a sharp 50% decrease in hepatic GSH levels however the coumarin supplementation completely prevented NDEA-induced GSH depletion in rat liver. A severe increase in ALT levels to 97% after NDEA administration was inhibited by Umb and Esc supplementation by 80% and 87%, respectively. Similarly, NDEA-induced reduction in serum albumin levels was inhibited by Umb and Esc by 2% and 11%, respectively. In the histopathological studies, NDEA treated mouse liver showed loss of cell architecture and characters of necrosis, but pre-treatment with Umb and Esc showed mild necrosis and a regeneration of hepatocytes was observed. In addition, the consumption of coumarins by rats showed a significant increase in the expression of antioxidant enzymes NQO1, HO1 and GSTP1 in rat liver. These results suggest that dietary supplementation of these coumarins protected rats against NDEA-induced oxidative damage and hepatotoxicity via enhancement of the endogenous antioxidant defences and scavenging NDEA-induced ROS production. The findings on the hepatoprotective effects of coumarins in this study are summarised in Table 7.1.

Table 7.1 - Hepatoprotective effects of coumarins

Assay/Parameter	**Hepatoprotective effects of coumarins on oxidative stress-induced toxicity in HepG2 cells and rat liver**		
	***In vitro* (HepG2)**	***In vivo* (Rat liver)**	
	Esculetin	**Esculetin**	**Umbelliferone**
Increase in cell viability	+++ (H_2O_2 toxicity)	++ (NDEA toxicity)	++ (NDEA toxicity)
Reduction of ROS generation	++ (H_2O_2 toxicity)	NA	NA
Reduction of GSH depletion	++ (H_2O_2 toxicity)	+ (NDEA toxicity)	+ (NDEA toxicity)
Reduction of apoptosis/necrosis biomarkers	+ (H_2O_2 toxicity)	NA	NA
Up-regulation of antioxidant enzymes	+++ (NQO1) ++ (SOD1)	+ (NQO1) + (HO1) + (GSTP1)	+ (NQO1) + (HO1) + (GSTP1)
Reduction of liver damage biomarkers	NA	++ (NDEA toxicity)	++ (NDEA toxicity)
Reduction of histological changes	NA	++ (NDEA toxicity)	++ (NDEA toxicity)
Reduction of lipid peroxidation	NA	++ (NDEA toxicity)	++ (NDEA toxicity)

NA: Not applicable

7.1.2 Coumarins protect against ROS-induced neurodegeneration

In view of the potent protective effects of esculetin and umbelliferone against ROS-induced hepatotoxicity, their effect on ROS-induced neurodegeneration was investigated. As described in chapter 5, the protective effects of esculetin against hydrogen peroxide and MPP^+-induced oxidative stress was investigated in SH-SY5Y neuroblastoma cell lines. The effect of hydrogen peroxide and MPP^+ on cell growth was determined by MTT assay and the IC_{50} were found to be approximately 0.38 ± 0.02mM and 0.025mM respectively. To evaluate the neuroprotective effect of esculetin, the cells were pre-treated with sub-toxic concentrations of esculetin (25µM), umbelliferone (25µM) and quercetin (40µM) for 8h and various biological markers of cellular oxidative stress were determined. The MTT assay showed that hydrogen peroxide and MPP^+ exposure is toxic to SH-SY5Y cells and the pre-treatment with esculetin (25-50µM) increased cell survival during hydrogen peroxide toxicity in a dose-dependent manner. However, esculetin pre-treatment did not show any increase in cell viability in MPP^+ toxicity. To determine the antioxidant properties of esculetin, generation of reactive oxygen species (ROS) was measured using a fluorescent probe and the results showed that 8h pre-treatment with esculetin and umbelliferone effectively scavenged the formation of intracellular ROS induced by hydrogen peroxide and MPP^+. Moreover, the redox status of the cell was measured by estimating the total glutathione levels and esculetin pre-treatment prevented GSH depletion in H_2O_2-induced stress in SH-SY5Y cells. Umb and Esc showed a similar preventive effect on GSH decrease in MPP^+-induced toxicity. Pre-treatment with Umb and Esc also inhibited increase in Bax expression after MPP^+ exposure in SH-SY5Y cells. Furthermore, esculetin and umbelliferone induced an increase in the expression of the antioxidant enzyme, copper/zinc superoxide dismutase (SOD1), in SH-SY5Y cells. These results suggest that esculetin and umbelliferone protect neuronal cells from hydrogen peroxide and MPP^+-induced oxidative damage by mechanisms including scavenging ROS, regulation of the cellular redox status, inhibition of apoptosis and induction of the antioxidant enzyme SOD1.

The neuroprotective effects of Umb and Esc in neuronal cells and their known capacity to cross the blood brain barrier led to further investigation on these compounds using *in vivo* neurodegeneration models. The study described in chapter 5 examines the neuroprotective effects of umbelliferone and esculetin against oxidative stress-induced neurotoxicity by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in C57BL/6J mice. MPTP causes nigrostriatal dopaminergic neurotoxicity and behavioural impairments in primates and rodents, and has been used as a model of Parkinson's disease for assessing neuroprotective agents. Previous studies suggest that reactive oxygen species and nitric oxide are involved in MPTP-induced neurotoxicity (Rossetti et al., 1988), (Przedborski and Vila, 2003). Dietary administration of umbelliferone and esculetin significantly attenuated MPTP-induced neurotoxicity in the Substantia Nigra but not striatum, as measured by tyrosine hydroxylase immunostaining. The coumarins prevented the MPTP-induced increase in 3-nitrotyrosine immunoreactivity, and MPTP-dependent GSH depletion. Umbelliferone and esculetin also inhibited the MPTP-dependent elevation in biomarkers of apoptosis and activation of JNK. These results suggest that umbelliferone and esculetin protect against MPTP-induced neurotoxicity in mouse brain by inhibiting ROS and peroxynitrite production, which leads to a reduction in JNK activation and apoptosis. It was also observed that the coumarins enhanced the antioxidant defence system by up-regulation of the protective enzyme NQO1 and GSTP1 in SNpc. These results show that relatively low doses of these compounds can lead to molecular and cellular changes within SNpc, enhancing oxidant defences and thereby protecting against neuronal cell death. The findings on the neuroprotective effects of coumarins in this study are summarised in Table 7.2.

7.1.3 Coumarins and the induction of NRF2-ARE-dependent antioxidant protective enzymes

The study in Chapter 3 indicates that esculetin treatment induced the expression of NQO1 in HepG2 by the activation of NRF2-ARE pathway. In line with these results, the capacity of the coumarin compounds including esculetin, umbelliferone, fraxetin

Table 7.2 - Neuroprotective effects of coumarins

Assay/Parameter	Neuroprotective effects of coumarins on oxidative stress-induced toxicity in SH-SY5Y cells and mouse brain		
	In vitro (SH-SY5Y cells)	*In vivo* (Mouse brain; MPTP toxicity)	
	Esculetin	Striatum	SNpc/midbrain
Increase in cell viability	++ (H_2O_2 toxicity) - (MPP^+ toxicity)	- (Esc,Umb:TH staining)	+ (Esc,Umb:TH staining)
		+ (Esc,Umb:Nissl staining)	+ (Esc, Umb:Nissl staining)
Reduction of ROS generation	+ (H_2O_2 toxicity) - (MPP^+ toxicity)	NA	NA
Reduction of GSH depletion	+ (H_2O_2 toxicity) + (MPP^+ toxicity)	++ (Esc, Umb)	+ (Esc) ++ (Umb)
Reduction of apoptosis/necrosis biomarkers	++ (Bax levels)	++ (Esc: Caspase 3) - (Umb: Caspase 3)	++ (Esc, Umb: Caspase 3)
		++ (Esc, Umb: Bax)	++ (Esc, Umb: Bax)
		+++ (Esc, Umb: cytosolic Cytochrome C)	NA
Up-regulation of antioxidant enzymes	+ (SOD1)	++ (Umb: NQO1) - (Esc, Umb: GSTP1)	+ (Esc, Umb: NQO1) + (Esc, Umb: GSTP1)
Reduction of peroxynitrite production	NA	++ (Esc, Umb)	+++ (Esc, Umb)
Activation of JNK pathway	NA	++ (Esc, Umb)	++ (Umb) - (Esc)

NA: Not applicable

and scopoletin to induce various antioxidant enzymes mediated by NRF2-ARE pathway was investigated in vital organs such as brain, liver and kidney, in mice and rats. The expression and activities of the antioxidant enzymes were measured by immunoblotting and enzyme assays, respectively. In addition, intracellular glutathione levels were also measured to establish the effect of these coumarins on the redox status of the cell. Dietary supplementation of above mentioned coumarins (0.5% w/w) to the rats for 7 days significantly increased the expression of NQO1, HO1, AKR1A3, AKR7A1 and AKR7A4 enzymes in liver. In addition, a significant increase in enzyme activities of NQO1, GST, AKR and GRed, and total glutathione levels were observed in the rat liver after treatment with coumarins. Parallel to these changes, consumption of coumarins also increased the expression of NQO1, GSTY'8c/GSTA3, HO1 AKR1A3, AKR7A1 and AKR7A4 in rat brain. Moreover, in rat kidney, coumarins elevated the expression of NQO1, GSTY'8c/GSTA3 and AKR7A4 enzymes. In mice, consumption of coumarins increased the expression and enzyme activities of NQO1 and GSTP1 in midbrain. However, no significant increase in total glutathione levels was observed in mouse midbrain after the coumarin diet. These results suggest that Umb and Esc treatment generally lead to enhancement of the endogenous antioxidant defences and these coumarin compounds possess potential therapeutic value against oxidative damage-induced liver, brain and kidney diseases.

7.2 Discussion

In the present study, the coumarin compounds esculetin and umbelliferone decreased ROS levels in cells after exposure to oxidants and ROS-inducing toxins. In human hepatoma HepG2 cells, esculetin showed a decrease in ROS levels equivalent to a known antioxidant quercetin (Tanigawa et al., 2007). Similarly, in neuronal cells subjected to oxidant and neurotoxin-induced oxidative stress, esculetin and umbelliferone exhibited ROS scavenging properties. Esculetin has been previously reported to exhibit strong quenching capacities against DPPH and hydroxyl radicals, whereas umbelliferone has been shown to scavenge superoxide radicals (Paya et al., 1992a, Lin et al., 2000). This ROS scavenging effect of esculetin and umbelliferone

appears to be due the phenolic hydroxyl group(s) of these coumarins and their trapping capacities of unpaired electrons by means of hydrogen atom donation from free hydroxyl radicals (Halliwell B., 2007).

Lipid peroxidation (LPO) products such as MDA, HNE, isoprostanes and acrolein are associated with cancer and neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease (Neeley and Essigmann, 2006) (Dib et al., 2002), (Zarkovic, 2003), (Lovell et al., 2001, Uchida et al., 1998). In the present study, the dietary administration of umbelliferone and esculetin reduced lipid peroxidation levels induced by NDEA in rat liver. Previous studies have reported that umbelliferone (Paya et al., 1992a, Lin et al., 2000) and esculetin (Paya et al., 1992a, Lin et al., 2000) inhibit lipid peroxidation in different tissues. This lipid peroxidation inhibition property of umbelliferone and esculetin may be attributed to their metal sequestering ability, which prevents the initiation of LPO process. In addition, the presence of *o*-dihydroxyl (catechol) group in esculetin may increase its anti-lipid peroxidation capacity (Paya et al., 1992a, Lin et al., 2000).

The redox environment of the cell which regulates the redox homeostasis is represented specifically by their glutathione levels (Droge, 2002, Schafer and Buettner, 2001). The present study shows that the coumarins umbelliferone and esculetin maintain intracellular glutathione levels, although their effect was slightly inferior compared to the known antioxidants such as BHA and quercetin. Previously, an increase in intracellular glutathione by esculetin has been reported in C57BL/6J mice (Martin-Aragon et al., 1998). Elevated total glutathione levels indicate enhanced expression and/or activity of key enzymes involved in glutathione biosynthesis, such as the catalytic subunit of glutamate-cysteine ligase, glutathione synthase and γ-glutamylcysteine synthetase (γ-GCS) (Lu, 2009) (Rodgers and Grant, 1998, Myhrstad et al., 2002, Scharf et al., 2003). In addition, earlier studies have shown that compounds such as isothiocyanate, diallyl sulphide, quercetin and a fraction of *Ginkgo biloba* extract increase γ-GCS activity and glutathione concentration in cell lines (Scharf et al., 2003, Rimbach et al., 2001). In line with this, the observed increase in GSH levels after coumarin treatment indicates that

additional glutathione is being synthesized within the cell following their pre-treatment, which might be mediated through the enhanced expression of enzymes involved in glutathione biosynthesis.

The mitochondrial pathway of apoptosis process is regulated by proteins of B-cell lymphoma 2 protein (Bcl-2) family, which contains pro-apoptotic and anti-apoptotic factors. The stoichiometry between these pro-apoptotic and anti-apoptotic Bcl-2 family members regulates the cellular commitment to apoptosis (Oltvai et al., 1993, Yang et al., 1995). Apoptosis can be detected by the increased expression of pro-apoptotic Bax, cytosolic Cytochrome-c and caspase-3 as well as decreased expression of anti-apoptotic Bcl-XL (Galan et al., 2001, Dietz et al., 2008, Tatton and Kish, 1997). Mounting evidence suggest that cell death-associated molecular mechanisms may participate in neuronal cell death in degenerative diseases (Rudin and Thompson, 1997, Pettmann and Henderson, 1998). In this study, the treatment of mice with Umb and Esc lowers the MPTP-induced elevation of Bax levels, inhibits the consequent Cytochrome-c release and decreases caspase-3 induction in dopaminergic neurons in mice. Previous studies show that compounds with antioxidant properties such as puerarin and (-)-deprenyl prevent neurotoxicity by inhibiting apoptosis in neuronal cell lines and animals models by decreasing Bax expression (Zhu et al., 2010, Magyar and Szende, 2004). These observations demonstrate that Umb and Esc can prevent neuronal apoptotic cell death by inhibiting the expression of pro-apoptotic Bax protein.

Numerous studies have reported that MAPKs can be induced and regulated by ROS, reviewed by Bubici and co-workers (Bubici et al., 2006). One pathway that contributes to the initiation of apoptotic cell death in neurodegeneration involves the activation of c-Jun n-terminal kinase (JNK), a component of the stress-activated protein kinase (SAPK) pathway (Levy et al., 2009). Pertinent to this study, JNK has been shown to be activated by ROS in neuronal cells (Maroney et al., 1999, Luo et al., 1998). Moreover, JNK can then activate proteins involved in cell death such as p53, Bcl-2/Bcl-xL, Bax, and caspase-1 (Seimiya et al., 1997, Levy et al., 2009), thus providing a link between elevated ROS, JNK activation and apoptotic cell death. The

present study reveals that treatment of mice with Umb and Esc reduces the MPTP-induced activation of JNK in mouse brain. A similar reduction in the activation of JNK was observed in mice administered with antioxidant compounds including NAC and ginsenoside (Chen et al., 2005). This evidence indicates that this inhibition of JNK activation by Umb and Esc may be due to their ability to attenuate MPTP-induced increase in ROS levels.

The capability of Umb and Esc to cross the blood brain barrier (Sundt and Anderson, 1980, Barber et al., 2009) in addition to their known ability to directly scavenge ROS (Paya et al., 1992b) may be one of mechanisms by which they contribute to neuroprotection. Similar protective effects against MPTP neurotoxicity have been observed following treatment of mice with a range of known antioxidant compounds including edaravone (Kawasaki et al., 2007), cytosine (Ferger et al., 1998), bromocriptine (Muralikrishnan and Mohanakumar, 1998), ginsenoside (Chen et al., 2005), deprenyl (Ebadi et al., 2002), and salicylic acid (Mohanakumar et al., 2000). The proposed mechanism of neuroprotection by umbelliferone and esculetin is illustrated in Fig 7.1.

Induction of endogenous antioxidant enzymes appears to be part of a generalised mechanism of protection against electrophiles and reactive oxygen species, thereby ameliorating the risk of damage to cellular components (Kensler, 1997, Talalay et al., 1995). Antioxidant responsive element (ARE) or electrophile responsive element (EpRE) is involved in triggering the transcription of a battery of genes encoding antioxidant and detoxification enzymes (Rushmore et al., 1991, Rushmore and Pickett, 1990). NRF2 is associated with Keap1 in the cytoplasm, and the ability of NRF2 to regulate the expression of protective genes is associated with its accumulation in the nucleus (Nguyen et al., 2009). The current study in Chapter 3 shows that treatment of HepG2 cells with esculetin increased the accumulation of NRF2 in the nucleus and simultaneously elevated the expression of NQO1 at both protein and mRNA levels. Previously, the polyphenol quercetin, a compound that has been reported to up-regulate NQO1 expression at both protein and mRNA levels in HepG2 cells was shown to accumulate nuclear NRF2 and increase NQO1 expression

(Tanigawa et al., 2007). This evidence suggests that esculetin-induced protection of HepG2 cells against oxidant-stress may be mediated by NRF2-dependant expression of NQO1 gene. The proposed mechanism of protection by esculetin and umbelliferone is illustrated in Fig 7.1.

In addition, the present study also highlights the capacity of the coumarins including umbelliferone, esculetin, fraxetin and scopoletin to induce various antioxidant and detoxifying enzymes mediated by NRF2-ARE pathway, in vital organs including brain, liver and kidneys in mice and rats. The enzymes up-regulated by these coumarins include NAD(P)H: quinone oxidoreductase 1 (NQO1), heme oxygenase 1 (HO1), glutathione-S-transferases (GSTP1 and GSTY'8c/GSTA3), and aldoketo reductases (AKR1A3, AKR7A1 and AKR7A4). From these observations it can be speculated that the induction of these variety of enzymes by the coumarin compounds can protect against oxidative damage in different tissues. This protective effect of these coumarin compounds may be attributed to their capacity to enhance antioxidant defences by up-regulation of NRF2-ARE dependant antioxidant enzymes.

Currently, several clinical trials are in progress to investigate the chemopreventive effects of resveratrol (trans-3,4',5-trihydroxy stilbene), a phytoalexin derived from skin of grapes and other fruits. Evidence from numerous *in vitro* and *in vivo* studies has confirmed the potential of resveratrol as an anticancer and neuroprotective agent, which modulates various targets and signalling pathways (Athar et al., 2007, Albani et al., 2010). Similar to some flavonoids and other antioxidant enzyme inducers resveratrol is also reported to induce endogenous antioxidant system via activation of the antioxidant transcription factor NRF2. Studies show that resveratrol up-regulates the expression of NRF2 target genes including NQO1, HO1 and γ-GCS (Ungvari et al., 2010). However, resveratrol is also reported to act through several other mechanisms including modulation of inflammatory responses (Zhang et al., 2010) and activation of the sirtuin family member SIRT1. Umb and Esc, which also act through similar antioxidant enzyme up-regulation mechanism, are hence potential candidates for further investigation as chemopreventive agents.

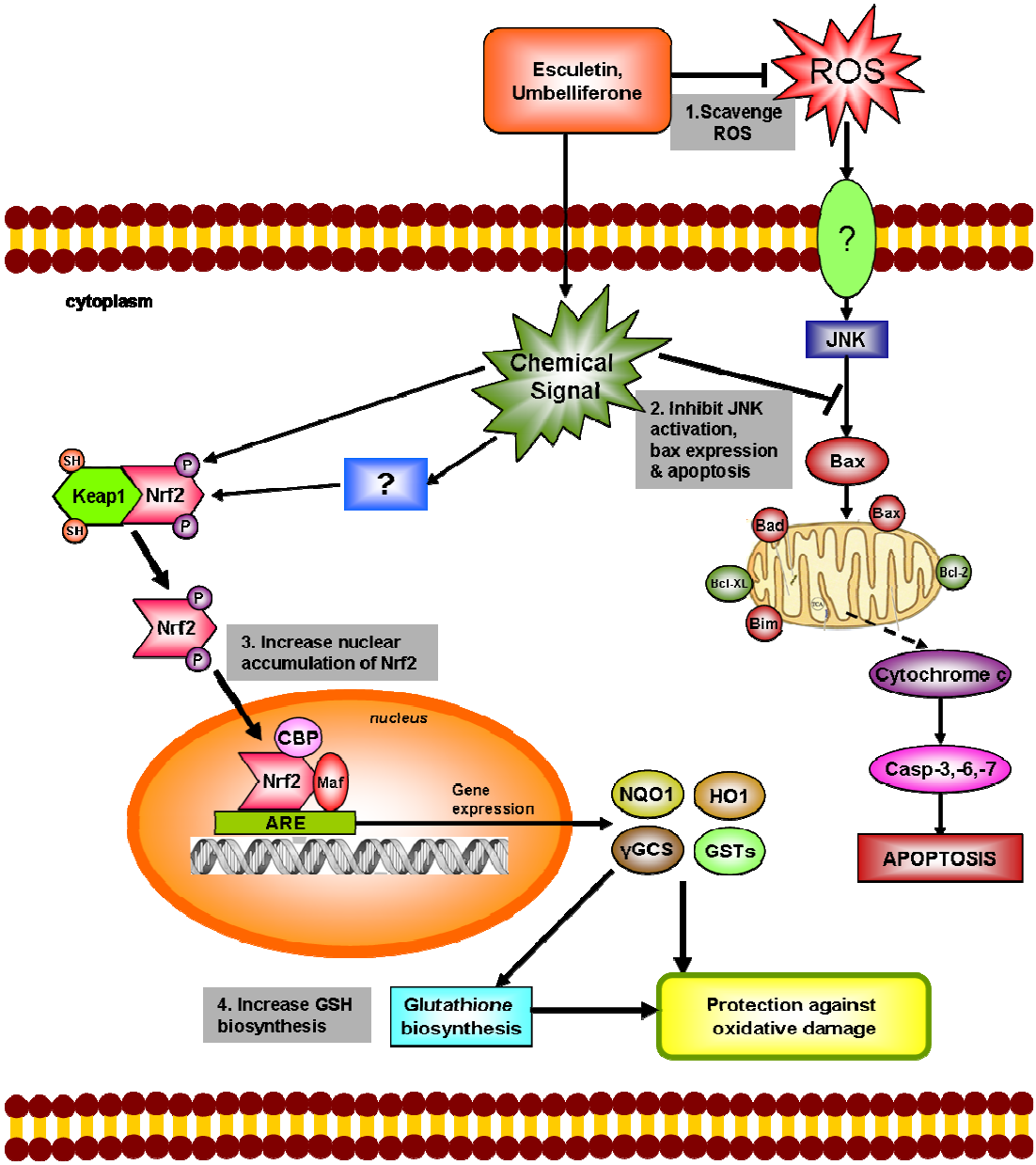


Fig. 7.1 - Proposed mechanism of protection by esculetin and umbelliferone against oxidative damage. The proposed mechanisms are noted in grey boxes.

In conclusion, the present study shows that the dietary coumarin compounds, umbelliferone and esculetin, have the potential to protect or delay oxidative damage and related hepatotoxicity or neurotoxicity in human cell lines and rodent models. The study also provides a mechanistic insight into the molecular mechanisms of the protective effects of these coumarins, which appears to be through enhancement of the endogenous antioxidant defence systems in addition to their known antioxidant properties. Interestingly, due to their capacity to cross the blood brain barrier and low toxic profile, these coumarins can be used as neuroprotective agents, which should benefit patients from chronic side effects, if any. Moreover, owing to their simple and stable chemical structures, the therapeutic treatment with these compounds can be cost effective.

Future studies would be aimed at exploring the protective effects of these coumarins in NRF2 knockout transgenic mice to confirm the relationship between their protective effect and expression of NRF2-ARE dependant antioxidant enzymes. In addition, work can be done to target these compounds at vital sources of ROS production such as mitochondria to study their beneficial effects.

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Appendix A

Published Research Outputs

1. Subramaniam, SR and Ellis, EM. (2011) Esculetin-induced protection of human hepatoma HepG2 cells against hydrogen peroxide is associated with the NRF2-dependant induction of the NAD(P)H: quinone oxidoreductase gene. *Toxicology and Applied Pharmacology,* 250, 130-6. http://dx.doi.org/10.1016/j.taap.2010.09.025
2. Subramaniam, SR and Ellis, EM. Neuroprotective effects of Esculetin on Hydrogen Peroxide-induced Oxidative Stress in Human Neuroblastoma Cells. *In Proceedings: British Pharmacological Society Summer Meeting 2009,Edinburgh.* http://www.pa2online.org/abstracts/Vol7Issue2abst056P.pdf
3. Subramaniam, SR and Ellis, EM. Umbelliferone and Esculetin Prevent 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-Induced Nigrostriatal Neurotoxicity in C57BL/6J Mice. *World Parkinson's Conference, Glasgow, Sep 2010. In abstracts: Movement Disorders, Vol. 25, Suppl. 3, 2010, S724.* http://dx.doi.org/10.1002/mds.23389
4. Subramaniam, SR and Ellis, EM. Up-regulation of Endogenous Antioxidant and Phase II Enzymes by Dietary Supplementation of Scopoletin, A Plant Derived Coumarin, in Rat Liver: Possible Role in Protection against Chronic Diseases with an Underlying Oxidative Stress, *WorldPharma, Copenhagen*, 2010. *In Abstracts: Basic & Clinical Pharmacology & Toxicology, 107 s1, 2010, 595-596.* http://onlinelibrary.wiley.com/doi/10.1111/j.1742-7843.2010.00600.x/pdf
5. Subramaniam, SR and Ellis, EM. Protective effects of Esculetin on Hydrogen Peroxide-induced toxicity in HepG2 Cells. *In Abstracts: The British Toxicological Society: Autumn meeting, Liverpool Hope University, Liverpool, Sep 2008, 31.*

Appendix B

Preparation of Buffers and solutions for SDS-PAGE and Western blotting:

SDS-PAGE

2× LSB (Laemmli's Sample Buffer): 1M Tris-Cl pH 6.8, 3.13 ml; SDS 2g; Glycerol 9 ml; 2-Mercaptoethanol 5 ml; 2% Bromophenol blue, 1 ml; dH_2O, 32 ml.

4× Resolving Buffer (Bottom Gel): Tris base, 90.8 g; SDS, 2 g. 450 ml of dH_20 was added and the pH adjusted to 8.8 with concentrated HCl. The solution was made up to 500 ml with dH_20 and then filter sterilized.

4× Stacking Buffer (Top Gel): Tris base, 15.14 g; SDS, 1 g. 200 ml dH_20 was added and the pH adjusted to 6.8 with concentrated HCl. The solution was made up to 250 ml with dH_20 and then filter sterilised.

1× Running Buffer: Tris base, 3 g; Glycine, 14.4 g; SDS, 1 g; made up to 1 litre with dH_20.

Generally, 10% acrylamide gels were used. The composition of gels was as follows:

Resolving Gel (10% Gel):

30% Acrylamide/bisacrylamide solution	: 6.6ml;
4× Resolving Buffer	: 5.0 ml
Ammonium persulphate (APS, 100mg/ml)	:100µl;
TEMED	: 10µl
dH_2O	: 8.2ml.

Stacking Gel (5%Gel):

30% Acrylamide/bisacrylamide solution	: 1.64ml
4 x Stacking Buffer	: 2.5ml
dH_2O	: 5.86ml
Ammonium persulphate (100 mg/ml)	: 60µl
TEMED	: 10µl

WESTERN BLOTTING

1 x Transfer Buffer: Glycine, 14.4 g; Tris base, 3 g; 800 ml dH_2O and made up to 1 litre with methanol.

10 x TBS (Tris-buffered Saline): 1 M Tris.Cl pH7.5, 100 ml; 4M NaCl, 375 ml.

1 x TBSTween (0.2% Tween): 10 x TBS, 100 ml; Tween 20, 2 ml; made up to 1 litre with dH_20.

Blocking Solution (10%): 5 g dried skimmed milk powder (Marvel) or bovine serum albumin (BSA); made up to 50 ml with 1 x TBSTween.