PROPERTIES OF PHENOLIC COMPOUNDS AND THEIR RELATIONSHIP WITH THE PREVENTION OF UV-A MEDIATED DAMAGE IN HUMAN SKIN FIBROBLASTS

BY

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ABSTRACT

UVA irradiation penetrates deep into the epidermis producing reactive oxygen species (ROS) that are responsible for damaging cellular components. Many phenolic compounds scavenge ROS and chelate transition metal ions that promote oxidation. In this thesis the effect of five polyphenols, namely, epicatechin gallate (ECG), luteolin, luteolin-4'-O-glucoside, luteolin-7-O-glucoside and trolox were investigated in terms of their ability to modulate UVA-mediated oxidative damage in human skin fibroblasts (HSF) at two different doses of irradiation (250 and 500 kJ/m²).

The cytotoxicity of each compound was evaluated by both the MTT and LDH assays using a polyphenol concentration of 30 μM which was not cytotoxic. At both doses of UVA irradiation, an increase in apoptosis was observed. Each of the phenolic compounds tested reduced the extent of apoptosis except for luteolin-4'-O-glucoside and trolox.

UVA irradiation of HSF increased lipid peroxidation (PV and MDA-TBARS) Overall, luteolin and its glucosides demonstrated the greatest protection in terms of reducing lipid peroxidation at both UVA doses. These flavones were more effective than ECG, which in turn was more protective than trolox. Trolox only reduced PV at the lower irradiation dose.

UVA irradiation caused an increase in protein cross-linkage (dityrosine formation) shown by increased fluorescence, and in collagen I and IV degradation in HSF. All (poly)phenols reduced protein cross-linkage at the high UVA dose but did not prevent collagen I and IV degradation.

Both luteolin and its 7-O-glucoside interacted effectively with Fe³⁺ and Cu²⁺ indicating the importance of both 3,4-dihydroxy group and the 4-oxo group on the B and C rings, respectively. Luteolin-4'-O-glucoside showed a lower interaction with
the two transition metals highlighting the importance of the B ring catechol group. ECG also interacted effectively with Cu$^{2+}$ but not Fe$^{3+}$ whereas trolox showed the least interaction with these metal ions.

These results show that UVA causes apoptosis and damage to lipids and proteins in HSF. This damage may be reduced by the inclusion of luteolin and its associated glucosides, as well as ECG, to HSF prior to irradiation. However, trolox was generally ineffective. Overall the efficacy of the (poly)phenols investigated in terms of their ability to prevent UVA induced damage was related to their structural features and their ability to interact with metal ions.
ACKNOWLEDGEMENTS

In the name of Allah, most Gracious, most Merciful, I praise and thank Allah, the Cherisher and Sustainer of the world, most Gracious, most Merciful Master of the Day of Judgment.

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I furthermore dedicate this complete work, to my late father Abdulaziz Rahimuddin, who would be incredibly proud of me, on completion of this thesis.
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<tr>
<td>-CH-</td>
<td>Carbon Radical</td>
</tr>
<tr>
<td>O₂</td>
<td>Singlet Oxygen</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired Immunodeficiency Disease Syndrome</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis Of Variance</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium Persulfate</td>
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<tr>
<td>ATP</td>
<td>Adenosine Tri-Phosphate</td>
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<tr>
<td>BHA</td>
<td>Butylated Hydroanisol</td>
</tr>
<tr>
<td>BHT</td>
<td>Butylated Hydroxytolune</td>
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<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<tr>
<td>C</td>
<td>Catechin</td>
</tr>
<tr>
<td>CAT</td>
<td>Catalase</td>
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<tr>
<td>-CH₂-</td>
<td>Methylene Group</td>
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<tr>
<td>Cu, Zn-SOD</td>
<td>Copper, Zinc-Superoxide Dismutase</td>
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<tr>
<td>DAPI</td>
<td>4',6-Diamidino-2-Phenyindole Dilactate</td>
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<td>Desferrioxamine</td>
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<td>Dityrosine</td>
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<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
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<td>DNA</td>
<td>Dideoxy Ribonucleic Acid</td>
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<td>DPBS</td>
<td>Dulbecco's Phosphate Buffer Saline</td>
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<td>dUTP</td>
<td>Deoxyuridine Triphosphate</td>
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<td>EC</td>
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<td>ECM</td>
<td>Extracellular Materix</td>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
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<td>EGC</td>
<td>Epigallocatechin</td>
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<td>EGCG</td>
<td>Epigallocatechin Gallate</td>
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<td>ELISA</td>
<td>Enzyme Linked Immunosorbent Assay Technique</td>
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<td>ER</td>
<td>Endoplasmic Reticulum</td>
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<td>ESR</td>
<td>Electron spin Resonance</td>
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<td>FAD</td>
<td>Flavin Adine Dinucleotide</td>
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<td>FBS</td>
<td>Fetal Bovine Serum</td>
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<td>Fe²⁺</td>
<td>Ferrous Ion</td>
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<td>Fe³⁺</td>
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<td>F</td>
<td>Ferritin</td>
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<td>GC/MS</td>
<td>Gas Chromatography/Mass Spectrometre</td>
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<td>GPx</td>
<td>Glutathione Peroxidase (GPx)</td>
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<td>GSH</td>
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<td>GSSG</td>
<td>Oxidized Glutathione</td>
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<td>HDL</td>
<td>High Density Lipoprotein</td>
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<td>HEPES</td>
<td>N-(2-hydroxyethyl) Piperazine-N-2-Ethanesulfonic Acid</td>
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<td>HO⁻</td>
<td>Hydroxyl Radical</td>
</tr>
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<td>HO₂⁻</td>
<td>Hydroperoxyl Radical</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
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<td>HRP</td>
<td>Horseradish Peroxidase</td>
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<tr>
<td>HSF</td>
<td>Human Skin Fibroblasts</td>
</tr>
<tr>
<td>IEC</td>
<td>International Equipment Company</td>
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<tr>
<td>INT</td>
<td>2-P-(Iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
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<tr>
<td>KDa</td>
<td>Kilo Dalton.</td>
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<td>L</td>
<td>Fatty Acid.</td>
</tr>
<tr>
<td>L•</td>
<td>Fatty Acid Radical.</td>
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<tr>
<td>LDH</td>
<td>Lactate Dehydrogenase.</td>
</tr>
<tr>
<td>LIP</td>
<td>Labile Iron Poole.</td>
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<tr>
<td>LOO•</td>
<td>Peroxyl Radical.</td>
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<tr>
<td>LOOh</td>
<td>Lipid Hydroperoxides.</td>
</tr>
<tr>
<td>LP</td>
<td>Lipid Peroxidation.</td>
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<tr>
<td>MDA</td>
<td>Malondialdehyde.</td>
</tr>
<tr>
<td>MDA-TBARS</td>
<td>Malondialdehyde Thiobarbituric Acid Reactive Substance.</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimum Essential Medium Eagle.</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix Metalloproteinase.</td>
</tr>
<tr>
<td>MTT</td>
<td>Tetrazolium salt,-(4,5-Dimethylthiazol-2-yl)-2,5,Diphenyltetrazolium Bromide.</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium Chloride.</td>
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<tr>
<td>NADH</td>
<td>Nicotinamide Adenine Dinucleotide, reduced.</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide Adenine Dinucleotide Phosphate, reduced.</td>
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<tr>
<td>NaF</td>
<td>Sodium Floride.</td>
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<td>NMR</td>
<td>Nuclear Magnetic Resonance.</td>
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<td>NO•</td>
<td>Nitric Oxide Radical.</td>
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<td>NTA</td>
<td>Nitrilotriacetate.</td>
</tr>
<tr>
<td>O2•</td>
<td>Oxygen.</td>
</tr>
<tr>
<td>O2•</td>
<td>Superoxide Radical.</td>
</tr>
<tr>
<td>OFN</td>
<td>Oxygen Free Nitrogen.</td>
</tr>
<tr>
<td>ONOO•</td>
<td>Peroxynitrite.</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffer Saline.</td>
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<td>PUFA</td>
<td>Polyunsaturated Fatty Acids.</td>
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<td>PV</td>
<td>Peroxide Value.</td>
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<tr>
<td>R•</td>
<td>Free Radical.</td>
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<td>Fatty Acid.</td>
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<td>Alloxyl Radical.</td>
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<td>ROO•</td>
<td>Peroxyl Radical.</td>
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<tr>
<td>ROS</td>
<td>Reactive Oxygen Species.</td>
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<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfate.</td>
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<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulfate-Polyacrilamide Gel Electrophoresis.</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of Mean.</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide Dismutase.</td>
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<tr>
<td>TBA</td>
<td>Thiobarbituric Acid.</td>
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<td>TBA-MDA</td>
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<td>TBARS</td>
<td>Thiobarbituric Acid Reactive Substances.</td>
</tr>
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<td>TdT</td>
<td>Terminal Deoxynucleotidyl Transferase.</td>
</tr>
<tr>
<td>TEAC</td>
<td>Trolox Equivalent Antioxidant Activity.</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N′,N′-tetra-Methyl Ethylene Diamine.</td>
</tr>
<tr>
<td>TEP</td>
<td>1,1,3,3-Tetraethoxypropane.</td>
</tr>
<tr>
<td>TNF-R</td>
<td>Tumour-Necrosis Factor Receptor.</td>
</tr>
<tr>
<td>TUNEL</td>
<td>TdT-mediated dUTP Nick End Labeling.</td>
</tr>
<tr>
<td>TyrO•</td>
<td>Tyrosine Radical.</td>
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<td>TyrOH</td>
<td>Tyrosine.</td>
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<td>UV</td>
<td>Ultraviolet.</td>
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<td>UVA</td>
<td>Ultraviolet A.</td>
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<td>Acronym</td>
<td>Abbreviation</td>
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<tr>
<td>UVB</td>
<td>Ultraviolet B.</td>
</tr>
<tr>
<td>UVC</td>
<td>Ultraviolet C.</td>
</tr>
<tr>
<td>UVR</td>
<td>Ultraviolet Radiation.</td>
</tr>
<tr>
<td>WB</td>
<td>Western Blot.</td>
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<tr>
<td>XOD</td>
<td>Xanthine Oxidase.</td>
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<tr>
<td>α-TOH</td>
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CHAPTER 1
1. INTRODUCTION

1.1 Skin composition and structure

Skin consists of three main layers, the epidermis, dermis, and the subcutaneous tissue (Fig. 1.1, Curtis, 1979). The epidermis is the outer layer of skin which contains no blood vessels and is entirely dependent on the underlying layer, the dermis, for nutrition (Hobson, 1991; Snell, 2000).

![Fig. 1.1 (A) Cross-section of the skin of a human foot. (B) Schematic diagram of the main skin layers (Alberts et al, 1994).](image)

The epidermis contains different types of cells with different functions (Fig. 1.2). These cells include keratinocytes, pigment-containing melanocytes, antigen-processing Langerhans' cells, and the pressure-sensing Merkel cells (Hobson, 1991).
Fig. 1.2 Schematic diagrams showing the cell types of the mammalian skin (Alberts et al., 1994).

The second skin layer is the dermis. It is separated from the epidermis by the basement membrane (Cross and Mercer, 1993). The primary function of the dermis is to sustain and support the epidermis. It also participates in the exchange of metabolites between blood and tissues, fat storage, protection against infection and tissue repair (Monteiro-Riviere, 1991). The dermis regulates heat through a network of tiny blood vessels that nourish the skin and provide protection for the cellular and fluid systems. The dermis consists of a matrix of connective tissue in which fibers such as collagen and elastin, are embedded in a gel-like ground substance. Like the epidermis, the dermis contains different types of cells with different functions. These include fibroblasts, mast cells, macrophages, plasma cells, fat cells, and leukocytes (Monteiro-Riviere, 1991).

The third layer of the skin is the subcutaneous tissue specialized in the fat formation. In addition to providing protection and insulation, the subcutaneous tissue serves as a depository for reserve fuel and vitamin D manufacture (Diffey, 1990).
1.2 Collagen

1.2.1 Collagen structure

The collagen molecules consist of three polypeptide chains called α-chains. These chains consist mainly of glycine as one third of all residues (Galloway, 1980), proline, hydroxyproline and hydroxylysine, which are necessary for intermolecular cross-linking (Neville, 1993). These chains are wound around one another in a ropelike superhelix (Alberts et al., 1994). Collagen accounts for approximately 30% of all protein in the human body (Lin and Liu, 2005) and is the most abundant protein in the dermis (Pilcher et al., 1998) constituting of 70% of the dry weight of skin (Petersen et al., 1992).

1.2.1.1 Collagen I

Collagen I is the major component of the extracellular matrix (Kaji et al., 1992) which is mainly synthesized by fibroblasts especially cultured human fibroblasts (Karn et al., 2001; Miltyk and Palka, 2000). A number of the collagens, including types I, II, and III, are described as fibrillar collagens because they assemble into rigid, cable-like fibrils, which in turn assemble into thicker fibers (Ottani et al., 2002). The fibrils are further strengthened by covalent cross-links between lysine and hydroxylysine residues on adjacent collagen molecules which gives the tertiary structure (Ottani et al., 2002).

1.2.1.2 Collagen IV

Unlike collagen I, type IV collagen is a non-fibril collagen which is the major component of basement membranes (Graham et al., 1997). Basement membrane
plays an important role in biological functions such as cell adhesion, cell differentiation and tissue repair (Hiki et al, 2003). The major function of the basement membrane of human skin is to attach the skin layers, the epidermis and the dermis to each other and to provide resistance against external forces (Gayraud et al, 1997). This membrane is produced by resident fibroblasts (Rittie and Fisher, 2002).

In addition to the triple helical structure, type IV collagen contains non-helical segments along the molecule and globular domains at each end. This collagen contains disulphide bonds formed by cysteine residues (Deyl et al, 2003). The non-helical segments give the molecule flexibility, while the globular ends serve as sites of interaction between molecules that give the complex its network like character (Karp, 2002; Soder and Poschl, 2004). Type IV collagen is known to have six genetically different α (IV) chains, from α1 (IV) to α6 (IV) (Deyl et al, 2003). The most abundant form is composed of two α1 (IV) chains and one α2 (IV) (Mahmoodian and Peterkofsky, 1999). The novel α chains from α3 (IV) to α6 (IV) chains are not found in all basement membranes (Deyl et al, 2003; Soder and Poschl, 2004).

1.2.2 Fibroblasts and collagen synthesis

Skin fibroblasts are specialized in collagen biosynthesis (Kama et al, 2001). They are found among the spaces between the connective tissue fibers (Jenkins, 1991). The most characteristic feature in the cytoplasm of these cells is the abundance of rough endoplasmic reticulum (ER) that is involved in the synthesis of collagen proteins (Neville, 1993).
Inside the fibroblasts, individual collagen polypeptide chains are synthesized on membrane-bound ribosomes. These chains are then injected into the lumen of the ER as pro-α chains. In the lumen of the ER, each pro-α chain combines with two others to form a triple stranded helical molecule known as procollagen (Solomon et al, 1999). The process of collagen secretion begins when fibroblasts release collagen components, into the extracellular space to form the characteristic collagen fibers. Fibroblasts secrete procollagen out of the cell by secretory vesicles which are seen all along the cell membrane of the fibroblast. Outside the cell, procollagen is converted to collagen by specific proteolytic enzymes (Monteiro-Riviere, 1991; Alberts et al, 1994) in a process that occurs close to the plasma membrane (Alberts et al, 1994). When a tissue is injured, the fibroblasts migrate into the wound, proliferate, and produce large amounts of collagenous matrix, which helps to repair the damaged tissue (Alberts et al, 1994; Shreiber and Tranquillo, 2001).

1.2.3 Enzymes involved in collagen biosynthesis and degradation

Prolidase [E.C.3.4.13.9] is a cytosolic enzyme involved in collagen biosynthesis (Karna et al, 2001). The biological function of the enzyme involves the metabolism of collagen degradation products and recycling of proline for collagen resynthesis (Surazynski et al, 2001). An alteration of collagen metabolism is known to be a pathological factor in some disorders including skin ageing (Kaji et al, 1992).

The degradation of extracellular matrix collagen is accomplished largely by a family of zinc-containing enzymes called matrix metalloproteinases (MMPs) (Lei et al, 1996; Rittie and Fisher, 2002). These enzymes are either secreted into the extracellular space or bound to external surface of plasma membrane. Gelatinases (MMP-1, MMP-2, MMP-3 and MMP-9) may cleave both type IV collagen
(Kobayashi et al, 1996; Lei et al, 1999) and the α-chain of interstitial collagen I (Scharffetter-Kochanek et al, 1993). These enzymes are found in human keratinocytes, human fibroblasts (Kobayashi et al, 1996) and the epidermis (Pilcher et al, 1998).

1.3 Ultraviolet radiation and its effects on skin cells

The sun is the major source of ultraviolet radiation (UVR). Depending on the wavelength, UVR can be divided into three categories: UVA, UVB and UVC. UVA, also called black light (Inal and Kahraman, 2000), ranges between 320-400 nm (Diffey, 1990; Henningsen, 1991; Roenigk, 1995; Stray et al, 1997; Debuys et al, 2000; Didier et al, 2001). UVB ranges between 280-320 nm (Finlay-Jones and, Hart, 1997; Chen et al, 1998; Duthie et al, 1999). Finally, UVC ranges between 100-280 nm. The biologically reactive solar UVR that attacks the skin is limited to UVA and UVB (Saliou et al, 1999). Stratospheric ozone and atmospheric moisture effectively block wavelengths shorter than 290 nm together with 70-90% of UVB (Duthie et al, 1999; Debuys et al, 2000).

UV radiation has a wide range of effects on human skin. The direct effects of UVR on the human body are limited to the surface skin, since it has a low power of penetration. The direct effects include reddening of the skin (erythema) (Saliou et al, 1999), pigmentation development (suntan), premature ageing such as wrinkles, blotches and laxity and, carcinogenic changes (Kligman, 2000). Erythema can be mild, causing only redness and tenderness, or they can be severe producing swelling, and sloughing of the outer skin. Skin burning has a protective effect on the tissues preventing a mutated cell from proliferating and initiating cancer (Stray et al, 1997; Stahl et al, 2000). Tanning is a natural body defense relying on melanin. Melanin
prevents significant DNA damage and protects the skin from further injury (Duthie et al, 1999). Solar UVR is known to constitute a major risk factor in the development of human skin cancer (Didier et al, 2001). Constant exposure to the sun causes skin changes commonly associated with the three basic skin cancers: basal-cell carcinoma, squamous-cell carcinoma and melanoma (Stray et al., 1997; Emri et al, 2000).

The extent of the solar UVR biological effect on the skin is determined by the dose and duration of the radiation, the area of skin exposed and the genetics of the individual (Saliou et al, 1999). More recently, personal exposure to UV has increased due to more “sunshine” holidays, the wearing of minimal clothing outdoors (Duthie et al, 1999), the use of sunbeds for cosmetic tanning and the insufficient use of sunscreens or more sun exposure depending on the protective effect of sunscreen (Nohynek, and Schaefer, 2001). In addition, some industrial UV exposure can occur, for example with welding arcs or tungsten halogen lamps (Duthie et al, 1999).

1.3.1 Ultraviolet A (UVA)

On the earth’s surface, UVA radiation constitutes 96% of the terrestrial UV solar energy (Han et al, 2004), while the most energetic component, UVB radiation is about 1.5-5.0% of the total (Stray et al, 1997; Ahmad, 2001). While UVB radiation may only reach the epidermis and upper dermis, UVA is a more penetrating radiation that elicits its effects in the dermis (Saliou et al, 1999; Duthie et al, 1999) (Fig.1.3).
1.3.2 Cytotoxicity induced by UVA radiation

UVA generates free radicals in skin which accelerates skin ageing, in a process known as photo-ageing (Leccia et al., 1998; Kitazawa and Iwasaki, 1999). UVA was involved in the degenerative changes in the skin related to ageing through the formation of reactive oxygen species (ROS) such as H$_2$O$_2$ (Masaki et al., 1995). The ROS are responsible for the induction of damage to DNA (Stray et al., 1997) lipids proteins (Saliou et al., 1999) and the damage to the extracellular matrix integrity and collagen degeneration (Philips et al., 2003). Singlet oxygen (¹O₂) and hydrogen peroxide (H$_2$O$_2$) are thought to be the most important ROS generated intracellularly by UVA, promoting biological damage in exposed tissues (Soriani et al., 1998; Pourzand et al., 1999; Stahl et al., 2000). ¹O₂ plays a major role in both UVA cytotoxicity and UVA mutagenicity in mammalian cells (Stray et al., 1997). These ROS may cause oxidation of lipids and proteins that in turn affect DNA repair, induce matrix metalloproteinases, and result in skin photoageing and carcinogenesis.
UVA increases the levels of collagenase, an extracellular matrix degrading enzyme involved in skin photo-ageing (Soriani et al, 1998).

The contribution of UVA to carcinogenesis has been emphasized in many studies (Didier et al, 2001; Leccia et al, 1998). UVA is able to create mutations, some of which may lead to malignant transformation. Stray et al, (1997) showed a significant decrease in skin cell survival as a function of UVA dose. It was found by Emri et al (2000) that a low dose of UVA (150 kJ/m²) induces chromosomal aberration in human fibroblasts, which may contribute to skin carcinogenesis. Studies on cultured human cells showed that exposing human skin DNA to UVA induces DNA lesions. This includes DNA single strand breaks, oxidizing purine (A or G) nucleic acids (Debuys et al, 2000), DNA-to-protein cross links, and a low level of directly produced photoproducts, such as pyrimidine dimers (Stray et al, 1997; Saliou et al, 1999). Modified DNA bases may result in mutations, deletion, or gene amplification as a first step of carcinogenesis (Diplock, 1998).

UVA irradiation causes cytotoxicity (Didier et al, 2001) and results in lipid peroxidation in human skin fibroblasts (Obermüller et al, 1999). It increased malondialdehyde (MDA) and ROS in human skin fibroblasts. Also UVA has been reported to affect different skin cells such as the depletion of Langerhans' cells causing the skin to be immunologically unresponsive (Duthie et al, 1999). A study by Han et al, (2004) showed that the exposure of fibroblasts to UVA altered the activity of various enzymes responsible for the protection of the cell against oxidative stress such as superoxide dismutase (SOD), glutathione peroxidase (GSH), catalase (CAT) and xanthine oxidase (XOD).
It was reported that the exposure of cultured human cells to low, moderate and high doses of UVA radiation causes oxidative damage to lysosomes which leads to ferritin (Ft) degradation and the consequent rapid release of potentially harmful free iron into the cytosol (Pourzand et al, 1999). Iron accumulation is known to occur under pathological conditions in many inflammatory skin diseases or in human skin chronically exposed to UVA (300 kJ/m^2) light (Giordani et al, 2000). The presence of excess iron also has been demonstrated in a variety of skin disorders such as psoriasis, venous ulceration, and atopic eczema (Pourzand et al, 1999).

1.3.3 Ultraviolet B and ultraviolet C radiation

UVB radiation produces the most harmful effects for skin. Acute negative effects of UVB radiation include inflammation, sunburn, pigmentation, and hyperplasia (Debuys, et al, 2000). Chen et al (1998) demonstrated the increase of skin thickness and the decrease of skin elasticity after UVB irradiation. Also UVB is the major cause of non-melanoma skin cancer; it acts both as a tumor initiator and tumor promoter in human skin keratinocytes cell line (Takema and Imokawa 1999). In addition, UVB irradiation leads to cell proliferation in vivo, which may lead to tumorigenesis (Chen et al, 1998). Brancaleon et al (1999) found that irradiation in the UVB region is more effective in producing the change in skin components in contrast to UVA.

Although UVC radiation is also a potent photocarcinogen, its biological significance may be less important, since it does not reach the Earth’s surface (Chen et al, 1998; Ahmad, 2001).
UVR also has positive effects on the human body. UVB radiation stimulates the production of vitamin D in the skin surface (Debuys et al., 2000). In addition, UVA can be used as a therapeutic agent for some diseases such as psoriasis. Finally, UVC, has bactericidal capabilities at wavelengths of 260-280-nm, thus it is useful as both a research tool and a sterilizing technique.

1.4 Free radicals

The existence of an organism in an O$_2$ environment is associated with the generation of ROS which lead to free radical formation, even under physiological conditions (Diplock et al., 1998). Free radicals are chemical species possessing one or more unpaired electrons (Hughes, 1999) which are capable of independent existence and are generally highly reactive (Cheeseman and Slater, 1993).

The most important free radicals include the superoxide radical (O$_2^-$), hydroxyl radical (•OH), peroxyl radical (ROO•), hydroperoxyl radical (HOO•) and the alkoxyl radical (RO•). The reactive non-radical species include hydrogen peroxide (H$_2$O$_2$), singlet oxygen (•O$_2$), and transition metals such as ferrous ions (Fe$^{2+}$). Superoxide radical anion (O$_2^-$) is considered important since other reactive intermediates are formed in reaction sequences starting with it (Diplock et al., 1998). An example is the generation of H$_2$O$_2$ in biological systems via the reduction of O$_2^-$, (eq-1) (Cheeseman and Slater, 1993).

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$  \text{eq-1.}$$

Once hydrogen peroxide (H$_2$O$_2$) is inside the cell, it reacts with iron ions to form much more damageing •OH (eq-2). This reaction is known as the iron-catalyzed Haber-Weiss reaction (Cheeseman and Slater, 1993; Hughes, 1999).
\[ H_2O_2 + Fe^{2+} \rightarrow \cdot OH + OH^- + Fe^{3+} \quad \text{eq-2.} \]

In addition, the \( \cdot OH \) radical can be produced by ultraviolet light in the sunlight exposed skin as in eq-3 (Halliwell and Gutteridge, 1999).

\[ H_2O_2 \rightarrow 2 \cdot OH \quad \text{eq-3.} \]

Free radicals may occur during normal cellular processes such as electron transfer reactions (Halliwell and Gutteridge, 1999; Hursting et al, 1999) or phagocytosis (Guyton and Hall, 1996). The major source of free radicals in cells is the electron leakage from the electron transport chains of mitochondria and the endoplasmic reticulum (Maziere et al, 1999). The produced free radicals such as the superoxide radicals (\( O_2^- \)) can be transformed to \( H_2O_2 \) by superoxide dismutase (SOD) ether in mitochondria or cytoplasm (Gyulkhandanyan et al, 2003).

Neutrophils, macrophages and peroxisome enzymes may also produce large quantities of \( O_2^- \), \( H_2O_2 \), and \( OH^- \), all of which are lethal to most bacteria even in small quantities (Pruitt et al, 1986; Guyton and Hall, 1996). Also, free radical production in cells can be greatly increased by xenobiotic compounds such as carbon tetrachloride, or external factors such as UVR (Hursting et al, 1999). Free radicals are also found or generated through environmental pollution, cigarette smoke, automobile exhaust fumes and pesticides.

Singlet oxygen (\( ^1O_2 \)) is suggested to be formed in light exposed tissue (Xia et al, 2006). It can interact with other molecules, preferably PUFAs, either by transferring its energy or by combining chemically (Diplock et al., 1998).
Free radicals are oxidizing agents that react with most of the cell components and lead to lipid (Nohl, 1993; Ossola and Tomaro, 1998) DNA (Gurr et al, 2005) and protein oxidation (Biswas et al, 2005). Enrichment of body systems with natural antioxidant may keep the production of free radicals to the minimum (Cheeseman and Slater; 1993; Tiwari, 2001). The reduction in free radicals may protect from a variety of disorders; these include skin inflammation (Wei et al, 2002) cardiovascular diseases (Santos and Moreno, 2001) inflammatory bowel disease (Wendland et al, 2001).

1.5 Lipid structure and function

Lipids are water insoluble components present in cell membranes and can be extracted by nonpolar solvents; they consist mainly of carbon, hydrogen and oxygen molecules. Lipids include fats, phospholipids, carotenoids, steroids and waxes. Lipids are used for energy storage, as components of cellular membrane, and some are important hormones such as prostaglandin. Fats consist of a glycerol moiety joined to one, two, or three fatty acids. Fatty acids with one double bond are called monounsaturated fatty acids, while those with more than one double bond are polyunsaturated fatty acids (PUFAs). Phospholipids consist of a glycerol molecule attached to two fatty acids, and a phosphate group linked to an organic compound usually containing nitrogen. Cell membranes contain large amounts of PUFAs. Linoleic acid (Mead et al, 1986) and arachidonic acid (Halliwell and Gutteridge, 1999) are the major PUFA found in cell membranes.
1.5.1 Lipid photooxidation mechanisms by UVA and UVB

The mechanism of lipid photoxidation has been described by Girotti, (2001). UVA has been shown to be a source of oxidative stress to skin via the interaction with intracellular chromophores or sensitizers with ROS generation. Debuys et al (2000) defined chromophores as molecules that absorb the energy of photons. UVA radiation may cause lipid oxidation by two proposed mechanisms. The first mechanism occurs as a consequence of the reaction of UVA sensitzers and ground state molecular oxygen ($^3\text{O}_2$) (type II mechanism) (Fig 1.4). UVA absorbing sensitizers include the tetrapyrroles (FAD, FMN) and the reduced pyridine nucleotides (NADH, NADPH), riboflavins melanin precursors and more recently collagen (Wondrak, et al, 2004). Singlet oxygen ($^1\text{O}_2$) is generated by energy transfer in this process. $^1\text{O}_2$ which is a powerful oxidant capable of reacting with a hydrogen atom abstracted from a lipid (RH) and or electron transfer to a reductant producing reduced oxygen $\text{O}_2^-$ (type I mechanism). RH might be a reductant, located in the aqueous compartment such as NADPH, ascorbate glutathione or a membrane associated species, which is probably not a lipid itself. $\text{O}_2^-$ produces $\text{H}_2\text{O}_2$ and both can react with transition metal ions such as iron and copper to generate highly reactive species such as HO•. Lipid oxidation can be terminated by chain breaking antioxidants, depletion of oxidized lipids and depletion of oxidizable lipids such as polyunsaturated fatty acids (PUFA). Lipid peroxidation mechanisms triggered by UVA irradiation and the role of antioxidants is summarized in Fig. 1.4.
Fig. 1.4 Lipid peroxidation mechanism induced by UVA irradiation and antioxidants that interact to protect cells. Antioxidants include: catalase, superoxide dismutases (SOD), ferritin, and α-tocopherol (α-Toc), reduced glutathione (GSSH). RH= Lipid or other electron transfer reductant.

1.5.2 Mechanism of lipid oxidation

Lipid peroxidation is a very destructive chain-reaction that can proceed in three stages (Nohl, 1993). These stages are the initiation stage, the propagation stage and the termination stage (Mead et al, 1986; Halliwell and Gutteridge, 1999) (Fig. 1.5). The initiation stage starts when a free radical (R*) oxidizes the target a PUFA formed LH. In this reaction, a hydrogen atom is abstracted from a methylene group (-CH₂-) of the fatty acid leaving behind an unpaired electron on the carbon (-•CH⁻).
carbon radical can combine with oxygen to form a peroxyl radical (LOO•) (Howell and Saeed, 1999).

Fig. 1.5 The process of lipid peroxidation. (Adapted from Cheeseman and Slater, 1993; De Zwart et al, 1999).
In the propagation stage, the LOO* can abstract another H from another lipid molecule to produce a lipid hydroperoxide (LOOH) and a fatty acid radical (L•). This leads to an autocatalytic chain reaction. The hydroperoxide (LOOH) formed in this stage is unstable and degrades to various secondary products many of which are toxic. They decompose on heating, exposure to UV light or in the presence of transition metal ions such as iron (II) (Halliwell and Gutteridge, 1999). These secondary products include various mutagens and carcinogens such as fatty acid hydroxides, cholesterol hydroxides, endoperoxides, fatty acids epoxides, enals and other aldehydes including malondialdehyde (MDA), ketones and lactones (Howell and Saeed, 1999).

The third step in the lipid oxidation mechanism is the chain termination reaction. This can be achieved by the reaction of two free radicals together (eq-4) (Howell and Saeed, 1999).

\[
L• + L• \rightarrow L-L
\]

eq-4.

The self reaction of peroxy radicals is unlikely to be a favoured reaction until significant levels have accumulated within the membrane and peroxidation is already extensive (Halliwell and Gutteridge, 1999). Also peroxides within membranes can be removed by conversion to alcohols by phospholipid hydroperoxide glutathione peroxidase (GSH). Another possible mechanism for the termination reaction is the reaction of the peroxides with protein molecules. This reaction can cause cross linking and severe damage to proteins (Saeed and Howell, 1999). Finally, free radicals can react with antioxidants to terminate reactions.
1.5.3 Effects of lipid oxidation on cell membrane

Lipid peroxidation processes can cause damage to membrane lipids and decrease membrane fluidity (Sicinska et al, 2006). This leads to an easier exchange of phospholipids between the two monolayers, and increases the leakiness of the membrane bilayer to substances that do not normally cross it (Halliwell and Gutteridge, 1999). Continued oxidation of fatty acid side chains and their fragmentation to produce aldehydes and hydrocarbons such as pentane will eventually lead to a loss of membrane integrity.

1.6 Metal ions

1.6.1 Iron ions

Iron in the human body is stored and transported bound to proteins. Iron containing proteins can be divided into haem proteins, iron-sulfur proteins and non-haem proteins (Crichton, 2001). Haem proteins in the cytochromes function as electron transporters in both mitochondria and endoplasmic reticulum. Also, haem proteins act as oxygen carriers, activators of molecular oxygen, e.g. peroxidases and catalase.

The second type of iron containing protein is iron-sulfur protein; they contain iron atoms bound to sulfur covalently. They participate in electron transport as ferredoxins or act as enzymes such as succinate dehydrogenase and aconitase (Rawn, 1983).

The third type of iron bound protein is non-haem non-iron sulfur proteins. This group includes enzymes such as lipoxygenases and hydroxylases as well as the iron transport protein, transferritin (Crichton, 2001). Transferritin is bound to two
Fe$^{3+}$ ions and is able to transport iron to be utilized or stored in ferritin which binds up to 4500 ferric atoms (Lehninger, 1984); this requires oxidation of Fe$^{2+}$ to Fe$^{3+}$ in a process mediated by caeruloplasmin (La Fontaine et al, 2002).

1.6.1 Labile iron pool (LIP)

For iron to act as an oxidant it must be in its free form. Before extracellular iron is absorbed and utilized, it enters a pool of cytosolic free iron or labile iron pool (LIP) (Arredondo and Nunez, 2005). In mammals, dietary Fe$^{3+}$ is first reduced by ferrireductases to arrive within cells as Fe$^{2+}$ (La Fontaine et al, 2002; Crichton, 2001). Iron enters this transit pool not only from outside the cell, but also as a result of endogenous haem breakdown and the mobilization of ferritin iron (Kushner et al, 2001; Weinberg 1999). This intracellular iron is thought to be responsible for oxygen-mediated iron toxicity and is probably the major site of iron chelation by different chelators (Fernaeus et al, 2005). This pool is in steady state equilibrium, loosely bound to low molecular weight compounds such as ATP, citrate (Reelfs et al, 2004), and albumin (Kushner et al, 2001; Fernandez-Real et al, 2002; Dominici et al, 2003). The concentration of cellular non-bound iron is between 0.2-1.5 μM (Crichton, 2001). In inflammation, this concentration may rise to 7-12 μM (Benvenisiti-Zarom, 2005).

The presence of excess iron has also been demonstrated in a variety of skin pathologies that involve inflammatory responses such as psoriasis and atopic eczema (Reelfs et al, 2004) and skin carcinogenesis (Bhasin et al, 2003). Also excessive iron in specific tissues leads to infectious diseases such as AIDS (Weinberg, 1999), diabetes (Fernández-Real et al, 2002), Parkinson’s disease (Pennathur et al, 1999; Gotz et al, 2004) Alzheimer’s and other CNS diseases (Cui et al, 2005).
1.6.2 Copper ions

Copper is a trace element that is important for its catalytic function as a cofactor for proteins including mitochondrial respiratory chain complex IV and the antioxidant enzyme, Cu, Zn-SOD. Copper proteins are widely distributed in living organisms, and have two main functions electron transfer and dioxygen transport and activation. Similar to ferritin, caeruloplasmin is the principal copper-containing plasma glycoprotein. The level of non-caeruloplasmin-bound copper (II) is 0.25 μM bound to histidinate (Silwood et al, 2004). Caeruloplasmin consists of six copper atoms and plays a key role in cellular iron metabolism (Aouffen et al, 2004) in which it oxidizes the Fe$^{2+}$ to Fe$^{3+}$. Also, dietary copper is essential for the function of over 30 proteins including SOD cytochrome c oxidase (Arredondo and Nunez, 2005) and the cardiovascular system in humans and animals (Schuschke, 1997).

However, copper is also cytotoxic due to its ability to produce ROS via the Fenton reaction (Gyulkhandanyan et al, 2003). Cu$^+$ catalyses the Fenton reaction with H$_2$O$_2$ just as Fe$^{2+}$ does. Iron and copper can generate the reactive •OH through the Haber-Weiss reactions (Cheeseman and Slater, 1993; Hughes, 1999; Fernandez et al, 2002). It has been reported that exposure of caeruloplasmin to H$_2$O$_2$ induces a release of free copper ions from the protein, generating oxidative damage (Aouffen et al, 2004). On account of its capacity to catalyse the production of highly toxic hydroxyl radicals, the management of free cellular copper is of great importance. Metal chelation is an important attribution in the antioxidant mechanism of some phenolic compounds in which damage to cell components can be prevented. Antioxidants interact with metal ions to produce complexes that prevent the generation of free radicals that damage target biomolecules (Fernandez et al, 2002).
1.6.3 Mechanism of oxidative damage caused by metal ions (Fenton reaction)

The mechanism in which free iron can cause oxidative damage to DNA and other cellular components is described in Figure 1.6. During normal metabolism, molecular oxygen (O₂) is metabolized into reactive ROS via successive reactions. These reactions are catalyzed by a series of enzymes involved in oxidative damage. O₂⁻⁻ is produced as a byproduct of O₂ reduction with the electron transport chain. O₂⁻⁻ can oxidize ferritin and liberate Fe^{2+} from the protein (Henle and Linn, 1997). Also, O₂⁻⁻ dismutation releases H₂O₂ leading to the Fenton reaction. The Fenton reaction was first described in 1894, in the process of tartaric acid oxidation by Fe^{2+} and H₂O₂ (Henle and Linn, 1997). Also, O₂⁻⁻ dismutation by SOD leads to the release of H₂O₂ causing the Fenton reaction. In addition to the formation of H₂O₂ by endogenous metabolism, it is formed exogenously by neutrophils in the defense process (Polla and Polla, 2003). Fe³⁺ produced by the Fenton reaction is reduced back by NADH thus replenishing Fe^{2+}. Glutathione (GSH), one of the cellular antioxidants which cannot cross plasma membranes, is oxidized in this process (Dominici et al., 2003). Iron (II) and copper (I) ions have a major role in the production of the very reactive HO• through the Fenton and Haber-Weiss reactions (Punchard and Kelly, 1996; Fernandez et al., 2002).

In addition, metal ions bind the fatty acids of the cell membrane causing lipid peroxide decomposition to alkoxy (LO•) and peroxyl radicals (LOO•) through chain-propagation (Erba et al., 2003; Ozgová et al., 2003) (eq-5 and 6).

\[
\text{LOOH} + \text{Fe}^{3+} \rightarrow \text{LO}^\cdot + \text{Fe}^{2+} + \text{H}^+ \quad \text{eq-5.}
\]

\[
\text{LOOH} + \text{Fe}^{2+} \rightarrow \text{LOO}^\cdot + \text{Fe}^{3+} + \text{OH}^\cdot \quad \text{eq-6}
\]
Fig. 1.6 Oxidative damage to DNA and other cellular components caused by the Fenton reaction, SOD = Superoxide dismutase; GSH = glutathione peroxidase; GSSG = Oxidized glutathione. Red = Iron and iron mediated mechanism (Henle and Linn, 1997; Polla and Polla, 2003).
Ferrous iron has the capacity to reduce oxygen to superoxide radical, a reaction that, in aerobic organisms (eq-7). Several reductants or antioxidants (ascorbate, thiol compounds) can restore ferrous iron from ferric iron (eq-8). Two molecules of superoxide can dismutate, spontaneously or enzymatically, yielding molecular oxygen and \( \text{H}_2\text{O}_2 \) (eq-9) which can propagate lipid oxidation or further quenched by antioxidants.

\[
\text{Fe}^{2+} + \text{O}_2 \rightarrow \text{Fe}^{3+} + \text{O}_2^- \quad \text{eq-7}
\]

\[
\text{Fe}^{3+} + \text{A}^- \rightarrow \text{Fe}^{2+} + \text{A}_{\text{ox}} \quad \text{eq-8}
\]

\[
\text{O}_2^- + \text{O}_2^- \rightarrow \text{O}_2 + \text{H}_2\text{O}_2 \quad \text{eq-9}
\]

1.7 Apoptosis

Apoptosis is programmed cell death that is a normal process in cell development (Banerjee et al, 2005). Stimulation of apoptosis with defective DNA repair mechanisms and cell cycle alteration (Assefa et al, 2005) can contribute to several disorders such as cancer, AIDS (Allen et al, 1997), autoimmune diseases, degenerative disorders, ageing, liver and heart diseases (Ducasse et al, 2005) and viral infections (Farinati, et al, 2001). Apoptosis is caused by specific activation of a family of intracellular cysteine proteases known as caspases; with at least 14 members identified (Leung et al, 2005). Among them, the caspase-3 is required for many of the nuclear changes associated with apoptosis, including DNA fragmentation (Leung et al, 2005). Apoptosis is controlled by a network of positive and negative signal pathways. This could be via the intrinsic or extrinsic pathways. In the intrinsic pathway, mitochondria release caspase activating proteins into the
cytosol, thereby triggering apoptosis. The extrinsic pathway is regulated by proteins such as Fas and the tumour-necrosis factor receptor (TNF-R) inducing apoptosis at the cell surface. Apoptosis is characterized by cell shrinkage, plasma membrane blebbing (loss of asymmetry), protease and endonuclease activation causing DNA fragmentation (Allen et al, 1997). In contrast, necrosis (non-programmed death), is characterized by cell swelling, lysis and inflammatory response (Girotti, 2001).

1.7.1 Mechanisms of UV radiation induced apoptosis

UV radiation generates ROS that play a key role in oxidative damage of DNA and cellular proteins. UV light can also damage nuclear DNA due to direct absorption (Fig. 1.7). Caspase activation in response to UV radiation occurs mainly via the intrinsic, mitochondria-driven pathway (Lee et al, 2002). Also, caspases can be activated directly by cell surface death receptors at high doses of UV radiation or indirectly through induction of the secretion of cathepsins. The P53 protein is activated in response to DNA damage and mediates either cell cycle arrest or apoptosis depending on the extent of the damage (Assefa et al, 2005; Zhang, 2006).
Fig. 1.7  Mechanisms of UV radiation induced apoptosis (Assefa et al., 2005). ROS= Reactive oxygen species, P$^{53}$= Sequence-specific transcription factor and tumor suppressor protein.

1.8 UVA and protein damage

Exposure of skin to ultraviolet A (UVA) can lead to the formation of ROS and H$_2$O$_2$ (Yan et al, 2005). The ROS generated as by-products of UVA and UVB may cause modifications to amino acids of proteins in the epidermis, dermis (Sander et al, 2002) and stratum corneum (Thiele et al, 1999). Superoxide anion, O$_2^-$ is produced by energy transfer from several endogenous UV-absorbing chromophores, including NADH/NADPH, tryptophan, and riboflavin to molecular oxygen. O$_2^-$ is then converted by SOD to H$_2$O$_2$, which in the presence of transition metals such as iron and copper can undergo conversion to the highly reactive HO•. In turn, these
free radicals may cause many modifications in proteins such as protein cross linking and collagen degradation through activation of interstitial collagenases (Polte and Tyrrell, 2004).

The exposure of proteins to radical attack in the presence of O₂ results in multiple changes in the target molecule. These modifications include the oxidation of side chain groups, backbone fragmentation, both intra- and intermolecular cross-linking of peptides and proteins (Shen et al, 2000). Protein unfolding changes the hydrophobicity and conformation which alter the susceptibility to proteolytic enzymes (Davies et al, 1999; Kato et al, 2001; Sander et al, 2002; Heinecke et al, 2002). Ultimately, these processes result in the loss of structural or enzymatic activity of the protein and hence biological perturbations (Headlam and Davies, 2004). Oxidized proteins in the form of protein carbonyls have been detected after UVA irradiation with a dose of 770 kJ/m² in human stratum corneum (Thiele et al, 1999) and in human skin fibroblasts irradiated with 100, 250, 500 kJ/m² (Sander et al, 2002). Irradiation of human skin fibroblasts with UVA (250 kJ/m²) caused the oxidation of proteins and involved iron (Vile and Tyrrell, 1995). In addition, exposure of proteins to oxidized lipids is known to alter the amino acid in proteins sequence and protein conformation, leading to protein cross-linking (Saeed et al, 1999). Oxidized proteins can also react with aldehydes produced during lipid oxidation (Fletcher et al, 1973).

1.8.1 Mechanisms of protein oxidation and dityrosine formation

There are three mechanisms of protein oxidation and dityrosine formation. The first involves free metal ions such as copper and iron ions in vivo and in vitro (Kato et al, 2001). The H₂O₂/Cu⁺⁺ system cause dityrosine formation which is not
affected by atmospheric oxygen and starts with H$_2$O$_2$. The mechanism of dityrosine formation by metal-catalyzed oxidation is still unknown in detail (Kato et al, 2001).

The second mechanism depends on myeloperoxidase which is a haem protein secreted by activated phagocytes. This enzyme uses H$_2$O$_2$ to convert the aromatic amino acids (e.g. tyrosine) into reactive intermediates that promote oxidation of proteins and lipids. $o,o'$-Dityrosine formation begins when H$_2$O$_2$ is produced by phagocytes (Heinecke, 2002) or UVA (Yan et al, 2005). The mechanism is illustrated in Fig. 1.8 in which myeloperoxidase reacts with H$_2$O$_2$ to form compound I. Compound I is reduced back \textit{in vitro} to its native oxidation state by electron donors such as antioxidant phenols. The tyrosyl radical initiates lipid peroxidation by abstracting hydrogen from bis-allylic methylene groups of polyunsaturated fatty acids (Fig. 1.9). Three products may be formed in this mechanism; these are protein tyrosyl radical, protein-bound dityrosine and dityrosine. The radical then diffuses out of the enzyme’s active site. When the tyrosyl radical encounters a protein containing tyrosine, it abstracts an electron forming protein tyrosyl radicals and regenerates L-tyrosine. In addition, tyrosyl radical may act as a physiological catalyst for the initiation of lipid peroxidation \textit{in vivo}. 
Fig. 1.8 The mechanism of dityrosine formation in which myeloperoxidase is involved.

Fig. 1.9 Lipid peroxidation by the tyrosyl radical (Heinecke, 2002).
The third mechanism involves HO• (eq-10-11) (Audette et al 2000). Tyrosyl radical forms o,o'-dityrosine as the major product. The stability of o,o'-dityrosine compounds to acid hydrolysis makes them potentially useful markers of protein oxidation in vitro (Pennathur et al, 1999).

\[ \text{OH}^+ + \text{TyrOH (tyrosine)} \rightarrow \text{TyrO}^+ (\text{phenoxy radical}) + \text{H}_2\text{O} \quad \text{eq-10} \]

\[ 2 \text{TyrO}^+ \rightarrow \text{DiTyr (dityrosine)} \quad \text{eq-11} \]

Di-tyrosine is formed via dimerization of phenoxy radicals, and the yield of this product depends on the radical flux; its formation therefore, depends on both the overall extent of the oxidation insult and the rate of radical formation. Thus the interruption or blocking of such radicals by (poly)phenols that act as antioxidants may be useful in reducing protein cross linkage in terms of dityrosine formation resulting from UVA irradiation.

1.9 Antioxidants

Antioxidants can scavenge ROS, which are known to degrade lipid constituents of cell membranes, proteins, and DNA (Kligman, 2000). According to this property, antioxidants are reported to have beneficial effects on the prevention of cancer (Lu and Foo, 2001), wound repair, inflammation, and the improvement of the ageing process (Kligman, 2000).

1.9.1 Natural antioxidant defense system

Daily, humans are exposed to a large number of carcinogenic stimuli such as UVR and small amounts of many naturally occurring mutagens and carcinogens that are present in the normal diet which produce ROS (Conney et al, 1992). The human diet contains a large number of different compounds that possess antioxidant
activities that can scavenge ROS based on their structural properties. The most prominent are ascorbate (vitamin C) (Hughes, 1999), α-tocopherol (vitamin E) (Wolf et al., 1998; Stahl et al., 2000; Peus, et al., 2001), flavonoids (Wang et al., 2000) and green tea polyphenols (Tang et al., 2000; Jung and Ellis, 2001).

In general, the natural antioxidant defense systems are involved in prevention of the generation of ROS in vivo or to intercept with that are produced. Antioxidants exist in both the aqueous and membrane compartments of cells and can be enzymes or non-enzymatic compounds (Hughes, 1999). Antioxidant enzyme defense systems include catalase (CAT) (EC 1.11.1.61), glutathione peroxidase (GPx) (EC.1.11.1.9) and superoxide dismutase (SOD) (EC1.15.1.1). These enzymes can safely decompose peroxides, particularly H₂O₂ and scavenge free radicals such as superoxide radical (Hughes, 1999).

Vitamin E is a generic name for all naturally occurring tocopherol derivatives. D-α-tocopherol exhibits the highest antioxidant activity among tocopherols (Diplock et al., 1998). Vitamin E acts as an effective lipophilic antioxidant and radical scavenger and stabilizes the cellular membrane (Fig 1.15) (Peus et al., 2001).

![Fig. 1.10 The structure of Vitamin E (Ishige et al., 2000).](image)
Vitamin E contains phenolic hydroxyl group acting as a shield from oxidation by ROS. Also vitamin E is optimally positioned in the membrane with its side chain in the hydrophobic region of the membrane structure (Fig.1.11) (Diplock et al, 1998). Vitamin E plays a major role in maintaining cell membrane integrity by limiting lipid peroxidation by ROS (Hughes, 1999). D-α-tocopherol minimizes UV

Electron micrograph of a cell membrane

Fig. 1.11 The composition of cell membrane. Adapted from Halliwell and Gutteridge, (1999).
radiation induced skin erythema, formation of sunburn (Eberlein-König et al, 1998) cell tumorigenesis, lipid peroxidation of the epidermis, inhibition of DNA synthesis, and immunosuppression (Hughes, 1999; Peus et al, 2001).

The tocopheroxyl radical is less reactive than peroxy radical towards adjacent PUFA (Peus et al, 2001). Thus it acts as a chain breaking antioxidant and so terminates lipid peroxidation (Hughes, 1999). It is known that the antioxidants, vitamin C and E, stored in the horny layer, are degraded by UV light and can be depleted in photo-damaged skin (Kligman®, 2000). In addition, irradiation of human skin with UVA and UVB can reduce plasma total caroten e levels in vivo (El-Habit et al, 2000; Trekli et al, 2003). Part of this reduction is attributed to indirect photo-decomposition of plasma carotenoids resulting from interactions with ROS generated in blood or skin (White et al, 1988).

Vitamin C plays an important role in the synthesis of collagen, the fibrous material that makes up about 95% of the dermal matrix and whose loss with photo ageing mainly accounts for laxity and sagging of skin. Vitamin C tissue levels decrease with age, and it is considered as a corrector for wrinkled photoaged skin (Kligman®, 2000). Also vitamin C regenerates d-α-tocopherol consumed, during the process of protecting lipids from oxidation by (Eberlein-König et al, 1998). Also, pyruvate, an end product of glycolysis, is considered as antioxidant since it neutralize $H_2O_2$ non-enzymatically to water, preventing oxidative damage (Gyulkhandanyan et al, 2003).
1.10 (Poly)phenols

The main classes of (poly)phenols are: phenolic acids, flavonoids, and the less common stilbenes and lignans. The consumption of (poly)phenol rich foods or beverages has been reported to play a role in the prevention of cancers, stroke, heart disease and inflammation (Tapiero et al., 2002). Phenolic acids account for approximately one third of total (poly)phenols, and flavonoids account for the remaining two thirds (Tapiero et al, 2002).

1.10.1 Flavonoids

Flavonoids are plant secondary metabolites widely distributed in the plant kingdom. Flavonoids are subdivided into seven classes: flavones, isoflavones, flavanones, flavanols, flavonols, chalcone, and anthocyanins.

The difference in these compounds is dependant on the structure and conformation of the heterocyclic oxygen ring (C ring) of the basic molecule (Fig. 1.12). Flavonoids associate with the surface of the cell membranes to protect the polar phospholipids from oxidation (Matsushita et al, 2000). When either fed or topically applied to irradiated skin, flavonoids were photoprotective to skin (Saija et al, 1998; Aquino et al, 2002) and reduced skin inflammation (Chi et al, 2003).
1.10.2 Tea Polyphenols

The beneficial effects of green and black tea are generally attributed to the antioxidant activity of their (poly)phenols known as tea flavonoids (Majchrzak et al., 2004). There are three main categories of tea: green (unfermented), oolong (semi-fermented) and black (fully fermented). These categories result from different processing procedures (Wang et al., 2000).

Gallocatechins are the major constituents of green tea. These polyhydroxybenzene derivatives are shown to be effective antioxidants in vivo and in vitro, this is due to favorable one-electron donation properties. Of particular importance is the ability to repair the vitamin E radical, which is a unique characteristic of the gallocatechins. In addition, tea catechins are very efficient...
scavengers of biologically damaging oxyl species, such as the superoxide, \(O_2^{-}\), and \(^1O_2\) (Javanovic et al., 1998).

1.10.3 Epicatechin gallate

Green tea is particularly rich in flavonoids (Dulloo et al., 1999) such as the polyhydroxylated catechins (Hodgson et al., 2000). Catechins are colorless, astringent, water-soluble, readily oxidizable (Jung and Ellis, 2001), and have a high antioxidant capacity (Lotito and Fraga, 2000). The catechins differ in the number and position of the hydroxyl groups in the molecule (Fig.1 13). The gallate group in position 3 is relevant to the antioxidant capacity of catechins as suggested by the fact that (−)-epicatechin gallate (ECG) and (−)-epigallocatechin gallate (EGCG) are the most effective antioxidants among catechins (Lotito and Fraga, 2000). Tea catechins are flavanols and comprise 20-30% of the dry weight of green tea (Wang et al., 2000). The major catechins in fresh tea leaves and green tea according to their antioxidant activity are (−)-epicatechin gallate (ECG), (−)-epigallocatechin gallate (EGCG), (+)-epigallocatechin (EGC), (+)-epicatechin (EC) and, (+)-catechin (C) (Samman et al., 2001). A cup of green tea (2.5g/200ml) may contain 90 mg of EGCG and about 20 mg each of ECG and EC (Dong et al. 1997). In addition, catechins are present in vegetables, red wine, fruits (Young et al., 1999), spices (Conney et al., 1992) and chocolate (Lotito and Fraga, 2000).

Many studies have indicated that polyphenolic compounds present in tea may reduce the risk of a variety of illnesses including coronary heart disease (Majchrzak et al., 2004) cancer (Kao et al., 2000; Tang et al., 2001), and skin cancer induced by chemical carcinogens (Conney et al., 1992). Green tea extracts were found to stimulate apoptosis of various cancer cell lines to protect against the development
and progression of skin tumors (Jung and Ellis, 2001). In addition, green tea flavonoids are associated with a reduced risk of cardiovascular disease by inhibition of lipoprotein oxidation (Hodgson et al, 2000) and cancer (Bedouei et al, 2005). Tea flavonoids also have anti-inflammatory, and metal-chelating activities since they inhibit the absorption of non-haem iron (Samman, et al, 2001). EGCG (0.1 μM) protected human skin fibroblasts (HSF) from ROS resulted after irradiation with 200 kJ/m² UVA (Lee et al, 2005). ECG (0.8 mg/ml) has anti-inflammatory effect on rat skin (Kapoor et al, 2004) and cartilage (20 μM) (Adcocks et al, 2002) in vitro. However, the catechins effects in vivo have not been established (Kapoor et al, 2004). Also, tea and its phenolic constituents have antioxidant activity in vitro that minimizes the oxidation of fatty acids through scavenging of free radicals and metal chelation (Conney et al, 1992).

Repeated application of EGCG inhibited tumor promotion of two tumor promoters operating through different mechanisms of action on mouse skin (Fujiki et al, 1992). EGCG also acts as a suppressor for oxyradical formation and can inhibit H₂O₂ formation by tumor promoters (Fujiki et al, 1992).

Green tea polyphenols have been shown to inhibit carcinogenesis in animal models including cancers in skin (Buetler et al, 2002), lung esophagus, stomach, small intestine, pancreas, mammary gland (Dong et al, 1997), liver and duodenum (Fujiki et al, 1992). Skin carcinogenesis caused by chemicals and UV light is most studied. Green tea polyphenols inhibited cell proliferation in various tumor cell lines in culture and stimulated apoptosis, in transformed cells but not in normal cells. Ahmad et al, (1997) have investigated the effects of green tea polyphenols and the major constituent, EGCG, on the induction of apoptosis (programmed cell death) and
regulation of cell cycle in human and mouse carcinoma cells. They found that green tea may protect against cancer by causing cell cycle arrest and inducing apoptosis.

**Flavanol**

![Flavanol structure]

**Flavones**

![Flavones structure]

**Phenolic compound**

![Phenolic compound structure]

Fig. 1.13 The structure of (poly)phenols used in this study.
1.10.3 Trolox

Trolox is a water-soluble vitamin E analogue (Young et al, 1999) (Fig.1.13). Trolox is known to exhibit antioxidant activity under a variety of conditions in various systems. It protects against harmful effects of UVB radiation in human skin keratinocytes mainly through an inhibitory effect on intracellular H$_2$O$_2$ generation (Peus et al, 2001) and oxidative damage of rat heme proteins caused by CBrCl$_3$ (Chen and Tappel, 1995).

Many studies use Trolox as a reference for antioxidant activity (Pellegrini, et al, 1999; Ishige et al, 2000; Lu and Foo, 2001). Trolox equivalent antioxidant activity (TEAC) is defined as the concentration of trolox with equivalent antioxidant activity to a 1mM concentration of substance under investigation. In this procedure a substance is compared to 1mM Trolox in its ability to suppress lipid peroxidation (Ishige et al, 2000).

Trolox may be less prominent in the membrane as compared with cytosol due to its high hydrophilicity (Massaeli et al, 1999). The antioxidant activity of trolox is mainly due to formation of a stable phenoxy radical due to the oxygen in the ring system also to its ability for transferring hydrogen atom to an active oxygen radical such as peroxy (Barclay et al, 1995).

1.10.4 Luteolin and luteolin glucosides

Luteolin, a component of the flavone subclass of flavonoids, usually occurs as glycosylated forms in red and green pepper (Kim et al, 2003), celery (Tapiero et al, 2002) camomile tea (Shimoi et al, 1998) and olive oil (Ciafardini and Zullo, 2002). It is reported to be antimutagenic and antitumorigenic due to the property of scavenging hydroxyl radicals (Shimoi et al, 1998) superoxide generation (Lu et al,
metal chelation involved in the free radical generation and modulation of the activity of different enzymes (Simonetti et al, 2005). These properties have been attributed to the presence of the phenolic hydroxyl groups (Paganga and Rice-Evans, 1997) and the OH• scavenging activity is directly related to the number of hydroxyl groups especially at the 3' positions. The substitution of phenolic groups by methylation for example (Shimoi et al, 1998) decreases the antioxidant activity (Paganga and Rice-Evans, 1997).

In food, most flavonoids are present as glycosides that are bound to a sugar moiety (Young et al, 1999) such as glucose and rhamnoglucose. Flavonoids must be absorbed to exert their antioxidant function. Yang et al, (1999) reported the absorption of flavonoids and their presence in human plasma in the glycosylated form. Shimoi et al, 1998 demonstrated that luteolin 7-O-glucoside was hardly absorbed glucosylated in the small intestine of the rat; it is first hydrolyzed by the intestinal microflora to produce the aglycone. Simonetti et al, 2005 reported both the presence of luteolin and the glucosylated forms in human plasma but at very low concentrations (0.1 μmol /l) dependent on the type of food. Luteolin showed anticancer effects in human skin tumor cells at 20 μM (Lee et al, 2004), influence the downstream effectors of apoptosis in human umbilical vein cells (Bagli et al, 2004) and antiallergic activity in human basophil cell line at 30 μM (Hirano et al, 2006). Luteolin also reduced inflammation in lung tissue (Tormakangas et al, 2005) and in rat fibroblasts (Kim et al, 2003).
1.11 Objectives

A review of the above literature indicates the need to investigate the effect of selected antioxidants to counteract UV irradiation on skin cells. The objectives of this study were:

- To investigate the effect of the UVA on human skin fibroblasts (HSF) viability and the effect of 5 (poly)phenols in reducing apoptosis caused by irradiation (chapter 3).
- To study the effect of UVA irradiation on lipids in HSF with and without (poly)phenols (chapter 4).
- To examine the ability of these (poly)phenols to chelate ferric and copper ions (chapter 5).
- To investigate the effect of UVA on cell protein and the effect of (poly)phenols to reduce protein cross-linkage in irradiated skin cells (chapter 6).
2. GENERAL MATERIALS AND METHODS

2.1 Skin cell studies

2.1.1 Culture of human skin fibroblasts (HSF)

2.1.1.1 Materials

Minimum Essential Medium Eagle (MEM), (with non-essential amino acids, Earle’s salts and L-Glutamine, without sodium bicarbonate; trypsin-EDTA solution; cell culture water; dimethyl sulfoxide (DMSO); foetal bovine serum (FBS) heat inactivated; non essential amino acids solution (100X); N-(2-hydroxyethyl) piperazine-N-(2-ethanesulfonic acid) (HEPES) buffer (1M) and sodium bicarbonate were all purchased from Sigma Co. Limited, (St. Louis, USA). Penicillin/streptomycin (10,000 units/ml penicillin G and 10,000 μg/ml) in 0.85% saline were obtained from Gibco BRL, UK. Ethanol (70%) was purchased from Hayman Limited, Essex, England. A Cold centrifuge (B-22M) was purchased from International Equipment Company, Massachusetts, USA. Centrifuge tubes 50 ml, 25 and 75 cm² culture flasks were both from Corning USA, and a thermostatically controlled incubator (5% CO₂ 37° C) was from Hotpack Inc, USA.

2.1.1.2 Method

A litre of the growth medium MEM was prepared by dissolving 9.7 g, the content of one vial, in 900 ml of culture water. To this solution was added 2.2 g sodium bicarbonate, 200 mM L-glutamine (10 ml), non-essential amino acids (10 ml), HEPES buffer (10 ml), and penicillin/streptomycin (10 ml). The pH of the
medium was adjusted to 7.2 with the use of 1N HCl or IN NaOH. The medium was then filtered with 0.22 μ filter into a sterile bottle. Sterile FBS (100 ml) was added to the growth medium. The transfer medium used in the collection of skin cells contained 25% (w/v) penicillin/streptomycin antibiotic without FBS.

2.1.2 Primary cell culture

Preparation of human skin fibroblasts from newborn infants was based on the method of Tyrrell and Pidoux (1986). The foreskin of one normal healthy male infant aged 2 weeks was obtained during circumcision surgery. The foreskin was collected in 20 ml transferring medium.

The foreskin was washed twice with transferring media. The tissue was cut into 2 mm² pieces and the inner part of the tissue dermis was taken. The dermis was dissociated in 15 ml of 0.25 % trypsin 0.02 % containing EDTA solution by stirring for 30 min at room temperature. The solution was transferred to a 50 ml centrifuge tube containing 15 ml growth medium and centrifuged for 10 min at 4°C and 300 x g. The supernatant was discarded and the pellet was washed again. The pellet was then reconstituted in 10 ml growth medium, and transferred to 25 cm² culture flasks and covered with 20 ml growth medium to allow growth of primary fibroblast. The primary cultures were allowed approximately 3 weeks to attach and 6 weeks to become confluent. They were incubated in 5 % CO₂ at 37°C. Cells were collected by trypsinization on passage 2 and kept at -80°C. The next day they were transferred to liquid nitrogen in cryogenic vials.
2.1.3 Routine cell culture

Human skin fibroblasts (HSF) were grown in MEM containing 10% FBS. Cells were grown in monolayers starting with a cell concentration of $0.75 \times 10^6$ cells in 75 cm$^2$ flasks. Cells were subcultured at 3 day intervals by trypsinization. HSF were kept in a humidified atmosphere in 5% CO$_2$ at 37 °C.

2.1.4 Cell count

Counting cells with trypan blue provides an assessment of both total and viable cell counts. Cells that take the dye are considered dead, while cells that exclude trypan blue are considered viable (Mather and Roberts, 1998).

2.1.4.1 Materials

Trypan blue 0.4% (w/v in PBS); trypsinized cells suspended in culture media; haemocytometer slide with cover slip, tally counter, and inverted microscope, Hund Wetzlar GmbH, Germany.

2.1.4.2 Method

The cell suspension (50 μl) was diluted 1:1 with 0.4% trypan blue. Cells were mixed carefully with a micropipette. A drop of the mixture was placed on the haemocytometer slide using a pipette. The total cell number was counted in 4 chambers with a total volume of 0.1 mm$^3$ or $10^{-4}$ ml. The cell number/ml was calculated as follow:

$$\text{Cell number/ml} = \text{dilution factor} \times \text{average count} \times 10^4.$$
The total number of cells was obtained by multiplying cell number/ml with the original volume in which cells were suspended (10 ml).

2.1.5 Cell freezing

2.1.5.1 Materials

MEM, DMSO; FBS heat inactivated were purchased from Sigma Co Lmtd (St. Louis, USA), cryogenic vials (2 ml) were purchased from Nalgene Cryovail, N.Y., USA, and a liquid nitrogen facility was used for cell storage.

2.1.5.2 Method

The freezing medium was prepared by mixing DMSO: FBS: growth medium in a ratio of 1:3:6. Three flasks of the required passage were trypsinized and collected in a 50 ml centrifuge tube and counted in trypan blue (section 2.1.4). Cells were centrifuged at 120 x g for 10 min. The pellet was resuspended in the freezing medium at suitable density (2-3 x 10^6). Vials were stored at 4° C for 30 min, and then moved to -80 °C freezer. On the following day, vials were transferred to the liquid nitrogen container.

2.1.6 Cell thawing

The cell vial was immersed in a 37° C water bath and shaken until thawed. The content of the vial was transferred to 25 cm^2 flask with MEM (10 ml). Confluent cells were divided into 0.75 x 10^6 cell/75 cm^2 flask.
2.1.7 Cells and condition of culture

HSF derived from a child foreskin explants, were used. Cells were cultured in MEM supplemented with 10% v/v heat inactivated FBS (Sigma, St. Louis, USA), glutamine (3mM) sodium bicarbonate (0.2% w/v), penicillin, and streptomycin (10,000 units/ml penicillin G and 10,000 µg/ml). Cells were grown in T75 flasks and were passaged by trypsinization every 3 days, which involved removal of the media, a single wash with trypsin and gentle detachment by incubation for 5 min at 37°C in 0.25 % w/v trypsin. Cells used for experiments were seeded at 0.75 x 10^6 on a 10 cm culture dishes to reach a confluence of 80-90 % on the day of experiment. Cells for experiments were between passages 9-14.

2.2 Irradiation of human skin fibroblasts (HSF)

2.2.1 Materials

Cells were always irradiated before each determination by using Sellamed UV lamp 3000, purchased from Sellas Medizinische Geräte GmbH, (Gevelsberg, Germany). The lamp gave a spectral range of 340-440 nm while the other spectral portions are reduced to a minimum through three different filters. In addition it was equipped with three ventilators, 2 ventilators for cell cooling and one for device cooling. The radiation intensity was measured using UVA radiometer, purchased from Cole-Parmer, (Chicago, USA).
2.2.2 Methods

Two different doses were used in this study, 250 and 500 kJ/m². These doses represent 0.5 and 1 of the minimal erythemal dose respectively (Kambayashi et al, 2001; Offord et al, 2002). Irradiation dose of 0-100 kJ/m² is considered to mimic natural exposure to the sun (Tallec et al, 1998). A physiological dose of 250 kJ/m² UVA is equivalent to the amount of UVA that reaches the skin during 70 min of sunlight exposure at noon during a cloudless summer day at northern latitude of 35° (Vile and Tyrrell, 1995; Trekli et al, 2003). The exposure time (T) was calculated for each dose using the following formula:

\[ T = \frac{D}{E \times 60/1000} \]

\( T = \) exposure time (min.)

\( D = \) dose (J/cm²)

\( E = \) radiation intensity (mW/cm²).

The time of exposure of each UVA dose was calculated with the above equation using the average of triplicate radiation intensities. Cells were UVA irradiated on passages 9-14 in 20 x 100 mm sterile tissue culture dishes, Falcon NJ, USA. Fibroblasts were seeded at 0.75 x 10⁶/plate. Before irradiation, cells were washed twice with PBS with Ca²⁺ and Mg²⁺ and irradiated in 10 ml PBS.
2.3 Non-irradiated controls

Control fibroblast plates were washed twice with PBS with Ca\(^{2+}\) and Mg\(^{2+}\) and plates were covered with aluminum foil and kept at room temperature in 10 ml PBS with Ca\(^{2+}\) and Mg\(^{2+}\).

2.4 Cells treatment with (poly)phenols

(Poly)phenols (3 mM) were dissolved in absolute methanol and were added to the plates 18 h before irradiation and incubated at 37° C. The final concentration of each antioxidant was 30 \(\mu\)M. The volume of methanol added was 1 \%(v/v). Cells were washed twice with PBS with Ca\(^{2+}\) and Mg\(^{2+}\) and UVA irradiated.

2.5 Mycoplasma test

Mycoplasmas are a group of bacteria, which frequently contaminate cell cultures (Tang et al, 2000). They are not always obvious as bacterial contamination and usually primary cultures are free of them (Adams, 1980). Skin fibroblasts were screened for mycoplasma using a mycoplasma detection kit. Cells were tested on passage 3 and 4. The detection kit was based on ELISA, which contains polyclonal antibodies for the detection of the most common mycoplasma species contaminating mammalian cell cultures. These species are \textit{M. arginini}, \textit{M. hyorhinis}, \textit{A. laidlawii}, and \textit{M. orale}. 
Fig. 2.1 The of principle of the mycoplasma detection kit.

2.5.1 Materials

The mycoplasma Detection kit was purchased from Roche, (Germany). Sodium azide was from Sigma, UK. Microtiter immunoplate, Nunc GmbH, Denmark.

2.5.2 Method

Working solutions were prepared as stated on the kit. Step I was to coat the microtiter plate wells with 4 different coating antibodies (250 μl) in duplicates. The plate was covered tightly and incubated for 2 h. at 37° C. Each well was washed with washing buffer with sodium azide (1 g/l) to avoid microbial contamination. Step II involved adding the samples, and the negative and positive mycoplasma controls (200 μl). Samples include: cell suspension, medium with FBS, FBS-free medium and trypsin. Washing buffer was used as negative mycoplasma control while the positive control was provided with the kit. The plate was incubated overnight at 2-8° C. In step III, 4 different detection
antibodies were applied (200 µl) and the plate was incubated for 2 h at 37° C. The plate was washed thoroughly 4 times with washing buffer described by the manufacturer and streptavidin-AP enzyme was added (200 µl), and incubated for 1 h at 37° C. The final step (V) was to add the substrate nitrophenolphosphate (4-NPP) (200 µl). The plate was read after 30 min visually.
CHAPTER 3
3. CYTOTOXICITY OF UVA AND ANTIOXIDANTS ON SKIN FIBROBLASTS

3.1 Apoptosis and the effect of UVA and (poly)phenols

Apoptosis has been induced by a variety of oxidizing conditions, including \( \text{H}_2\text{O}_2 \) treatment. UVA may cause apoptosis in cells (Han et al, 2004; Assefa et al, 2005). UVA (100 kJ/m\(^2\)) induced apoptosis in human skin keratinocytes (Banerjee et al, 2005; Valencia and Kochevar, 2006). The dose of 100-1200 kJ/m\(^2\) UVA leads to apoptosis by inducing \( P^{53} \) in human melanoma cells (Zhang, 2006).

Flavonoids are (poly)phenolic compounds widely distributed in plants (Cheng et al, 2005). Their antioxidants effects are well established in reducing cancer (Simonetti et al, 2005). They are reported to inhibit DNA fragmentation and apoptosis (Girotti, 2001; Shi et al, 2005). Green tea (poly)phenols, including catechins are reported to inhibit oxidant-induced DNA strand breakage in cultured lung cells (Mukia et al, 2005). Antioxidants may prevent apoptosis by means of suppression or scavenging ROS and metal chelation. In cultured HeLa cells ECG inhibited apoptosis by 50 % at 1 \( \mu \text{M} \) D-galactosamine by quenching \( \text{O}^{2-} \) (Katunuma et al, 2006). The green tea flavonoid, epigallocatechin gallate (EGCG, 10 \( \mu \text{M} \)), which is similar in structure to ECG with extra OH group in 5' position, reduced apoptosis in neuronal cells through scavenging ROS (Choi et al, 2001). Apoptosis was suppressed in rats’ cerebellum when injected with 2.5 mg/kg of trolox (Usuki et al, 2001). Luteolin is considered as anti-tumor (Kanadaswami et al, 2005) with promising contribution against cancer (Horinaka et al, 2005). Luteolin increased apoptosis by influencing the downstream
effectors of apoptosis at 10-50 μM in human cancer umbilical vein cells (Bagli et al, 2004) and human cervical cancer HeLa cells (Horinaka et al, 2005).

### 3.2.1 Methods for apoptosis determination

Cell death or apoptosis can be evaluated by the quantification of plasma membrane damage. Many methods are used to determine cell death. These methods are based on the uptake or exclusion of dyes by active cells. Dyes such as trypan blue (Gille et al, 2002), eosin-haematoxylin (Ehemann et al, 2003), Hoechst (Fabian et al, 2003) propidium iodide (Brana et al, 2002) or ethidium bromide and 4',6-diamidino-2-phenylindole dilactate (DAPI) (Gichner et al, 2005) have been used.

A second group of assays is based on the release of radioactive isotopes such as $^{51}$Cr, $^3$H-thymidine, $^3$H-proline, $^{75}$Se-methionine or fluorescence dyes followed by flow cytometry such as using annexin V. A third type of assay is based on measuring the cytoplasmic enzyme activity released by damaged cells such as alkaline and acid phosphatase, glutamate and pyruvate transaminase (Halliwell and Gutteridge, 1999) and aspartate aminotransferase (Feutren et al, 1984). In addition, apoptotic cells can be identified by DNA fragmentation methods such as the comet assay (single-cell gel electrophoresis) (Fabian et al, 2003) and DNA laddering on agarose gels by electrophoresis. Also, apoptosis can be detected by immunohistochemistry using antibodies to detect factors involved in apoptosis (Brana et al, 2002; Ducasse et al, 2005) or even by new developed methods such as electrical evaluation of cell membrane integrity (Huang et al, 2003).
However, each method has advantages and disadvantages. Dye exclusion methods may be an insensitive and misleading measure of membrane integrity and do not allow the processing of large sample numbers (Allen et al., 1997; Walsh et al., 1998; Fabian et al., 2003). Enzymes activity assays are very difficult because of the low amount of released enzymes present in many cells and the difficult kinetic calculations required to quantitate most enzyme activities. Using radioactive isotopes in most of enzymes methods and the high spontaneous release of most labels from the pre-labeled target cells are the disadvantages of these methods. The comet assay provides qualitative answers about the presence or absence of apoptosis but the qualitative analyses of apoptotic changes are difficult (Fabian et al., 2003). DNA laddering is only suitable to groups of cells in which a relatively high percentage is simultaneously undergoing apoptosis (Gille et al., 2002). The disadvantages of immunohistochemistry techniques are the requirement for high quality antibodies reacting specifically with individual proteins cleaved in apoptosis; it is also a time consuming and mainly qualitative assay (Kohler et al., 2002).

In this study, two simple methods were used to determine the degree of cell viability after UVA and (poly)phenols addition. These were metabolic reduction of a tetrazolium salt to formazan dye (MTT assay) and the release of lactate dehydrogenase (LDH) from membrane damaged cells (LDH release assay). These methods were used to analyze cell viability, (poly)phenols and UVA cytotoxicity on human skin fibroblasts. In addition DNA fragmentation as a hallmark of apoptosis was detected by the TUNEL reaction. All these assays
are widely used as a simple and sensitive method which does not require sophisticated equipment.

3.2.1.1 The tetrazolium salt, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

The MTT assay is used to measure the cytotoxic effects of many compounds on cultured cells as mentioned by Olivier et al (2000) and, Surazynski et al (2001). They measured the cytotoxicity of daunorubicine, an antitumor agent, and melatonin on normal HSF and neuroblastoma cells. Also the MTT assay was used to determine the effects of UV irradiation on cells (Kitazawa and Iwasak, 1999), they measured the effects of UVB on murine dermal fibroblasts.

The MTT assay has been used to determine the protective or the cytotoxic effects of flavonoids on cells by many researchers (Sasaki et al, 2003). Rodgers and Grant, (1998) used the MTT dye to test the cytotoxicity of a range of flavonoids including quercetin and epicatechin (0-200 μM) on human breast cancer cells (2.5 x 10^6 cell/cm^2) incubated for 2 h with MTT.

3.2.1.2 Lactate dehydrogenase (LDH)

Lactate dehydrogenase (LDH) is a stable cytoplasmic enzyme found in all cells. It is quickly released upon damage to the plasma membrane and this strongly correlates with the number of lysed cells (Tipton et al, 2003). Like the MTT assay, this test is widely used in cytotoxicity studies (Benvenisti-Zarom, et al 2005). The normal plasma membrane is impermeable to LDH, but damage to the cell membrane results in a change in membrane permeability and subsequent
leakage of LDH into the extracellular fluid. In vitro release of LDH from cells provides an accurate measure of cell membrane integrity and cell viability. As a result, the release of LDH has proved to be a popular and reliable test for cytotoxicity in both immunological studies, where it has superseded tests using the release of radioactive chromium as an assay for cellular cytotoxicity, and in biocompatibility studies, where it has now become an important in vitro screening test. In order to assess the effects of a biomaterial on mammalian cell culture, cells are exposed to a variety of concentrations of test material over a period of days. The release of LDH into the culture supernatant correlates with the amount of cell death and membrane damage, providing an accurate measure of the cellular toxicity induced by the test substance. The assay is based upon a coupled enzymatic assay involving the conversion of a tetrazolium salt, 2-P-(iodophenyl)-3-(p-nitrophenyl) -5- phenyl tetrazolium chloride (INT), into a formazan product. The reaction is catalyzed by LDH released from the cells and diaphorase present in the assay substrate mixture. In the assay, the formazan concentration is determined by measuring the optical absorbance at 492 nm in a 96 well plate.

3.2.1.3 TdT-mediated dUTP nick end labeling (TUNEL) technique

The TUNEL reaction is widely used to detect apoptosis in keratinocytes (Kawashima et al, 2004) human lymphocytes (Ehemann et al, 2003), chondrocytes (Gille et al, 2002) and sperm cells (Enciso et al, 2005). The TUNEL reaction has several advantages (Otsuki et al, 2003). Firstly, the TUNEL method is applicable to any kind of materials: cultured cells, tissues and blood samples. Secondly, when a tissue contains a few apoptotic cells, the TUNEL
method easily detects apoptotic cells easily than electrophoresis of extracted DNA. Thirdly, the TUNEL assay enables us to topographically visualize the location of individual positive cells in tested materials. Fourthly, the TUNEL assay can detect apoptotic cells at a relatively early stage of apoptosis, because of the increase in the number of sites of free-3' OH DNA ends yielded during the apoptotic process which preceded the morphological changes of apoptosis. On the other hand, disadvantages (Allen et al, 1997) include the fact that it may be difficult to differentiate positive nuclei from the background and there may be nonspecific staining unless the procedure is optimized. Thus in this study, the method was optimized to overcome this disadvantage at the outset.

The objectives of this chapter are:

- To test five different concentrations (1-100 µM) of (poly)phenols to determine their cytotoxicity on fibroblasts and to confirm, by both MTT and LDH tests, that the chosen concentration of (poly)phenols (30 µM) was non-toxic for HSF.
- To determine the effect of UVA in inducing apoptosis in HSF and the ability of 5 (poly)phenols to prevent apoptosis was investigated using TUNEL assay.
3.2 Materials and Methods

The MTT and the release of lactate dehydrogenase (LDH) assays were used to measure the cytotoxicity of the 5 (poly)phenols (1-100 μM) on HSF, and to study the effect of UVA (250 and 500 kJ/m²) on the viability of these cells.

3.2.1 Cell culture was performed as described in section 2.1.7.

3.2.2 Cell treatment with (poly)phenols was undertaken as described in section 2.4.

3.2.3 UVA irradiation of HSF was performed as described in section 2.2.

3.2.4 Non-irradiated controls were tested as described in section 2.2.3.

3.2.5 MTT Assay

3.2.5.1 Principle

The tetrazolium salt, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay is dependant on the conversion of the soluble yellow MTT to the insoluble purple formazan by the mitochondrial enzyme, succinate dehydrogenase in metabolically active cells. This enzyme causes the cleavage of the tetrazolium ring in the structure of MTT to form formazan. Water insoluble formazan is largely impermeable to cell membranes and it accumulates within healthy cells (Jouvet et al, 2000). When formazan is solubilized in isopropanol, it produces a purple color, which is measured using a simple colorimetric assay. The intensity of the color is proportional to the amount of living cells present.
3.2.5.2 Materials

Tetrazolium salt, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (MTT) (Thiazolyl Blue 98%), minimal essential medium Eagle (MEM) and Dulbecco’s phosphate buffer saline (DPBS) (10x) without Ca$^{2+}$ and Mg$^{2+}$ (PBS) were obtained from Sigma, St. Louis, USA. Isopropyl alcohol, and methanol were purchased from BDH, Poole, England. The 96-well cell culture clear flat bottomed plates were obtained from Nunc Ltd. Denmark. Syringe filters 0.22 μm were purchased from Millipore, Bedford, Massachusetts, USA. (-)-Epicatechin gallate (ECG) (98% HPLC grade) and trolox 97% were purchased from Sigma Aldrich, St. Louis, USA. Luteolin, luteolin-4’-O-glucoside and luteolin-7-O-glucoside were obtained from Extrasynthese, Genay, France.

3.2.5.3 Method

Human fibroblasts were seeded into 96-well microtiter tissue culture plates with 200 μl MEM containing 2 x 10^4 cells/well. Cells in each well were allowed to attach for 24 h. MTT (final concentration 5 mg/ml) was dissolved in sterile DPBS (pH 7.4) in a water bath at 37° C. The solution was filtered into 10 ml tubes with 0.22 μm filter and stored at 4° C for further use. According to the
protocol (Sigma), MTT was added to each well which was equal to one tenth the original culture volume (20 μl). Cells were incubated with the dye for 2 h at 37°C in 5% CO2. At the end of the incubation period the culture medium was aspirated off and isopropanol (200 μl) was added to each well. The formazan crystals produced were dissolved completely by shaking the plate for 30 min at room temperature. The resultant color was measured at 570 nm and at 650 nm (as a reference) using isopropanol as a blank.

3.2.5.4 MTT and cell density determination

Different cell densities were seeded and incubated with MTT to find the optimum cell concentration that would give the best relationship between absorbance and cell density. These densities were 5, 10, 15, 20, 25 and 30 x 10^3 cells/well. In addition, different incubation times for the MTT test were studied including 1, 2, 3, and 4 h as described in section 3.2.5.3.

3.2.5.5 MTT and UVA irradiated human skin fibroblast

Dermal fibroblasts were seeded in 96-well culture plates at 2 x 10^4 cells/well and incubated for 24 h at 37°C and 5% CO2. Before irradiation the medium was removed by suction and cells were gently washed twice with PBS with Ca^{2+} and Mg^{2+}. Cells were irradiated in 200 μl PBS with Ca^{2+} and Mg^{2+} as described in section 2.2. Immediately after irradiation with the two UVA doses (250, 500 kJ/m²), MTT determination was performed (section 3.2.5.3).
3.2.5.6 MTT and (poly)phenols concentration

The cytotoxic effect of different concentrations ranging from (1-100 μM) of trolox, ECG, luteolin, luteolin-4'-glucoside, and luteolin-7-O-glucoside on fibroblasts was studied. Stock solution (10 mM) of each antioxidant was prepared in methanol. Fibroblasts were seeded in a 96 well microtiter plate at 2 x 10^4 cells/200 μl medium. After 24 h, 2 μl of each antioxidant were added to each well to give a final concentration of 1, 3, 10, 30, and 100 μM respectively. The cytotoxicity of methanol on fibroblasts was also determined by the addition of 2 μl of methanol to the cells (final concentration of methanol 1%). MTT determination was performed after 18 h incubation at 37°C and 5% CO₂. Absorbance was read at 570 and at 650 nm as reference background with a microtiter plate spectrophotometer and results were expressed as % control.

3.2.6 Lactate dehydrogenase (LDH) assay

3.2.6.1 Principle

Lactate dehydrogenase release was determined by a colorimetric assay for the quantification of cell death and cell lysis, based on the measurement of the enzyme released from the cytosol of damaged cells into the supernatant. The test include two steps, in the first step LDH reduces NAD⁺ to NADH + H⁺ by oxidation of lactate to pyruvate. In the second step, the catalyst (diaphorase) transfers 2 H from NADH/H⁺ to the tetrazolium salt INT which is reduced to formazan (Fig. 3.2.)
3.2.6.2 Materials

The following were purchased: MEM from Sigma St. Louis, bovine serum albumin (BSA) (w/v) from Gibco, Grand Island, USA; Triton X-100 solution from Bio-Rad Richmond, California, USA; hydrochloric acid from BDH Poole England, 37°C incubator from Hotpack Philadelphia Pennsylvania USA; centrifuge with rotor for microtiter plates, and cold centrifuge from International Equipment Company (IEC) B-22M Needham Massachusetts, USA; microtiter plate reader with 490 nm filter, and 96-well microplates clear flat bottomed from Nunc, Denmark; cytotoxicity detection kit (LDH) from Roche, Germany; (poly)phenols were purchased as in section 3.2.5.2.

3.2.6.3 Method

Fibroblasts, passages 9-14, were collected by trypsinization and washed with the assay medium MEM containing (1% w/v) FBS. Each well of the 96-well microtiter tissue culture plate were seeded with $2 \times 10^4$ cells in 200 μl of assay
medium and incubated for 24 h at 37°C. This cell number was the same as that used for the MTT assay and was also the cell number recommended by the kit manufacturer. After 24 h, the growth medium was removed and replaced with fresh assay medium (200 µl) to remove the LDH released from the cells during the overnight incubation step. Each phenolic compound was added (2 µl) at 5 different concentrations from a stock solution so the final concentration was (1, 3, 10, 30, 100 µM). As in the MTT assay, the effect of methanol as a solvent for the phenolic compounds was also tested. The maximum LDH release was determined (high control) in which cells were solubilized with a final concentration of 1% (w/v) Triton X-100. Spontaneous LDH release (low control) was determined by incubating the cells with the assay medium. The cells were incubated with the phenolic compounds for 18 h. Thus, the LDH assay specifically measured the ability of each concentration of the test compound to damage or protect the integrity of the plasma membrane. The assay medium on its own was used as a background control and subtracted from all other values. Cell-free supernatants (100 µl) were removed carefully and transferred to clean 96-well flat bottom plates. LDH activity was assayed in the supernatant by the addition of 100 µl reaction mixture containing the INT, which was reduced to the red formazan salt. The reaction mixture was incubated for 30 min at 25°C and protected from light. Absorbance was read at 490 nm and at 650 nm as reference.

**Calculation**

\[
\text{% cytotoxicity} = \frac{\text{experimental value} - \text{low control}}{\text{high control} - \text{low control}} \times 100.
\]
3.2.6.4 LDH and UVA irradiated cells

Irradiation was performed as in section 3.2.3. LDH release was determined immediately after irradiation with the two UVA doses (250, 500 kJ/m²).

3.2.6.5 LDH and (poly)phenols

The cytotoxic effect of different concentrations of trolox, ECG, luteolin, luteolin-4'-glucoside, and luteolin-7-O-glucoside on fibroblasts was studied. Cell culture and (poly)phenols treatment were performed as described previously for MTT (section 3.2.4). The LDH determination was performed after 18 h incubation at 37° C and 5 % CO₂.

3.2.7 Cell death detection by fluorescence microscopy, (TUNEL) reaction

Apoptosis resulting from UVA irradiation (250 and 500 kJ/m²) as well as from the incubation of human skin fibroblasts with 30 μM of (poly)phenols for 18 h was investigated by the TdT-mediated dUTP nick end labeling (TUNEL) technique.

3.2.7.1 Principle

The assay was first described by Gavrieli et al, (1992). Cleavage of cellular DNA during apoptosis yields double stranded, low molecular weight DNA fragments and single strand breaks in high molecular weight DNA. The principle of this assay is to identify these breaks by labeling of DNA strand breaks at the free 3'-OH DNA by terminal deoxynucleotidyl transferase (TdT).
This is called TdT-mediated dUTP nick end labeling (TUNEL) technique. The reaction mixture also contains fluorescein labels which are incorporated into nucleotide polymers and can be detected by fluorescence microscopy. The main advantage of this test is that it preferentially labels apoptosis in comparison to necrosis, thereby discriminating apoptotic cell from necrotic cell.

3.2.7.2 Materials

*In situ* cell death detection kit, fluorescein and DNase I, grade I were purchased from Roche, Germany, and 96-well microplates (clear flat bottomed) from Nunc, Denmark. Dulbecco's phosphate buffer saline (DPBS 10x) with and without Ca\(^{2+}\) and Mg\(^{2+}\), Tris-HCl buffer and bovine serum albumin BSA, powder, were obtained from Sigma, St. Louis, USA. Triton X-100 solution was purchased from Bio-Rad Richmond, USA. Paraformaldehyde powder 95-100 %, purchased from Panreac PRS, Barcelona Spain. Olympus microscope BH2 U-PMTVC, Japan, connected with Nikon camera FX-35A HFXII, Japan was used. The scale slide was purchased from Graticules Ltd. Tonbridge, Kent, England and analyzed with Image-pro plus 5.1, Silverspring, MD, USA. (Poly)phenols were purchased as in section 3.2.5.2.

3.2.7.3 Method

Cells were trypsinized, counted and seeded in 96-well plates. Each well contained 2 x 10^4/ 200 μl MEM. Cells were incubated at 37° C for 24 h. Fixation solution (pH 7.4) was prepared by dissolving 2 g of paraformaldehyde in PBS and heated at 75° C until dissolved. The solution was filtered to remove crystals which gave false results in the negative control. Washing buffer was
PBS (pH 7.4) and the permeabilisation solution contained 0.1% Triton X-100 in 0.1% sodium citrate. All reagents were freshly prepared on the day of the experiment. The TUNEL reaction mixture was prepared immediately before use and kept on ice until use. After the cells were irradiated or treated with (poly)phenols, the PBS was removed and cells were left to dry at room temperature (25° C). Cells were fixed with 200 µl paraformaldehyde solution for 30 min, rinsed with PBS and permeabilised with 200 µl of permeabilisation solution 10 min at 4° C. The wells were washed twice and the reaction mixture was added and incubated for 1 h at 37°C incubator in the dark. Each well was washed three times before analysis by fluorescence microscopy.

3.2.7.4 (Poly)phenols treatment of the cells

After cell attachment each (poly)phenol was freshly prepared as the stock solution (3 mM) and added to the cells to give a final concentration of 30 µM for 18 h. Cells were further incubated for 18 h before irradiation with the two UVA doses (250, 500 kJ/cm²).

3.2.7.5 UVA irradiated cells

Each plate was irradiated as described in section 3.2.3.

3.2.7.6 Positive controls

To create positive controls, cells were treated with DNase I to digest DNA to nucleosome sized fragments (Gichner et al, 2005). DNase I grade I vial contained 20000 units was dissolved in 500 µl buffer containing 50 mM Tris-HCl (pH 7.5), 1 mg/ml bovine serum albumin (BSA). The enzyme solution was
divided into eppendorff tubes and kept at -20°C for one month. On the day of the experiment, the enzyme was dissolved and 50 μl was added to each well of the positive control. The activity of the enzyme was 2000 U/ml. The cells were incubated with the enzyme for 10 min at room temperature to induce DNA strand breaks prior to labeling procedures.

3.2.7.7 Negative controls

Instead of adding the TUNEL reaction mixture to each well, cells were incubated in the label solution (50 μl/well) for 1 h at 37°C in the dark.

3.2.8 Statistical analyses

All experiments were performed at least three times. For the 96-well microtiter tissue culture plates, 6-8 replicate wells were used per category. The data were presented as the arithmetic mean percentages ± the standard error of mean (SEM). For significant differences between control and experimental values the P-value between groups was determined using SPSS (version 11.5) one way analysis of variance (ANOVA) followed by Bonferroni test at significance level of ≤0.05.

3.2.9 Microscopy and photographing

Observations and photographs for controls, UVA irradiated and UVA irradiated cells treated with (poly)phenols were made using Nikon FX-35A HFX camera, under 20 x lens using Kodak 400 35mm film. The slide stage scale was obtained from Graticules Ltd. (Tonbridge, Kent UK) and analyzed with Image-pro plus 5.1 Silverspring from Mediacybernetics, (MD USA).
3.3 Results

3.3.1 MTT Assay

3.3.1.1 Cell density and incubation time

The incubation time of HSF with the MTT was determined since the MTT uptake is dependant on the cell type and the cell density (Raspoting et al., 1999). Figure 3.3 shows the average of three independent MTT assays to test 4 incubation times (1-4 hours). A linear correlation ($R^2=0.99$) (Table 3.1) was obtained with 1 and 2 h incubation time. Also the relationship between with 6 different cell densities 5, 10, 15, 20, 25, 30 x 10^3 cell/well and MTT uptake is shown in Figure 3.4. The best linearity was demonstrated with a cell density of 15, 20 and 25 x 10^3 cell/well ($R^2=0.99$). The density 20 x 10^3 cell/well and 2 h incubation time was used throughout this study.

Fig. 3.3 The relationship between 4 incubation times with the amount of MTT uptake by human skin fibroblasts.
Fig. 3.4 The relationship between different cell densities and the amount of MTT uptake by human skin fibroblasts.

Table 3.1. The linear correlation ($R^2$) for (a) incubation times and (b) cell densities.

<table>
<thead>
<tr>
<th>Incubation time (h)</th>
<th>$R^2$ (correlation)</th>
<th>Cell density x $10^3$</th>
<th>$R^2$ (correlation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.99</td>
<td>5</td>
<td>0.95</td>
</tr>
<tr>
<td>2</td>
<td>0.99</td>
<td>10</td>
<td>0.98</td>
</tr>
<tr>
<td>3</td>
<td>0.93</td>
<td>15</td>
<td>0.99</td>
</tr>
<tr>
<td>4</td>
<td>0.89</td>
<td>20</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30</td>
<td>0.98</td>
</tr>
</tbody>
</table>

3.3.1.2 Cytotoxicity of UVA irradiated and non-irradiated HSF measured by MTT

The effects of UVA (250 and 500 kJ/m$^2$) on HSF survival was determined by measuring the intensity of the insoluble formazan accumulated in viable cells for plate photograph Fig 3.5 (A, B, C). As shown in Fig 3.6 non-irradiated control cells were considered as 100% survival. The average ± SEM of cell survival was 16% ± 1.5 when fibroblasts were irradiated with 250 kJ/m$^2$ UVA. Cell viability was decreased to 7 ± 1.6 when the UVA dose was doubled. In
other words 84 % cell death is caused by UVA dose of 250 kJ/m² compared with 93 % at 500 kJ/m².

Fig. 3.5. The accumulation of formazan after 2h incubation with MTT. (A) Non irradiated controls with high formazan accumulation. (B) Fibroblasts irradiated with 250 kJ/cm² UVA with less formazan accumulation. (C) Fibroblasts irradiated with 500 kJ/cm² UVA with the least formazan accumulation.

Human skin fibroblast cultures treated with MTT inspected using a Leica inverse microscope (Leica, Wetzlar, Germany) equipped with a Minolta camera (Konica Minolta, Tokyo, Japan).
Fig. 3.6 Changes in the percentage of cell survival of human skin fibroblasts irradiated with 2 UVA doses measured by MTT reduction and LDH assay (mean ± SEM) of three different experiments (n=3-4). Controls are considered as 100% survival, *p< 0.05 compared to controls. **p< 0.05 when the two UVA doses are compared.

3.3.1.3 Determination of cytotoxicity of 5 (poly)phenols on fibroblasts by MTT

The cytotoxicity of different concentrations of (poly)phenols (1-100 μM) was determined. Since all (poly)phenols are dissolved in methanol, the cytotoxicity of methanol on HSF was also determined. Methanol as a solvent for (poly)phenols was found to be non-toxic to the fibroblasts at the concentration used in the experiment (1% methanol in the culture medium). The average cell survival for 15 different experiments was 100.4 ± 2% (n=12-14). There were no significant differences between control fibroblasts and cells incubated with methanol, p=0.83.

The incubation of ECG for 18 h with HSF resulted in a significant increase in cell survival as shown in Table 3.2 which was enhanced with an increased amount of ECG in the culture medium. When the Bonferroni test was applied to results it revealed that the addition of ECG at low concentration from
1-10 μM did not affect the viability while the addition of 30 and 100 μM significantly increased cell survival by 23 and 50% respectively.

The addition of trolox had no significant effect on cell survival and had no effect at 30 μM (100 ± 2.7 %) on cell viability even at the highest concentration used (100 μM).

Luteolin, showed no significant difference at low concentrations (1-10 μM). At 30 μM luteolin there was significant increase in cell survival to 118.6 ± 3.1. In contrast to ECG, luteolin caused a significant drop in cell viability at 100 μM to 85.3 ± 4.8 (Table 3.2)

In contrast to the aglycon, luteolin, luteolin-4'-O-glucoside caused no significant increase of cell survival compared to controls and no toxic effect were obtained at 30 μM. Luteolin-7-glucoside showed an antioxidant effect by significantly increasing cell viability even at a low concentration 10 μM when compared to control to 124.6 ± 2.3. This increase was dose dependent reaching a maximum at 100 μM (128.3 μM) (Table 3.2).
Table 3.2 Viability of confluent human skin fibroblasts treated with different concentrations of the (poly)phenols detected with MTT and LDH. Values are Mean ± S.E.M from three independent experiments are presented, (n=6-8).

<table>
<thead>
<tr>
<th>Substance: ECG (μM)</th>
<th>Cell viability (% of control)</th>
<th>p-significance Compared to controls</th>
<th>Cell viability (% of control)</th>
<th>p-significance Compared to controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100.0 ± 0.0</td>
<td></td>
<td>100.0 ± 0.0</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>93.0 ± 1.1</td>
<td>NS</td>
<td>118.2 ± 2.4</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>3</td>
<td>102.0 ± 2.6</td>
<td>NS</td>
<td>116.0 ± 0.8</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>10</td>
<td>102.0 ± 2.8</td>
<td>NS</td>
<td>118.4 ± 1.6</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>30</td>
<td>123.0 ± 1.1</td>
<td>0.016</td>
<td>121.8 ± 1.9</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>100</td>
<td>150.0 ± 8.3</td>
<td>0.001</td>
<td>120.0 ± 2.7</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Substance: Trolox (μM)</th>
<th>Cell viability (% of control)</th>
<th>p-significance Compared to controls</th>
<th>Cell viability (% of control)</th>
<th>p-significance Compared to controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100.0 ± 0.0</td>
<td></td>
<td>100.0 ± 0.0</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>102.6 ± 3.9</td>
<td>NS</td>
<td>115.2 ± 2.1</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>3</td>
<td>97.6 ± 4.2</td>
<td>NS</td>
<td>115.8 ± 2.0</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>10</td>
<td>97.6 ± 4.2</td>
<td>NS</td>
<td>116.4 ± 2.1</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>30</td>
<td>100.6 ± 2.7</td>
<td>NS</td>
<td>116.6 ± 2.4</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>100</td>
<td>111.0 ± 6.8</td>
<td>NS</td>
<td>114.2 ± 2.6</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Substance: Luteolin (μM)</th>
<th>Cell viability (% of control)</th>
<th>p-significance Compared to controls</th>
<th>Cell viability (% of control)</th>
<th>p-significance Compared to controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100.0 ± 0.0</td>
<td></td>
<td>100.0 ± 0.0</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>95.0 ± 1.4</td>
<td>NS</td>
<td>110.6 ± 1.6</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>3</td>
<td>100.0 ± 1.8</td>
<td>NS</td>
<td>112.0 ± 2.2</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>10</td>
<td>103.0 ± 3.0</td>
<td>NS</td>
<td>111.0 ± 1.4</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>30</td>
<td>118.6 ± 3.1</td>
<td>0.004</td>
<td>111.8 ± 2.1</td>
<td>&lt;0.0001</td>
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<tr>
<td>100</td>
<td>85.3 ± 4.8</td>
<td>0.024</td>
<td>109.6 ± 0.5</td>
<td>0.002</td>
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</table>

<table>
<thead>
<tr>
<th>Substance: Luteolin-4-O-glucoside (μM)</th>
<th>Cell viability (% of control)</th>
<th>p-significance Compared to controls</th>
<th>Cell viability (% of control)</th>
<th>p-significance Compared to controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100.0 ± 0.0</td>
<td></td>
<td>100.0 ± 0.0</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>113.3 ± 6.6</td>
<td>NS</td>
<td>118.0 ± 1.8</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>3</td>
<td>110.0 ± 5.7</td>
<td>NS</td>
<td>120.0 ± 3.1</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>10</td>
<td>101.3 ± 4.7</td>
<td>NS</td>
<td>117.2 ± 3.6</td>
<td>0.001</td>
</tr>
<tr>
<td>30</td>
<td>117.0 ± 11.1</td>
<td>NS</td>
<td>117.0 ± 3.2</td>
<td>0.001</td>
</tr>
<tr>
<td>100</td>
<td>120.6 ± 17.7</td>
<td>NS</td>
<td>118.6 ± 2.2</td>
<td>≤ 0.0001</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Substance: Luteolin-7-O-glucoside (μM)</th>
<th>Cell viability (% of control)</th>
<th>p-significance Compared to controls</th>
<th>Cell viability (% of control)</th>
<th>p-significance Compared to controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100.0 ± 0.0</td>
<td></td>
<td>100.0 ± 0.0</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>113.3 ± 4.7</td>
<td>NS</td>
<td>106.0 ± 1.0</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>3</td>
<td>110.3 ± 3.7</td>
<td>NS</td>
<td>108.7 ± 0.7</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>10</td>
<td>124.6 ± 2.3</td>
<td>0.002</td>
<td>107.5 ± 0.8</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>30</td>
<td>126.6 ± 2.0</td>
<td>0.001</td>
<td>106.5 ± 0.2</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>100</td>
<td>128.3 ± 3.5</td>
<td>≤ 0.0001</td>
<td>107.2 ± 0.4</td>
<td>&lt; 0.0001</td>
</tr>
</tbody>
</table>
3.3.2 LDH assay

3.3.2.1 Cytotoxicity of UVA irradiated and non-irradiated HSF measured by LDH

In lactate dehydrogenase (LDH) leakage assay the integrity of the outer cellular membrane was tested (i.e. a damaged membrane leaks LDH into the medium). Cell viability was determined after HFS were irradiated with 250 and 500 kJ/m². As shown in Table (3.2) cell survival was not changed with UVA irradiation measured by LDH leakage indicating that there was no significant detection of LDH leakage during UVA irradiation.

3.3.2.2 Determination of cytotoxicity of 5 (poly)phenols on fibroblasts by LDH

Since all (poly)phenols were dissolved in 100 % methanol, the cytotoxicity of methanol on HSF was tested. Like the results obtained from the MTT, LDH assay gave no significant difference in cell viability compared to controls when cells incubated for 18 h in culture medium with 1% methanol. There was no significant difference between control fibroblasts and cells incubated with methanol (p=0.8). The average cell survival for 15 different experiments was 103.6 ± 0.98 % (n=8-12).

Cytotoxicity induced by (poly)phenols at concentrations between (1-100 μM) was determined after 18 h incubation. All concentrations of the flavonoid ECG showed a protection effect on HSF and this protection was increased with increasing the ECG dose Table (3.2). Multiple comparisons gave no significant differences between these concentrations.
Trolox raised cell viability significantly compared to controls Table 3.2. Again, these different concentrations were similar in their effects on HSF. Luteolin and its glycosylated forms were similar to trolox and ECG. All (poly)phenols had a significant increase in cell viability to different extents however, there were no significant differences between (poly)phenol concentrations used (Table 3.2).

3.3.3 Apoptosis

3.3.3.1 Effect of UVA irradiation on apoptosis induction in HSF

Positive and negative controls are shown in Figure 3.7, 1-2. It was also evident that deposits appeared similar to positive cells when solutions were not filtered (Figure 3.7, 3-4). All solutions were therefore filtered.

![Fig. 3.7 Apoptosis detected by Tunnel reaction showing 1, 2, 3, = -ve control, 4= + ve control, white bar = 25 micron.](image-url)
The effect of UVA irradiation on cell death induction was assessed in cultured HSF. The cells tested were pre-irradiated in 96-well plates with 2 UVA doses (250, 500 kJ/m^2). Control cells were treated in the same way as irradiated cells in term of washing and were kept in the culture cabinet at 25° C. The experiment was repeated 5 times on separate days each with 3 repetitions. The number of apoptotic cells were dramatically increased by UVA irradiation from 5.2 ± 1.2 to 13 ± 1.8 and 16 ± 2.5 at 250 and 500 kJ/m^2 respectively. (Table 3.3 and Fig 3.8-12, picture 1,3,5). The increase was significant compared to control cells (Table 3.3). Although there were differences in the number of apoptotic cells between the two UVA doses, this difference was not significant.

<table>
<thead>
<tr>
<th>Group</th>
<th>Apoptotic cells (n) Mean ± SEM</th>
<th>p-value#</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control HSF</td>
<td>5.2 ± 1.2</td>
<td>-</td>
</tr>
<tr>
<td>UVA 250 kJ/m^2</td>
<td>13 ± 1.8</td>
<td>0.044</td>
</tr>
<tr>
<td>UVA 500 kJ/m^2</td>
<td>16 ± 2.5</td>
<td>0.008</td>
</tr>
</tbody>
</table>

# compared to corresponding non-irradiated control, n= number.

3.3.3.2 Effect of 5 (poly)phenols on apoptosis prevention in HSF

As ROS are inducers of cell death, this raises the question as to whether (poly)phenols used could protect cells from apoptosis and/or necrosis induced by UVA. Normal human skin fibroblasts were treated with each (poly)phenol (final concentration 30μM) for 18 h prior to UVA irradiation. Fig 3.8 shows that the addition of ECG (30 μM) to control cells slightly increased the apoptotic cells from 8 ± 0.87 to 10.6 ± 1 but this was reported insignificant (Table 3.4). However, the addition of ECG (30 μM) to HSF 18 h prior to UVA irradiation dramatically decreased the number of apoptotic cells to levels even lower than the control non-irradiated cells (5.6 ± 1, 6.6 ± 1.2) compared to UVA irradiated cells (18 ± 2, 18 ± 1.5).
Table 3.4 Effect of ECG (30μM) incubated for 18 h on the apoptosis in human skin fibroblasts (HSF) irradiated with UVA (kJ/m²).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Apoptotic cells (n)*</th>
<th>Group comparison</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SEM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control HSF</td>
<td>8.0 ± 1.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Control HSF + ECG</td>
<td>10.6 ± 1.0</td>
<td>Compared to control cells</td>
<td>NS</td>
</tr>
<tr>
<td>HSF + 250 kJ/m²</td>
<td>18.0 ± 2.0</td>
<td>Compared to ECG + 250 kJ/m²</td>
<td>0.000</td>
</tr>
<tr>
<td>HSF + 500 kJ/m²</td>
<td>18.0 ± 1.5</td>
<td>Compared to ECG + 500 kJ/m²</td>
<td>0.000</td>
</tr>
<tr>
<td>ECG + 250 kJ/m²</td>
<td>5.6 ± 1.0</td>
<td>Compared to control + ECG</td>
<td>0.007</td>
</tr>
<tr>
<td>ECG + 500 kJ/m²</td>
<td>6.6 ± 1.2</td>
<td>Compared to control + ECG</td>
<td>NS</td>
</tr>
</tbody>
</table>

* n= number of cells.

Fig 3.8. The effect of ECG on the prevention of apoptosis of human skin fibroblasts after UVA irradiation. 1-control, 2-control cells with ECG (30 μM, 18 h), 3-UVA irradiated fibroblasts 250 kJ/m², 4-UVA irradiated fibroblasts 250 kJ/m² with ECG added (30 μM, 18 h), 5-UVA irradiated fibroblasts 500 kJ/m², 6-UVA irradiated fibroblasts 250 kJ/m² with ECG added (30 μM, 18 h). White bar = 25 micron.
The addition of trolox did not significantly modify the number of cells undergoing apoptosis. Actually, the incubation of trolox (30 μM) with HSF for 18 h resulted in an increase in apoptotic cells compared to control cells (6 ± 0.9, 10.6 ± 1.5). Also, the addition of trolox to UVA irradiated cells increased the number of cell death from 12 ± 0.6 to 18 ± 3.5 at 250 kJ/m². At the higher UVA dose the addition of trolox reduced the apoptotic cells from 20 ± 1 to 15 ± 3, both differences were not statistically significant (Table 3.5, Fig 3.9).

Table 3.5 Effect of trolox (30μM) incubated for 18 h on the apoptosis in human skin fibroblasts (HSF) irradiated with UVA (kJ/m²).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Apoptotic cells (n) * Mean ± SEM</th>
<th>Group comparison</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control HSF</td>
<td>6.0 ± 0.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control HSF + trolox</td>
<td>10.6 ± 1.5</td>
<td>Compared to control cells</td>
<td>NS</td>
</tr>
<tr>
<td>HSF + 250 kJ/m²</td>
<td>12.0 ± 0.6</td>
<td>Compared to trolox + 250 kJ/m²</td>
<td>NS</td>
</tr>
<tr>
<td>HSF + 500 kJ/m²</td>
<td>20.0 ± 1.0</td>
<td>Compared to trolox + 500 kJ/m²</td>
<td>NS</td>
</tr>
<tr>
<td>Trolox + 250 kJ/m²</td>
<td>18.0 ± 3.5</td>
<td>Compared to control + trolox</td>
<td>NS</td>
</tr>
<tr>
<td>Trolox + 500 kJ/m²</td>
<td>15.0 ± 3</td>
<td>Compared to control + trolox</td>
<td>NS</td>
</tr>
</tbody>
</table>

* n= number of cells.
Fig. 3.9 The effect of trolox on the prevention of apoptosis of human skin fibroblasts after UVA irradiation, 1-control, 2-control cells with trolox (30 μM, 18 h), 3-UVA irradiated fibroblasts 250 kJ/m², 4-UVA irradiated fibroblasts 250 kJ/m² with trolox added (30 μM, 18 h), 5-UVA irradiated fibroblasts 500 kJ/m², 6-UVA irradiated fibroblasts 250 kJ/m² with trolox added (30 μM, 18 h). White bar = 25 micron.

When luteolin was incubated with non-irradiated HSF it significantly increased the apoptotic cells from 7 ± 1 to 13 ± 1 (Table 3.6, Fig 3.10) After irradiation with 2 UVA doses, luteolin decreased the apoptotic cell number from 17 ± 1 to 6.5 ± 1 and 21 ± 0.4 to 7.5 ± 1 respectively.
Table 3.6 Effect of luteolin (30μM) incubated for 18 h on the apoptosis in human skin fibroblasts (HSF) irradiated with UVA (kJ/m²).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Apoptotic cells (n) *</th>
<th>Group comparison</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SEM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control HSF</td>
<td>7 ± 1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Control HSF +</td>
<td>13 ± 1</td>
<td>Compared to control cells</td>
<td>0.006</td>
</tr>
<tr>
<td>luteolin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSF + 250 kJ/m²</td>
<td>17 ± 1</td>
<td>Compared to luteolin + 250 kJ/m²</td>
<td>0.000</td>
</tr>
<tr>
<td>HSF + 500 kJ/m²</td>
<td>21 ± 0.4</td>
<td>Compared to luteolin + 500 kJ/m²</td>
<td>0.000</td>
</tr>
<tr>
<td>luteolin + 250 kJ/m²</td>
<td>6.5 ± 1</td>
<td>Compared to control + luteolin</td>
<td>0.000</td>
</tr>
<tr>
<td>luteolin + 500 kJ/m²</td>
<td>7.5 ± 1</td>
<td>Compared to control + luteolin</td>
<td>0.005</td>
</tr>
</tbody>
</table>

* n= number of cells.

Fig 3.10 The effect of luteolin on the prevention of apoptosis of human skin fibroblasts after UVA irradiation, 1-control, 2-control cells with luteolin (30 μM, 18 h), 3-UVA irradiated fibroblasts 250 kJ/m², 4-UVA irradiated fibroblasts 250 kJ/m² with luteolin added (30 μM, 18 h), 5-UVA irradiated fibroblasts 500 kJ/m², 6-UVA irradiated fibroblasts 250 kJ/m² with luteolin added (30 μM, 18 h). White bar = 25 micron.
The effects of the incubation of the 2 glycosylated forms of luteolin differ to a great extent. Luteolin-4'-glucoside addition to control cells did not increase the number of apoptotic cells from $4 \pm 0.5$ to $5.5 \pm 0.4$ (Table 3.7, Fig 3.11) when added to irradiated cells, this polyphenol caused in significant reduction in the number of apoptotic cells to the level of controls.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Apoptotic cells $(n)$ *</th>
<th>Group comparison</th>
<th>$p$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control HSF</td>
<td>$4 \pm 0.5$</td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>Control HSF +</td>
<td>$5.5 \pm 0.4$</td>
<td>Compared to control cells</td>
<td>NS</td>
</tr>
<tr>
<td>luteolin-4'-O-glucoside HSF+ 250 kJ/m²</td>
<td>$8 \pm 0.3$</td>
<td>Compared to luteolin-4'-O-glucoside + 250 kJ/m²</td>
<td>NS</td>
</tr>
<tr>
<td>HSF+ 500 kJ/m²</td>
<td>$10 \pm 1.0$</td>
<td>Compared to luteolin-4'-O-glucoside + 500 kJ/m²</td>
<td>NS</td>
</tr>
<tr>
<td>luteolin-4'-O-glucoside + 250 kJ/m²</td>
<td>$6 \pm 0.5$</td>
<td>Compared to control + luteolin-4'-O-glucoside</td>
<td>NS</td>
</tr>
<tr>
<td>luteolin-4'-O-glucoside + 500 kJ/m²</td>
<td>$9 \pm 1.0$</td>
<td>Compared to control + luteolin-4'-O-glucoside</td>
<td>0.05</td>
</tr>
</tbody>
</table>

* $n$= number of cells.
Fig. 3.11 The effect of luteolin-4'-O-glucoside on the prevention of apoptosis of human skin fibroblasts after UVA irradiation, 1-control, 2-control cells with luteolin-4'-O-glucoside (30 μM, 18 h), 3-UVA irradiated fibroblasts 250 kJ/m², 4-UVA irradiated fibroblasts 250 kJ/m² with luteolin-4'-O-glucoside added (30 μM, 18 h), 5-UVA irradiated fibroblasts 500 kJ/m², 6-UVA irradiated fibroblasts 250 kJ/m² with luteolin-4'-O-glucoside added (30 μM, 18 h). White bar = 25 micron.

The addition of 30 μM luteolin-7-O-glucoside to control cells had no significant effect on the number of apoptotic cells (1.3 ± 0.4 compared to controls 1 ± 0.6) (Table 3.8, Fig 3.12) when cells were irradiated with the 2 UVA doses, apoptotic cells number increased significantly to 11 ± 1.4 and 10 ± 1.4 respectively. Compared to luteolin-4'-O-glucoside, luteolin-7-O-glucoside had a
greater antioxidant activity in reducing cell death. Incubation of luteolin-7-O-glucoside prior to irradiation reduced the number of apoptotic cells to $2.2 \pm 0.5$, which increased to $6 \pm 2$ upon irradiation.

Table 3.8  Effect of luteolin-7-O-glucoside (30μM) incubated for 18 h on the apoptosis in human skin fibroblasts (HSF) irradiated with UVA (kJ/m²).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Apoptotic cells <em>(n)</em></th>
<th>Group comparison</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SEM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control HSF</td>
<td>1 ± 0.6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Control HSF + luteolin-7-O-glucoside</td>
<td>1.3 ± 0.4</td>
<td>Compared to control cells</td>
<td>NS</td>
</tr>
<tr>
<td>HSF+ 250 kJ/m²</td>
<td>11 ± 1.4</td>
<td>Compared to luteolin-7-O-glucoside + 250 kJ/m²</td>
<td>0.000</td>
</tr>
<tr>
<td>HSF+ 500 kJ/m²</td>
<td>10 ± 1.4</td>
<td>Compared to luteolin-7-O-glucoside + 500 kJ/m²</td>
<td>0.04</td>
</tr>
<tr>
<td>luteolin-7-O-glucoside + 250 kJ/m²</td>
<td>2.2 ± 0.5</td>
<td>Compared to control + luteolin-7-O-glucoside</td>
<td>NS</td>
</tr>
<tr>
<td>luteolin-7-O-glucoside + 500 kJ/m²</td>
<td>6 ± 2</td>
<td>Compared to control + luteolin-7-O-glucoside</td>
<td>0.000</td>
</tr>
</tbody>
</table>

* n= number of cells.
Fig. 3.12 The effect of luteolin-7-O-glucoside on the prevention of apoptosis of human skin fibroblasts after UVA irradiation, 1-control, 2-control cells with luteolin-7-O-glucoside (30 μM, 18 h), 3-UVA irradiated fibroblasts 250 kJ/m², 4-UVA irradiated fibroblasts 250 kJ/m² with luteolin-7-O-glucoside added (30 μM, 18 h), 5- UVA irradiated fibroblasts 500 kJ/m², 6- UVA irradiated fibroblasts 250 kJ/m² with luteolin-7-O-glucoside added (30 μM, 18 h). White bar = 25 micron.
3.4 Discussion

3.4.1 MTT and cell density determination

At the start, different cell densities were seeded to choose the best incubation period that gives the best relationship between the absorbance and cell density. An incubation time of 2 h and seeding cell density of $20 \times 10^3$ was chosen for the MTT method and used throughout the study. The incubation time of 2 h was also used by Didier et al., (2001) who studied the effect of UVA on human skin fibroblasts.

3.4.2 The effect of UVA on the viability of human skin fibroblasts (HSF)

ROS are produced following UVA irradiation and cause the accumulation of lipid peroxidation products with a consequent alteration in the structure and function of mitochondria (Somosy, 2000). These alterations may cause a decrease in cell viability, which is detected by MTT. The amount of UVA radiation penetrating the basal layer of the epidermis is approximately 30 % of the irradiation dose (Vile and Tyrrell, 1995).

In this study UVA irradiation of HSF reduced cell survival measured by MTT to 16 % and 7 % at 250 and 500 kJ/m$^2$ respectively. Comparing these results to the work of Han et al., (2004) who reported a 50% reduction in HSF irradiated with UVA (19 kJ/m$^2$) assessed by MTT using the same technique and cell density ($1.5-2 \times 10^4$ cell/well). These results considered high compared to the low UVA irradiation dose used. A study of Meewes et al., (2001) reported 10, 20, and 90% cell death of HSF irradiated with 900, 1200, 1500 kJ/m$^2$ UVA respectively. Also low percentage obtained with very high UVA dose may be due to that Meews have measured MTT cytotoxicity after 24 h after irradiation.
As reported by Tytus and Dobrucki (2000) formazan is accumulated as oval deposits (2-3 μm) in the region of plasma membranes and in the interior of cells. They also stated that the formation of formazan does not lead to immediate cell damage. MTT dye was not absorbed by normal cells as shown in Fig. 3.5.A. At 250 and 500 kJ/m² (Fig. 3.5.B and C) cells indicated a lower MTT intensity; however, the cells appeared intact because apoptosis events can start within the cells without the initial loss of membrane integrity (Allen et al., 1997). Thus damage in mitochondria may occur without affecting cell appearance. Also, reduced MTT may be produced in extracellular locations with dehydrogenase activity which may contribute to the total formazan production (Hansen et al., 1989). In addition, MTT reduction can be catalyzed by enzymes in the endoplasmic reticulum and cytosol as well as oxidoreductases associated with plasma membranes (Tytus and Dobrucki, 2000) which may contribute to high formazan accumulation.

On the other hand, a disadvantage of the MTT is that flavonoids such as luteolin can reduce MTT without living cells; therefore the results obtained may not reflect the true cell viability when using the MTT assay to determine the effects of flavonoids on cell growth. It has been suggested by Peng, et al., (2005) that flavonoid treated cells should be washed several times before the MTT assay. However, they used flavonoids at a high dose (25-200 μg) and for a longer incubation period with MTT (4-8 h) compared to our study. As the flavonoids concentration and incubation time with the MTT are both important factors, the present study used a low (poly)phenols concentration and short incubation time 2h compared to that reported by (Peng, et al., 2005).
The MTT assay appeared to be a sensitive indicator of cellular damage when combined with apoptosis results. The LDH release assay revealed no change in LDH during UVA irradiation. An explanation is that the amount of membrane damage caused by UVA was insufficient or the release of the enzyme is too low to affect the measurements of metabolic activity in the LDH assay. As considered by Feutem *et al*., (1984), the LDH assay has a low sensitivity in the detection of cell death. It could be suggested that LDH is estimated in both medium and cells after rupturing them by for example sonication. Also, it appears that UVA affects both mitochondria and cell nucleus before the cell membrane is damaged; this was clear in the results of MTT and apoptosis. In addition, sensitivity towards assays differs according to cell type. Tipton *et al*., (2003) used LDH and MTT (using 4 h incubation with MTT) assays for the determination of cytotoxicity of myrrh oil on gingival fibroblasts; they reported that the LDH assay was the more sensitive indicator of cellular damage compared with MTT and obtained different results when epithelial cells were used.

Actually both LDH and MTT assays are widely used in cytotoxicity studies. Variation in results may be due to reducing factors or the nature of the substances used in the research. For example, luteolin has the ability to oxidize NADH in the presence of \( \text{H}_2\text{O}_2 \) (Galati *et al*., 2002). Also reducing agents such as reduced nicotinamide adenine dinucleotide can increase \( \text{HO}^- \) generation by metal ions and by \( \text{H}_2\text{O}_2 \) interaction (Nocentini *et al*., 2001). Also a (poly)phenol may affect the enzyme activity as quercetin effectively inhibited LDH activity (Middleton *et al*., 2000). In contrast to our results UVA radiation (250 kJ/m\(^2\)) killed 15% of cultured HSFs and released 4% of the total LDH content of these fibroblasts in culture (Vile and Tyrrell, 1995). Membrane integrity was
determined by measuring LDH release from cells via the LDH assay with and without UVA, at a low dose of UVA 18 kJ/m² which did not cause membrane damage in fibroblasts (Philips et al, 2003).

3.4.3 Cytotoxicity of (poly)phenols measured by MTT

Loss of cell membrane integrity may be one of the direct consequences of lipid peroxidation (Cereser et al, 2001). The present study shows that ECG antioxidant activity started at 30 μM and the effect was increased with ECG concentration (30 and 100 μM). It can be concluded that ECG at 30 μM had antioxidant activity which increases with concentration as also reported by Lotito and Fraga, (2000). They used catechins as antioxidants in human blood plasma to delay lipid oxidation; they found ECG to be the most effective and its activity increased with increasing dose.

Trolox has no effect on HSF survival at 30 μM and may be needed at a higher concentration than 30 μM for antioxidant activity; this was obvious since trolox insignificantly started to increase cell viability from 100 ± 0 to 111 ± 6.8 % at 100 μM. Park et al, (2000) used trolox as an antioxidant in neuroblastoma cell lines at 50-500 μM concentrations and reported that at 400 μM trolox had a partial protective effect. Similar concentration of trolox (400 μM) reduced apoptosis 35 % when added to blastocytes incubated for 5 days and measured by the TUNEL assay.

Although luteolin resulted in an 18.6 % increase in cell survival compared to control at 30 μM measured by MTT, it showed prooxidant effect on HSF at 100 μM. Prooxidant activity of luteolin in 3 h incubation with 100 μM in hepatoma cells was also obtained (Michels et al, 2005). At 250 μM luteolin caused 4 fold increases in caspase3 activity in rat hepatoma cells incubated for 24
h (Michels et al, 2005) indicating increased prooxidant activity with increased concentration. The prooxidant activity of luteolin was documented (Lee et al, 2002; Miura et al, 2003; Cheng et al, 2005). The toxic effect of luteolin may be understood from its mechanism of action. Luteolin is able to inhibit the cell cycle and to induce apoptosis is contributed to it potential in cancer therapy.

The mechanism by which luteolin affect apoptosis in carcinogenic cells was discussed by Leung et al, (2005). Generally, cells with damaged DNA must arrest to allow repair of damage before replication. Thus luteolin induces DNA damage and cell cycle arrest in order to allow DNA repair. Therefore it was considered that the effect of luteolin inducing apoptosis was suspected to result from the antioxidant rather than the prooxidant action of luteolin (Leung et al, 2006). Also flavonoids act as antioxidant or prooxidant depending on the reaction condition (Miura et al, 2003). For example luteolin was examined at 40-80 μM in the presence and absence of 1 mM Ca^{2+} in (Lee et al, 2002). At 80 μM luteolin exhibited prooxidant activity due to the presence of metals ions. The results showed toxicity effects with quercetin, an analogue to luteolin with one additional OH at position 3, at 50 μM and above.

3.4.4 Cytotoxicity of (poly)phenols measured by LDH

Although a significant increase in cell viability was observed for all of the (poly)phenols used measured by LDH, it did not show the differences between concentrations of (poly)phenols added. This assay has been shown to be a relatively insensitive index for cytotoxicity assessment (Rodgers and Grant, 1998).
It appears that the structure of (poly)phenols affects the antioxidant activity. The catecholic hydroxyl group on the B ring is important for the antioxidant activity of luteolin (Miura et al., 2003). The catechol structure also exists in luteolin-7-O-glucoside. Thus glucosidation of the 4' hydroxyl group reduced the antioxidant effect of luteolin-4'-O-glucoside.

3.4.5 Effect of UVA on apoptosis in HSF measured by TUNEL reaction

Oxidative damage to cellular membranes is known to produce marked changes in molecular organization of lipids, resulting in increased membrane permeability and fragility. Such structural disorganization due to lipid peroxidation may result in leakage of cytoplasmic content from injured cells and may contribute to cell death (Cereser et al., 2001).

Due to the increase in the apoptotic cells, it appears that UVA irradiation has a role in ROS production in HSF, which in turn caused DNA damage followed by cell death. These ROS exacerbate formation of the highly reactive hydroxyl radical (HO•), which in turn can cause DNA strand breaks (Matsufuji et al., 2006; Wei et al., 2006), initiate lipid peroxidation and trigger apoptotic cellular death process. Also, the intracellular peroxide levels induce apoptosis (Lee et al., 2002). UVA produces O$_2$•$^-$ (Morliere and Santus, 1998) which in turn produces H$_2$O$_2$. However, H$_2$O$_2$ can cross membranes easily and is formed in human cells exposed to UVA. Also metals ions contribute to apoptosis formation, H$_2$O$_2$ can react with reduced transition metal ions (Fe$^{2+}$, Cu$^+$) to generate highly reactive HO• which are able to attack all components of DNA. Iron and copper are
commonly bound or very close to the DNA. DNA treated with $\text{H}_2\text{O}_2$ in the presence of iron and copper can produce single and double-strand breaks.

The apoptotic effect of two UVA doses on HSF was measured by the TUNEL reaction. Apoptosis increased by 2.5 and 3 times compared to control cells at 250 and 500 kJ/m$^2$ respectively. The extent of DNA breakage was UVA dose-dependent (Nocentini et al, 2001). In two recent studies, UVA-induced apoptosis in normal HSF was investigated using the Annexin V assay and gave a 3.6 times increase at a very low dose 19 kJ/m$^2$ (1.48 $\pm$ 0.11 control cells vs. 5.4 $\pm$ 0.12 UVA irradiated cells) (Han et al, 2004). Using the same technique by Didier et al, (2001) who measured apoptosis after cells were exposed to 50 and 100 kJ/m$^2$ reported 3.6 and 3.4 fold increase in apoptosis respectively (8.29 % for control cells, 29.9 and 28.6 % for 50 and 100 kJ/m$^2$, respectively). Annexin V method used may be more sensitive marker in apoptosis detection as described by Walsh et al, (1998).

### 3.4.6 Effect of (poly)phenols on apoptosis in HSF measured by TUNEL reaction

In this study, the possibility of photoprotective activity of 5 (poly)phenols supplemented exogenously on UVA-irradiated HSF were evaluated taking the induction of DNA damage as an indicator. These (poly)phenols are: ECG, trolox, luteolin, luteolin-7-O-glucoside and luteolin-4'-O-glucoside. The current study provided evidence that all (poly)phenols used, except trolox, could act as antioxidants and protected cells from oxidant damage and blocked apoptotic and necrotic cell death.
Green tea epicatechin, ECG exhibited the highest antioxidant activity compared to other (poly)phenols used when incubated with HSF 18 h before UVA irradiation. The number of apoptotic cells decreased significantly when compared to UVA irradiated cells at both doses.

The addition of trolox to cells had no effect on reducing apoptotic cell numbers. This matches the results obtained by Nocentini et al, (2001) who found that trolox by itself at 1-200 μM did not modify the integrity of DNA in non-irradiated HSF. In humans, the plasma concentration of the main form of vitamin E, α-tocopherol, is about 20-30 μM (Nocentini et al, 2001). This concentration is similar to that used in this study in which no cytotoxicity was observed when tested by MTT and LDH assays.

Upon UVA irradiation, cells treated with trolox exhibited an insignificant increase in DNA damage from 12 ± 0.6 in the control to 18 ± 3.5. Trolox has thus resulted in almost 1.5 times DNA damage compared to UVA (250 kJ/m²) irradiated cells. As measured by Nocentini et al, (2001) trolox increased DNA strand breaks in UVA irradiated HSF (50 kJ/m²). The extent of DNA damage was UVA dose dependant and damage also increased with increasing trolox concentration (10-200 μM). In this study doubling the UVA dose (500 kJ/m²) resulted in decrease of the number of apoptotic cells (Table 3.3); this may be explained by the fact that trolox needs a longer time to penetrate the lipids bilayers to act as an antioxidant (Matsufuji et al, 2006). Trolox had moderate water solubility and can be incorporated in both water and lipid compartments of cells (Poljsak et al, 2006). Also Nocentini et al, (2001) observed that the extent of DNA damage of HSF subjected to vitamin E, the lipophilic form of trolox, was at a lower degree than trolox.
Since trolox does not absorb in the UVA region, it may not act as a photosensitizers producing free radicals therefore, the increase of UVA-induced DNA damage suggests that in normal HSF, trolox, upon irradiation, facilitates the formation of oxygen species which are more reactive, or in greater quantity than those normally produced by UVA alone.

3.4.7 Conclusions

- UVA irradiation appeared to produce a great damage to HSF; it reduced cell viability to 84 and 93 % and caused 60 and 67 % apoptosis to cells at 250 and 500 kJ/m² respectively. These changes were detected by MTT and TUNEL assays but not with LDH assay.

- None of the added (poly)phenols had a toxic effect on cells when incubated with cells at 30 μM for 18 h. The (poly)phenols effect on HSF was either increasing viability as for ECG, luteolin and luteolin-7-O-glucoside or no effect at all as for trolox and luteolin-4'-O-glucoside.

- When the (poly)phenols were added to irradiated cells, interestingly, they showed reduction in apoptotic cell number except of trolox which had no significant change in cell viability or apoptosis. ECG reduced the apoptosis by 68% and 63% respectively when added to irradiated cells. Luteolin showed a different pattern; it showed a prooxidant effect without irradiation increasing apoptotic cells to 46 %. With UVA irradiation, luteolin decreased apoptosis 62 and 64 % respectively. The position of glucose moiety in the glycosylated forms of luteolin was important in the antioxidant activity. Luteolin-4'-O-glucoside reduced apoptosis by 25 and 10 % which was less active than luteolin-7-O-glucoside (80 and 40 %) due to the importance of 4'-hydroxyl group.
CHAPTER 4
4. EFFECT OF 5 PHENOLIC COMPOUNDS ON LIPID OXIDATION IN UVA IRRADIATED HUMAN SKIN FIBROBLASTS.

4.1 Introduction

4.1.1 Ultraviolet radiation A (UVA) effect on skin

UVA is very cytotoxic to human skin cells (Morliere and Santus, 1998; Merwald et al, 2005). It penetrates deep into the dermis, where it provokes dermal connective tissue alterations (Bonina et al, 1996) associated with photoageing and skin cancer (Assefa et al, 2005). UVA induces lipid peroxidation in cultured human skin fibroblasts (HSF) (Polte and Tyrrell, 2004). As a consequence of UVA, cell membrane damage (Morliere and Santus, 1998), DNA strands breaks, (Halliwell, 2000) and damage to lipids and proteins (Damiani et al, 2002; Kang et al, 2004) may occur.

Lipid peroxidation results from the interaction of ROS with double bonds in lipids of PUFA in cell membranes (Callaway et al, 1998; Maziere et al, 1999). ROS include free radicals (e.g. O$_2^*$ and OH$^*$), as well as non-radicals like H$_2$O$_2$ and $^1$O$_2$. ROS have been involved in causing a wide range of skin disorders such as inflammation, cancer (Jin et al, 2001) depigmentation (Schallreuter and Wood, 2001) and ageing (Richert et al, 2002). Evidence for the existence of hydroxyl and peroxyl radicals was demonstrated in the skin of live mice during UVA irradiation (Takeshita et al, 2005). Lipid oxidation pathways also involve iron (Polte and Tyrrell, 2004) and under the oxidative stress conditions, iron is released by reductants such as superoxide anion (O$_2^*$) and hydrogen peroxide (H$_2$O$_2$) from bound proteins (Callaway et al, 1998). These ROS propagate the peroxidation process and lead to the accumulation of lipid oxidation products.
such as peroxides; which, in turn, decompose mainly to MDA (Basu-Modak et al, 1996).

4.1.2 Malondialdehyde (MDA) and peroxidation products of lipids after UVA

Thiobarbituric acid reactive substances (TBARS) are considered as a biomarker of lipid peroxidation (Liu et al, 1997; Orhan, et al, 2004). Determination of TBARS is one of the simplest and most widely used methods for measuring lipid peroxidation in foods (Gutteridge and Quinlan, 1983); cell-membranes after UVA irradiation (Giordani et al, 2000); HSF after exposing to oxidants (Maziere et al, 1999; Cereser et al, 2001); and in studies investigating the role of antioxidants (Ferrali et al, 1997) in biological systems such as liposomes (Damiani et al, 2002) and liver extracts (Farinati et al, 2001). MDA arises largely from peroxidation of PUFAs containing more than two double bonds such as arachidonic and linolenic acid (Gutteridge and Howell, 1999). Determination of TBARS in biological samples relies on spectrophotometric detection at 532 nm (Vile and Tyrrell, 1995) or fluorescence assay (Lasch et al, 1997). However the HPLC method is more sensitive and specific than the spectrophotometric methods due to less interference from other substances (Punchard and Kelly, 1996). Although the MDA is only one of the secondary products derived from lipid peroxidation of PUFAs of membranes, it is often assumed that the main component of the TBARS is the MDA-thiobarbituric acid adduct (Cereser et al, 2001) according to the following equation:
After UVA irradiation, radical intermediates of lipids such as peroxides and their end product such as MDA are produced. Irradiation of HSF with 250 kJ/m² caused the production of H₂O₂ and MDA (Vile and Tyrrell, 1995; Masaki et al, 1995; Nocentini et al, 2001). Merwald et al, (2005) reported 10 fold increase in TBARS released in human skin carcinoma cells when UVA irradiated with 600 kJ/m². The content of free fatty acids in human stratum corneum as determined by Lasch et al (1997) is 20 wt % lipid thus indicating a large proportion of substrate for oxidation. MDA is toxic, mutagenic and carcinogenic (Burdan et al, 2001) and has been shown to be a good initiator of carcinogenesis in mouse skin (Shamberger, 1977).

4.1.3 Peroxidation products

Peroxides, generated as a result of UVA irradiation in HSF, has been determined by methods based on fluorescence detection. Masaki et al (1995) detected H₂O₂ by monitoring the change of non-fluorescent dihydro-rhodamine to fluorescent rhodamine by H₂O₂. Also Nocentini et al (2001) used the nonfluorescent diacetate which is converted by H₂O₂ in the presence of peroxidases to generate intracellular generation of green fluorescent fluorescein diacetate.
In the normal human body, peroxidases catalyze the reaction of SCN$^-$ with H$_2$O$_2$ according to eq-2. This reaction is may be important for non-immunoglobulin defense mechanisms especially in the human mouth; and may also limit the accumulation of toxic H$_2$O$_2$ in the range of 8-13 μM since human fibroblasts and other mammalian cells cannot tolerate H$_2$O$_2$ greater than 10 μM for extended periods of time (Pruitt et al, 1986).

\[ \text{SCN}^- + \text{H}_2\text{O}_2 \rightarrow \text{OSC}N^- + \text{H}_2\text{O} \quad \text{eq-2} \]

Aerobic cells including skin cells have evolved a complex antioxidant defense system. These defense systems include nonenzymatic antioxidants and include lipid soluble antioxidants such as vitamin E, and β-carotenoids; hydrophilic antioxidants include ascorbic and uric acid. Plasma proteins, glutathione and urate are considered as endogenous antioxidants (Middleton et al, 2000). Enzymatic antioxidants include superoxide dismutases (SOD), catalase, and glutathione peroxidase (GPx). Superoxide dismutases are metalloenzymes that catalyze the reduction of O$_2$ to H$_2$O$_2$. H$_2$O$_2$, produced by the reduction of O$_2$ is subsequently detoxified by catalase present in peroxisomes or by the selenoenzyme glutathione peroxidase located in the cytosol. UVA induced the mode and antioxidant defense system such as superoxide dismutase and glutathione peroxidase (Meewes et al, 2001; Merwald et al, 2005).

### 4.1.4 Effect of flavonoids on lipid oxidation

Dietary (poly)phenols are recognized as beneficial compounds against skin lipid oxidation resulting from UV irradiation (Bonina et al, 1996). Flavonoids are a group of (poly)phenolic compounds with varying structure and
characteristics that exert a wide range of antioxidant properties. The intake (Cornish et al, 2002) or topical application (Bonina et al, 1996) of flavonoid was reported to lower the ROS produced after UVA irradiation. Flavonoids can prevent lipid peroxidation at different stages, these include scavenging free radicals and (Afanasev et al, 1989) superoxide anion (Reddan et al, 2003) in the initiation stage or reacting with peroxyl radicals in the propagation stage, thus acting as a chain breaking according to eq-3 (Middleton et al, 2000). Furthermore, (poly)phenols may act as antioxidants by chelating metal ions (Ferrali et al, 1997; Fraga and Oteiza, 2002). Moreover, flavonoids can regenerate vitamin E which itself is involved in UV skin protection (Boelsma et al, 2001; Mukai et al, 2005).

\[
\text{LOO}^* + \text{FL-OH} \rightarrow \text{LOOH} + \text{FL-O}^* \quad \text{eq-3}
\]

The green and black tea constituent epicatechin gallate (ECG) (1-6 mM) is reported to protect against oxidative damage in biological systems by quenching \(^1\text{O}_2\) (Mukai, et al, 2005). Trolox showed antioxidant activities against lipid peroxidation when used alone (Benvenisti-Zarom et al, 2005), or with synergy of other antioxidants (equations-4-7) such as ascorbic acid (Juliano et al, 1999) and other tea catechins (Wie et al, 2006). Trolox may exhibit both prooxidant and antioxidant characteristics. For example, when trolox (0.75 mM) was incubated with LDL in Fe-ADP free radical generating system it increased MDA production whereas it showed an antioxidant effect at concentration of 1.3 mM (Massaeli et al, 1999).
LOO• + Trolox → LOOH + TX-sq•  
\text{eq-4}

\text{Peroxyl radical} \quad \text{Trolox-semiquinone radical}

LOO• + TX-sq• → TX-q  
\text{eq-5}

\text{Trolox-quinone}

TX-sq• + Antioxidant → Trolox + Antioxidant radical  
\text{eq-6}

Antioxidant radical + LOO• → LOOH + Antioxidant-q  
\text{eq-7}

The incorporation of luteolin as antioxidant showed antiallergic activity (Mastuda \textit{et al}, 2002) and anti-inflammatory effects (Kim \textit{et al}, 2003). Also, luteolin was protective against cytotoxicity of hydroperoxides (Sasaki \textit{et al}, 2003). Luteolin showed 3 times reduction of linoleic acid hydroperoxide when 50 μM was added to PC12 rat kidney cells (Sasaki \textit{et al}, 2003) and 20 μM luteolin modulated the transcription complex in rat fibroblasts resulting in reduced inflammation (Kim \textit{et al}, 2003). Luteolin (10-100 μM) protects against inflammation resulting from H$_2$O$_2$-induced oxidative damage when incubated with rat oligodendrocytes for 3 hours (Meeteren \textit{et al}, 2004) and retarded the formation of lipid peroxides in cotton seed oil at 0.1 mmol/l (Tsimogiannis and Oreopoulou, 2004). Luteolin-7-O-glucoside showed free radical scavenging properties in myocardial ischemia in rabbits (Rump \textit{et al}, 1994). Also, luteolin-7-O-glucoside significantly suppressed the superoxide generation induced in human neutrophils in concentration-dependent manner (0-50 μM) (Lu \textit{et al}, 2002). Luteolin-7-O-glucoside reported to improve the myocardial ischemia in rabbit heart due to free radicals scavenging properties.

Flavonoids that can scavenge radicals effectively usually give rise to semiquinone free-radicals eq-8. The aroxy radical, semiquinone free-radical, may react with a second radical (R•) acquiring a stable quinone structure.
4.1.5 Prooxidant effects of polyphenols

Although polyphenols and flavonoids are reported to prevent photioxidative skin damage (Bonina et al, 1996) they also may exhibit prooxidant effects which lead to tumor cell apoptosis (Galati et al, 2002). Its worth noting that the reduced forms of iron and copper produce HO• more efficiently upon reaction with H2O2 than the oxidized forms, so reducing agents such as (poly)phenols can increase HO• generation by metal ion and H2O2 interaction thereby increasing lipid oxidation and MDA production (Nocentini et al, 2001). However, the aroxyl radical can also interact with oxygen, generating quinones and O2• which may be responsible for the undesired pro-oxidant effects of flavonoids (Tiwari, 2001).

The objectives of this chapter are

- To monitor the effect of two doses of UVA, 250 and 500 kJ/m² on lipid oxidation products (hydroperoxides and MDA) in human skin fibroblasts.
To investigate the effects of 5 (poly)phenols namely epicatechin gallate (ECG), trolox, luteolin, luteolin-4'-O-glucoside and luteolin-7-O-glucoside (30 μM) incubated 18 h prior irradiation on the reduction of the lipid oxidation products.
4.2 Materials and Methods

4.2.1 Cell culture was performed as described in section 2.1.7.

4.2.2 Cells treatment with (poly)phenols was undertaken as described in section 2.4.

4.2.3 UVA irradiation of human skin fibroblasts (HSF) was performed as described in section 2.2.

4.2.4 Non-irradiated controls was tested as described in section 2.2.3.

4.2.5 Preparation of lipid extracts:

4.2.5.1 Materials

Epicatechin gallate (ECG) from green tea, 98% HPLC grade and Butylated hydroxytoluene (BHT) were purchased from Sigma St. Louis, 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic (trolox) acid 97% purchased from Sigma Aldrich, St. Louis. Luteolin (3',4',5,7-tetrahydroxyflavone), luteolin-4'-O-glucoside, and luteolin-7-O-glucoside were purchased from Extrasynthese, Genay, France. Chloroform analytical grade (AR) was obtained from LAB-Scan, Ireland. Methanol (HPLC grade) was from Fisher Scientific, U.K. Anhydrous sodium sulfate was obtained from Surechem Ltd, Needham Market Suffolk, England, A cold centrifuge IECB-22M, Massachusetts, USA, and sonicator Kinematica, GmbH, Kriens-Luzern, Switzerland were used.
4.2.5.2 Method

The method was based on a modified method of Bligh and Dyer (1959) described by Schreiner et al, (2000). For lipid extraction, HSF were prepared and UVA treated as described in section 4.2.3. HSF were trypsinized and collected in 15 ml centrifuge tubes. Cells were centrifuged (3000 x g) and the supernatant was discarded. Cells were decanted and the cell count and weight of the pellet were recorded. The extraction solution was prepared by mixing chloroform and methanol (2:1 v/v) BHT (0.1 mg/g cells) was also added to the extraction mixer to prevent further oxidation of cell extracts. The cells were mixed with 6 ml extraction mixture and homogenized for 1 min. This was followed by the addition of 4 ml of chloroform and 2 ml of distilled water and homogenized for another 30 sec. The extract was then sonicated, on ice for 1 min and centrifuged at 3000 x g for 10 min. at 4°C. Three layers were formed after centrifugation. The chloroform layer was collected by aspiration and dehydrated by the addition of anhydrous sodium sulfate (100-300 mg). The sample was filtered through Whatman paper number 1 under suction into 20 ml glass vials. The chloroform was evaporated under OFN at room temperature. The vial containing the lipid extract was weighed and wrapped in aluminum foil to prevent light induced lipid oxidation.

4.2.6 Determination of peroxide value (PV) by the thiocyanate method

4.2.6.1 Materials

(Poly)phenols purchased as in section 4.2.5.1. Ferrous chloride tetrahydrate; ferric chloride and hexane were purchased from Sigma St. Louis,
USA. Concentrated hydrochloric acid was from BDH, England. Absolute ethanol was from Hayman Ltd, England and ammonium thiocyanate from NTL, U.K. Oxygen free nitrogen (OFN) was obtained from Abdulla Hashim Company, Jeddah, SA. Spectrophotometer 1201 Shimadzu, Japan was used.

**4.2.6.2 Methods**

The thiocyanate method is based on Matsushita *et al* (2000). The colorimetric method for determining peroxide value was based on the oxidation of ferrous \( \text{Fe}^{2+} \) to ferric \( \text{Fe}^{3+} \) with hydroxides in the presence of thiocyanate ions according to the following equation

\[
\text{H}_2\text{O}_2 + \text{Fe}^{2+} + \text{SCN}^- \rightarrow \text{Fe(SCN)}_3 \quad \text{eq-9}
\]

(Red ferric thiocyanate)

**4.2.6.3 Standard curve preparation**

A standard curve of \( \text{FeCl}_3 \) in 3.5 % HCl in distilled water (v/v) was prepared over a range of 0-0.2 mg \( \text{FeCl}_3 \)/ml HCl. Absolute ethanol (5 ml) was pippeted into test tubes, which were previously washed with acetone and perfused with OFN. The second addition was 100 µl \( \text{NH}_4\text{SCN} \) 300 g/l (w/v) in distilled water; this was followed by the addition of 200 µl hexane. Each sample was mixed for exactly three min (slight alteration in the mixing period affected results). The sample was measured at 500 nm against ethanol as a blank.

**4.2.6.4 Sample treatment**

HSF, (controls, UVA irradiated, non-irradiated, and antioxidant treated) were trypsinized and collected in 15 ml centrifuge tubes. Lipid was
extracted as in section 4.2.5. Oxygen free nitrogen dried sample extracts were dissolved in 200 μl hexane. Absolute ethanol (5 ml) and ammonium thiocyanate (100 μl) were added to the sample and mixed. Ferrous chloride (100 μl) was added last. The sample was mixed and the absorbance was measured after exactly 3 min at 500 nm. The sample blank was treated exactly the same as the sample, except that it did not contain lipid extract instead 200 μl of hexane was added.

The PV was calculated in 1 g cells by the following equation:

\[
\frac{\text{Sample Absorbance} - \text{Sample blank Absorbance} - c/m}{55.84 \times \text{wt of lipid in (g)}}
\]

\(c\) = Intercept from the standard curve linear equation.

\(m\) = Slope of the standard curve.

55.84 = Molecular weight of Fe.

4.2.7 Determination of thiobarbituric acid reactive substances (TBARS)

4.2.7.1 Materials

(Poly)phenols purchased as in section 4.2.5.1. Thiobarbituric acid (TBA), butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and ethanolamine were purchased from Sigma St. Louis, USA. 1,1,3,3-tetraethoxypropane (TEP) and acetonitrile (HPLC grade) were purchased from Sigma Aldrich, Milwaukee, USA. Absolute ethanol was obtained from Hayman Limited, (Essex, UK). Concentrated hydrochloric acid and butanol were from BDH Poole, England. Sodium hydroxide was purchased from Pace Grove, UK. High performance liquid chromatography (HPLC) Waters Dual λ 2487
absorbance detector, 1525 binary pump and 717 plus autosampler (Breeze, Millford, USA); a cold centrifuge IECB-22M, Massachusetts, USA, and water bath from Precision Scientific Model 50, Illinois, USA were used.

4.2.7.2 Methods

4.2.7.3 Standard curve preparation

Malondialdehyde thiobarbituric acid reactive substances (MDA-TBARS) were determined according to Gutteridge and Quinlan (1983). A stock solution of TEP (1 μg/ml) was prepared for constructing the standard curve with a range of 0-1 μg/ml. To each tube of standard, 100 μl BHA (0.2 % w/v in ethanol) was added. This was followed by 500 μl of 25 % HCl in water. Finally 500 μl of TBA (1% w/v in 50 mmol NaOH) was added. After mixing for 20 sec, the tubes were incubated in a water bath at 80° C for 30 min. The tubes were cooled to room temperature and 2 ml of butanol was added to extract the TBARS (Cereser et al, 2001). The mixture was centrifuged at 3000 x g for 10 min. Two layers were formed, a pink top layer with chromagen and, a clear translucent layer at the bottom with inorganic solvent. The upper layer was collected and dried under OFN in a water bath at 37° C. For analysis, each tube was reconstituted with 1 ml mobile phase and mixed for 20 seconds.

4.2.7.4 Sample treatment

Skin fibroblasts (controls, UVA irradiated, non-irradiated, and antioxidant treated) were trypsinized and collected in 15 ml centrifuge tubes. After centrifugation, pellets were reconstituted in 1 ml PBS, counted and lysed by
sonication (Cereser et al., 2001) for 1 min. on ice. Samples were treated in the same way as the standards. The HPLC mobile phase was composed of 50: 50: 0.1 (v/v/v) acetonitrile/distilled water/ethanolamine. The sample injection volume was 100 µl and the flow rate was 1 ml/min for 30 min. The sample was measured at 532 nm. The column was 18 C 250 mm X 4.6 mm, 5µ (Novapack) from Waters. Peak integration (area) was performed using Waters software provided by the manufacturer.

4.2.8 Statistical analysis

For significant differences between control and experimental values The P-value between groups was determined using SPSS version 11.5 one way analysis of variance (ANOVA) followed by Bonferroni test. Significance of the effect had to be ≤ 0.05.
4.3 Results

4.3.1 Peroxide value (PV) determination

The PV was calculated in µg/g cells using a typical standard curve Figure (4.1). The data were presented as the arithmetic mean ± the standard deviation (SD).

![Typical standard curve](image_url)

Fig. 4.1 Typical standard curve represented as mean ± SD of FeCl₃ (0-200 µg/ml) for PV determination, R²=0.9993.

4.3.1.1 Effect of UVA on PV

Irradiation of HSF with 250 and 500 kJ/m² significantly increased PV from 71 ± 1.2 µg/g in control cells to 105 ± 4 and 116 ± 6 µg/g cells (Figure 4.2-4.6). When the results of the two UVA irradiation doses were compared, no significant difference was obtained (p=1.000).
Fig. 4.2 The PV of control human skin fibroblasts and fibroblasts irradiated with 250 kJ/m² and 500 kJ/m² UVA compared to fibroblasts UVA irradiated and incubated with (30 μM) ECG for 18 h. Experiments were repeated on three separate occasions in triplicate. Data are expressed as mean±SD.

4.3.1.2 Effect of (poly)phenols on PV in HSF

The addition of ECG to fibroblasts (Figure 4.2) caused a significant reduction in PV to 25.0± 1.1 μg/g cells (p<0.001) before irradiation. Cells incubated with ECG (30 μM) for 18 h resulted in reduction of PV to 86 ±17 and 93 ± 9 μg/g cells, when irradiated with 250 and 500 kJ/m² respectively. However, this reduction was not significant compared to irradiated cells at both doses (p=1.00, 0.8 for 250 and 500 kJ/m² respectively).

Trolox caused a reduction in PV from 71 ± 1.2 to 39 ± 4 μg/g cells when added to controls (Fig. 4.3). Trolox, gave no significant protection at 250 kJ/m² (105 ± 19 μg/g cells), but resulted in a significant decrease in PV at 500 kJ/m² (54 ± 5 μg/g cells, p<0.001).
Fig. 4.3 The PV of control human skin fibroblasts and fibroblasts irradiated with 250 and 500 kJ/m² UVA compared to fibroblasts UVA irradiated and incubated with (30 μM) trolox for 18 h. Experiments were repeated on three separated occasions in triplicate. Data are expressed as mean ± SD.

Luteolin treatment caused a 4 fold increase in PV compared to controls (310 ± 21 μg/g cells, p <0.0001). However, on irradiation, luteolin treated cells showed a significantly reduced level of PV, (51 ± 3 and 62 ± 5 μg/g cells, p<0.0001) compared to untreated cells at both UVA doses (Fig.4.4).

Luteolin-4'-O-glucoside caused a slight but insignificant increase in PV when added to HSF (86 ± 6 μg/g cells) (Fig.4.5). When luteolin-4'-O-glucoside was added to cells prior irradiation with 250 kJ/m², it lowered the PV (85 ± 3 μg/g cells) but this decrease was not significant compared to UVA irradiated cells (p=1.00). At the higher UVA dose, luteolin-4'-O-glucoside showed a decrease in PV from 116 ± 6 to 55 ± 10 μg/g cell (p <0.0001) compared to UVA 500 kJ/m² treated cells.

Luteolin 7-O-glucoside significantly lowered PV whether added as a control (23 ± 2 μg/g cells) or when cells were irradiated at 250 kJ/m² (60 ± 6 μg/g cells) and at 500 kJ/m² (60 ± 13 μg/g cells, p <0.0001) (Fig.4.6).
Fig. 4.4 The PV of control human skin fibroblasts and fibroblasts irradiated with 250 and 500 kJ/m² UVA compared to fibroblasts irradiated and incubated with (30 μM) luteolin for 18 h. Experiments were repeated on three occasions in triplicate. Data are expressed as mean ± SD.

Fig. 4.5 The PV of control human skin fibroblasts and fibroblasts irradiated with 250 and 500 kJ/m² UVA compared to fibroblasts irradiated and incubated with (30 μM) luteolin-4'-O-glucoside for 18 h. Experiments were repeated on three separate occasions in triplicate.

Fig. 4.6 The PV of control human skin fibroblasts and fibroblasts irradiated with 250 and 500 kJ/m² UVA compared to fibroblasts irradiated and incubated with (30 μM) luteolin-7-O-glucoside for 18 h. Experiments were repeated on three separate occasions in triplicate. Data are expressed as mean ± SD.
Table 4.1 The overall variation in PV (poly)phenols treated and untreated human skin fibroblasts compared by ANOVA followed by Bonferroni comparison at (0.05) significant level. NS = Not Significant.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% change compared to controls</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>250 kJ/cm²</td>
<td>47†</td>
<td>0.014</td>
</tr>
<tr>
<td>500 kJ/cm²</td>
<td>63†</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>ECG</td>
<td>65</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Trolox</td>
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<td>NS</td>
</tr>
<tr>
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<td>&lt; 0.0001</td>
</tr>
<tr>
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<td>17</td>
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</tr>
<tr>
<td>Trolox + 250 kJ/cm²</td>
<td>2†</td>
<td>NS</td>
</tr>
<tr>
<td>Luteolin + 250 kJ/cm²</td>
<td>51</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Luteolin-4'-O-glucoside + 250 kJ/cm²</td>
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<td>NS</td>
</tr>
<tr>
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<td>NS</td>
</tr>
<tr>
<td>Trolox + 500 kJ/cm²</td>
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</tr>
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<td>Luteolin + 500 kJ/cm²</td>
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<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Luteolin-7'-O-glucoside + 500 kJ/cm²</td>
<td>50</td>
<td>&lt; 0.0001</td>
</tr>
</tbody>
</table>

† Indicate increased levels were obtained.
4.3.2 Malondialdehyde Thiobarbituric acid (MDA-TBA) determination by HPLC

The effects of five (poly)phenols on the production of the secondary lipid oxidation product MDA by UVA (250 and 500 kJ/m²) was studied in HSF; it was expressed as μg/10⁶ cell using the standard curve in Figure (4.7).

Fig. 4.7 Standard curve represented as mean ± SD of 1,1,3,3, tetraethoxypropane (TEP) as a standard (0.0-0.1μg/ml) for TBARS-MDA calculation measured at 532 nm by HPLC, R=0.9994.

4.3.2.1 Effect of UVA on MDA-TBA in HSF

A significant increase in MDA-TBA (p<0.001) was obtained when HSF were UVA irradiated compared to controls from 0.85 ± 0.04 to 4.4 ± 0.16 and 5.1 ± 0.04 μg/10⁶ at 250 and 500 kJ/m², respectively (Figure 4.8). The two doses showed a significant difference in MDA-TBA when compared to each other (p<0.001).
4.3.2.2 Effect of (poly)phenols on MDA-TBA in HSF

The MDA-TBARS measured in cells incubated with 30 μM (poly)phenols for 18 h varied considerably. As seen in Figure 4.8, the addition of ECG to fibroblasts caused a significant reduction in MDA-TBA to 0.46 ± 0.02 μg/10^6 cells compared to controls (0.85 ± 0.04 μg/10^6 cells). ECG addition prior to UVA irradiation did not show significant changes at the 250 kJ/m^2 level (4 ± 0.08 μg/10^6 cell) but there was a significant decrease at 500 kJ/m^2 (2.9 ± 0.16), compared to UVA irradiated cells.

When trolox was added to cells without UVA irradiation it caused an insignificant decrease in MDA-TBARS level to (0.68 ± 0.02 μg/10^6 cell) compared to control cells (0.85 ± 0.04 μg/10^6 cell, p=0.145). The addition of trolox to UVA 250 and 500 kJ/m^2 irradiated cells significantly increased MDA-TBARS to (7.4 ± 0.21 and 6.6 ± 0.13 μg/10^6 cells) compared to control cells (Figure 4.9).
Luteolin showed an increase in MDA-TBARS when added to control cells (1.19 ± 0.4 μg/10^6 cell), but this increase was not significant (Figure 4.10, Table 4.2). On UVA irradiation, luteolin showed the greatest decrease in MDA-TBARS to (1.2 ± 0.01 and 1.9 ± 0.12 μg/10^6 cell) with 250 and 500 kJ/m² respectively.

![Fig. 4.9](image1)

**Fig. 4.9** The MDA-TBARS levels of control human skin fibroblasts and fibroblasts irradiated with 250 and 500 kJ/m² UVA compared to fibroblasts incubated in (30 μM) trolox for 18 h. Experiments were repeated on three occasions in triplicate. Data were expressed as mean ± standard deviation (SD).

![Fig. 4.10](image2)

**Fig. 4.10** The MDA-TBARS levels of control human skin fibroblasts and fibroblasts irradiated with 250 and 500 kJ/m² UVA compared to fibroblasts incubated in (30 μM) Luteolin for 18 h. Experiments were repeated on three occasions in triplicate. Data were expressed as mean ± standard deviation (SD).
Fig. 4.11 The MDA-TBARS levels of control human skin fibroblasts and fibroblasts irradiated with 250 and 500 kJ/m² UVA compared to fibroblasts incubated in (30 μM) Luteolin-4'-O-glucoside for 18 h. Experiments are repeated three times in triplicate. Data are expressed as mean ± standard deviation (SD).

The two glucosylated forms of luteolin demonstrated a different action on control cells (Figure 4.11, 4.12). Luteolin-4'-O-glucoside had no significant effect on controls (0.81 ± 0.6 μg/10⁶ cells, p=1.00), while luteolin-7-O-glucoside lowered MDA-TBARS significantly to 0.49 ± 0.09 μg/10⁶ cell (p=0.02). On irradiation luteolin-4'-O-glucoside significantly decreased MDA-TBARS from 4.4 ± 0.16 to 1.7 ± 0.03 at 250 kJ/m² and from 5.1 ± 0.04 to 2.6 ± 0.05 μg/10⁶ cell at 500 kJ/m² (p<0.00). Also, luteolin-7-O-glucoside showed a significant decrease in MDA-TBARS to similar values at both UVA doses. It reduced MDA-TBARS from 4.4 ± 0.16 to 2.1 ± 0.02 and from 5.1 ± 0.04 to 2.3 ± 0.18 μg/10⁶ cells at 250 and 500 kJ/m² respectively (p<0.00).
Fig. 4.12. The MDA-TBARS levels of control human skin fibroblasts and fibroblasts irradiated with 250 and 500 kJ/m² UVA compared to fibroblasts incubated in (30 μM) Luteolin–7-O-glucoside for 18 h. Experiments are repeated three times in triplicate. Data are expressed as mean ± standard deviation (SD).

Table 4.2 The overall variation in MDA in (poly)phenols treated and untreated human skin fibroblasts compared by ANOVA followed by Bonferroni comparison at (0.05) significant level, NS= Not Significant.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% change compared to controls</th>
<th>p-value</th>
</tr>
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</tr>
<tr>
<td>500 kJ/cm²</td>
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<td>ECG</td>
<td>36</td>
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<td>Trolox</td>
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<td>NS</td>
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<tr>
<td>Luteolin</td>
<td>29↑</td>
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↑ Indicate increased levels were obtained.
4.4 Discussion

4.4.1 UVA irradiation effect on lipid peroxidation production in HSF

UVA irradiation increased lipid oxidation products in HSF. UVA doses of 250 and 500 kJ/m² increased the production of hydroperoxides 1.5, 1.6 fold respectively and increased the MDA-TBARS levels 5 and 6 fold at the 2 UVA doses. UVA irradiation with 10 kJ/m² of human skin keratinocytes resulted in 5 μM H₂O₂ production (Shyong et al., 2003) and because H₂O₂ is an indirect marker of ROS, this study supports that ROS plays a crucial role in UVA-induced lipid oxidative damage in human skin fibroblast.

As recommended by Vile and Tyrrell (1995) the measured levels of TBARS in lipid extracts of cells were made after UVA irradiation, we have assumed that we have measured peroxidation of cell lipids and not oxidation products of other cell molecules that have been shown to interfere with the assay used. All chemical treatments of cells involved were followed by washing to ensure that as much as possible the effects of the agents were intracellular. The present study results of MDA-TBARS production after irradiation are nearly the same as Vile and Tyrrel, (1995). They obtained 4.8 and 6.6 fold increase in HSF irradiated with 250 and 500 kJ/m² respectively. In other studies, higher levels of MDA-TBARS were obtained. Irradiation of liposomes with very low dose of UVA, 0.75 mW/cm² for 30 min, caused 6-fold increase in the level of oxidation measured as TBARS (Damiani et al., 2002). Also, Merwald et al (2005) reported 3.5 times increase in human squamous carcinoma cell line at 200 kJ/m². A study by Basu-Modak et al., (1996) measured MDA-TBARS at 300 kJ/m² UVA had 18
fold increases in HSF compared to controls. The high levels obtained may be due to two factors. Firstly, these studies the MDA-TBARS was measured spectrophotometrically and the TBA mixture was boiled 15 min before butanol extraction instead of heating at 80°C in our method. As mentioned by Liu et al., (1997) the MDA-TBARS measured spectrophotometrically gives about two to six fold higher TBARS values due to non-specificity and artifactual formation of derivatives in the acid-heating step of the TBA assay. This problem was overcome by HPLC and incorporation of an antioxidant (BHT) in the extraction stage.

4.4.2 Effects of 5 (poly)phenols on reducing lipid peroxidation in HSF

The result of the addition of (poly)phenols (30) μM to HSF showed that all (poly)phenols used in the study had the ability to reduce the peroxidation products caused by UVA by different amount except for trolox.

4.4.2.1 Effect of ECG

In this study, ECG showed no effect on the products of lipid oxidation in irradiated cell except of reducing MDA-TBARS at 500 kJ/m². In another study, ECG inhibited the formation of MDA-TBARS in human blood plasma (0-100 μM) in a dose dependant manner and it was most effective compared to other catechins (Lotito and Fraga, 2000). In addition to the concentration factor, ECG properly shows antioxidant activity more in aqueous environment. Lotito and Fraga (2000) discussed the antioxidant activity of ECG as its capacity to scavenge water-soluble radicals preventing reactive species from reaching the lipid domains. Also it was suggested by Middleton et al (2000) that the
localization of ECG near the surface of phospholipids bilayers is suitable for scavenging aqueous oxygen radicals preventing the consumption of Vitamin E mainly through the gallate ring (Mukai et al, 2005).

4.4.2.2 Effect of trolox

Trolox effects fluctuated between having no effect, antioxidant and prooxidant effects. Trolox showed a decrease in PV and MDA-TBARS when added to control cells, but had no effect when added to cells irradiated with 250 kJ/m² as PV remained unchanged compared to irradiated cells. These results are similar to those obtained by Nocentini et al, (2001) in which trolox incubated for 24 h with HSF failed to decrease the production of H₂O₂ when these cells were irradiated by 50 kJ/m² UVA at high trolox concentration (200) μM.

Also, the antioxidant effect of trolox was shown in a significant decrease at the high UVA dose lowering PV significantly (Fig.4.3). This reduction may be explained by the longer irradiation time which allowed the hydrophilic trolox to penetrate through cell membranes and start its action as an antioxidant. Peroxyl radicals are known to be highly polarized and form initially in the non-polar lipid bilayers of membrane and diffuse rapidly towards the polar aqueous phase where they terminate or become trapped (Barclay et al., 1995). This diffusion-trapping mechanism may account for the very effective action of trolox in lowering PV in control and highly irradiated cells.

In this study, trolox did not reduce MDA in irradiated HSF. Moreover, its prooxidant activity doubled the effect of UVA in increasing the products of lipid oxidation. These results are similar to those reported by Nocentini et al, (2001)
in which trolox resulted in almost a doubling of DNA damage when used at 200 μM. Reducing agents such as trolox may increase damage through its ability to reduce Fe$^{3+}$ to Fe$^{2+}$ (Poljsak et al., 2006).

The increase of MDA in UVA treated HSF incubated with trolox may be due to the fact that trolox facilitated the formation of ROS which are more reactive or in greater amount than those normally produced by UVA alone (Nocentini et al., 2001).

The prooxidant characteristic of trolox was also reported by Massaeli et al. (1999) who reported a significant increase in MDA-TBARS formation up to 750 μM of trolox due to reduction of ferric ions to ferrous which can generate free radicals more rapidly. The same research group also showed no decrease in MDA-TBARS at concentrations as low as 30 μM of trolox. In our experiments it is worth noting that the prooxidant behaviour of trolox could not be attributed to the fact that trolox may act as a photosensitizer producing ROS by itself (Nocentini et al., 2001) since trolox does not absorb in the UVA range (maximum absorption at 288 nm, chapter 5). In contrast to results obtained in this chapter, trolox reduced TBARS formation in rat heart mitochondria in a dose dependant manner (0-60 μM). The dose of 60 μM reduced TBARS by 2 fold (Santos et al., 2001). Also, it was reported by Callaway et al., (1998) that trolox inhibited TBARS formation (100 %) in rat brain extracts at a very high concentration (1000 μM). These results emphasize that the concentration of trolox is critical to show its antioxidant effect. Also, Benvenisti-Zarom et al., (2005) reported a 90 % decrease in MDA stimulated by a drug, Clioquinol, in monolayer astrocytes when trolox (30 μM) was added. This may be due to the
difference of the cell type to those used in this study. In addition to the fact that trolox is an efficient radical scavenger in the aqueous phase at the water/lipid interface, it may also need the presence of synergetic antioxidant such as ascorbic acid to be regenerated (Chen and Tappel, 1995), thus producing maximum protection. On its own, trolox showed no protection (2.5-5.0 μM) in reducing LDL oxidation in human plasma unless added to another antioxidant, Raxofelast, (Iuliano, et al, 1999). Thus, the low potency found in the present study may be due to the absence of ascorbic acid which has been reported to be an important cofactor for increase of trolox antioxidant activity (De Mulder et al, 1995; Iuliano et al, 1999) as well as that of tea (poly)phenols (Majchrzak et al., 2004).

4.4.2.3 Effect of luteolin and luteolin glucosides

Surprisingly, luteolin showed a toxic effect on fibroblasts when added to control cells without irradiation. A similar prooxidant behavior was obtained with quercetin, a flavonoid that has a similar structure to luteolin with an extra OH on position 3. Yamashita, et al., (1999) indicated that luteolin has a dual function of anticarcinogenic and carcinogenic potential and that luteolin induces less damage to DNA compared to quercetin. Very recently it has been found that increased generation of H₂O₂ by flavonoids may be due to their oxidation in the culture media, which may not represent the real situation in vivo (Tiwari, 2001). Therefore, more studies are required to differentiate direct cellular effects of phenolic compounds from effects caused by H₂O₂ generation in the culture media and factual conditions in vivo. On UVA irradiation, luteolin showed 2 fold reduction in PV and 3 fold reduction in MDA-TBARS at both UVA doses. Similar antioxidant effect was obtained in which luteolin (50 μM) reduced
hydroperoxides in rat adrenal cells also, luteolin (2.5 – 25 µg/ml) reduced H_2O_2 in oligodendrocytes (Meeteren et al., 2004).

When the antioxidant activity of luteolin and its glucosides was compared, neither luteolin-4'-O-glucoside nor luteolin-7-O-glucoside showed prooxidant behavior as that of luteolin and as mentioned by Middleton et al. (2000) only the aglycon flavonoids exhibit mutagenic activity. It appears that the position of glycosylation also plays an important role in the antioxidant activity of a compound. This was obvious when comparing the antioxidant effects of luteolin-7-O-glucoside to luteolin-4'-O-glucoside. Luteolin-7-O-glucoside showed an antioxidant effect against peroxidation produced in human skin fibroblasts whether those cells were controls or UVA irradiated.

4.4.3 The effect of structure

The relationship between chemical structure and suppressing effect of flavonoids is not clear at present (Lu et al., 2002). The structural difference in the (poly)phenolic compounds used in this study in terms of number and arrangement of the hydroxyl group as well as glucosylation of these groups may affect their antioxidant activity (Rice-Evans et al, 1996; Mustada et al, 2002; Meeteren et al 2004). It is known that the hydroxylation positions in the A and B rings, the number of hydroxyl groups and the 4-keto group present on the C ring of a flavonoid are important for their antioxidant activity. The combination of the 3',4'-dihydroxyls (catechol moiety) in the B-ring with the double bond at C2-3 and C4-carbonyl moiety is considered to be essential structural requirement for aroxy radical stabilization.
In addition, the availability of phenolic hydrogen is important as donating radical scavengers may be used to predict the phenolic compound antioxidant activity (Rice-Evans et al., 1996); this may explain the weak effects of trolox which has only one hydroxyl group.

ECG which has 7 hydroxyl groups (4-OH group plus 3-OH on gallic acid on position 3) was more effective than trolox in reducing lipid oxidation. Luteolin has 4-OH groups equally distributed on the A and B rings showed more antioxidant behavior in reducing PV and MDA-TBARS than its glucosides which has one OH group occupied with glucose. When the molecular structures of the two luteolin glucodises are compared, the position of glucosylation was also found to contribute to the antioxidant activity. The 3',4' dihydroxy structure of luteolin at the B ring is essential to prevent \( \text{H}_2\text{O}_2 \) derived free radical induced cell damage (Meeteren et al., 2004). Thus the substitution of 4'-OH reduced luteolin 4'-O-glucoside compared to luteolin-7-O-glucoside.

In this study, luteolin and its glycosylated forms were more potent antioxidants in reducing UVA induced lipid oxidation products compared with ECG and Trolox. This potency may be due to the fact that luteolin, luteolin-4'-O-glucoside and, luteolin-7-O-glucoside absorb strongly in the UVA region. It was reported by Damiani et al. (2002) that the reasons for the protective effect against UVA, despite the formation of free radicals, is most probably due to the capability of a compound to absorb in the UVA region.

On the other hand, the glucosylation of flavonoids reduces their activity when compared to the corresponding aglycon (Mustada et al., 2002 and Rice-
Evans et al., 1996); this was confirmed in that luteolin was stronger than its glycosylated forms.

4.4.4 The effect of the partition coefficients of (poly)phenols

The greater effectiveness of luteolin compared with luteolin-7-O-glucoside in inhibiting lipid oxidation may be explained the fact that luteolin is more hydrophobic, with a partition coefficient of 22.2. The glycosylation at the 7-position would render the flavonoid more soluble in the aqueous environment (Brown and Rice-Evans, 1998).

The lipid bilayer is the first barrier that external agents encounter when coming into contact with cells. Hence, to have a protective effect, an antioxidant has to reside in close proximity to the membrane interior where lipid peroxidation chain propagation occurs. Partitioning of antioxidants and hydrogen bonding are important parameters that determine the antioxidant activity of a compound (Schwarz et al., 2000). Partition coefficients and the ease of diffusion into membrane’s bilayer may be important when considering the efficacy of luteolin as an antioxidant compared with its glucosylated forms. The partition coefficient of glucosylated (poly)phenols is less than the partition coefficient of the aglycon. An example, quercetin has a partition coefficient of 1.2 whereas the glucoside, quercetin-3-O-rhamnoglucoside, has a lower value (0.37) which suggests a greater hydrophilicity (Tapiero et al., 2002) (Table 4.3).
Table 4.3 The partition coefficient of some (poly)phenols.

<table>
<thead>
<tr>
<th>Antioxidant</th>
<th>Partition coefficient</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luteolin</td>
<td>22.2</td>
<td>Brown and Rice-Evans, 1998</td>
</tr>
<tr>
<td>Luteolin-4'-O-glucoside</td>
<td>Less than 22.2</td>
<td>Suggested by Tapiero et al., 2002</td>
</tr>
<tr>
<td>Luteolin-7-O-glucoside</td>
<td>Less than 22.2</td>
<td>Suggested by Tapiero et al., 2002</td>
</tr>
<tr>
<td>Epicatchingallate (ECG)</td>
<td>2.92</td>
<td>Caturla et al., 2002</td>
</tr>
<tr>
<td>Epigallocatechingallate (EGCG)</td>
<td>1.92</td>
<td>Caturla et al., 2002</td>
</tr>
<tr>
<td>Epicatchin (EP)</td>
<td>0.22</td>
<td>Caturla et al., 2002</td>
</tr>
<tr>
<td>Catechin (C)</td>
<td>0.2</td>
<td>Caturla et al., 2002</td>
</tr>
<tr>
<td>Trolox</td>
<td>2.90</td>
<td>Ancerewicz et al., 1998</td>
</tr>
</tbody>
</table>

It’s clear that lipophilicity or lipid solubility and antioxidant properties of (poly)phenols are strongly related (Ancerewicz et al., 1998). A lipophilic antioxidant such as vitamin E was more effective in lowering MDA-TBARS compared to the hydrophilic trolox (Massaeli et al., 1999). Vitamin E started its activity at 2.5 μM compared to 1300 μM for trolox. The hydrophilic antioxidant such as trolox has a lower ability to act as chain-breaking antioxidant because lipid oxidation occurs in the lipid phase and at the lipid surface (Schwarz et al., 2000).

Caturla et al (2003) compared the partition coefficient of different catechins. Catechins with galloyl moieties, especially ECG, showed higher partition coefficient (Table 4.3) compared to epicatechin (EC) and catechins (C). Thus ECG could penetrate to the deepest position in phospholipids of biological membranes and exhibited the highest antioxidant capacity against lipid peroxidation compared to these catechins. A study of the antioxidant activity of green tea polyphenols against lipid oxidation in canola oil tested by Chen and
Chan (1996) revealed that ECG was less active than EGCG and EC and that was explained by fewer hydroxyl groups present in ECG compared to EGCG. Moreover, the gallate group on ECG makes it more hydrophilic, (i.e. less soluble in oil) and therefore less effective against lipid oxidation compared to EC.

4.4.5 The prooxidant effect

(Poly)phenols act as both antioxidants as well as prooxidants dependent upon the environment they are exposed to. In this study, some (poly)phenols, such as luteolin and trolox, acted as a prooxidants. The classical antioxidants, α-tocopherol and vitamin C, have also been reported to behave in a similar fashion (Tiwari 2001). Concentration of an antioxidant is also considered important. Catecholamines such as 3,4-dihydroxy phenylalanine (DOPA) have prooxidant effects at low concentration (1-6 μM) and antioxidant effect at higher concentrations (Nguyen and Sok, 2004).

It is also important to note that flavonoids can be oxidized and may exert prooxidant effects *in vitro* under some assay conditions. However, most reported studies have emphasized their antioxidant effect as they may not meet similar conditions *in vivo* (Tiwari, 2001).

Two mechanisms for prooxidant behaviors were suggested by Galati *et al* (2002). The first is related to the ability of flavonoids to undergo autoxidation catalysed by transition metals to produce H_2O_2 via the Fenton reaction. The second mechanism involves peroxidase that catalyzes the oxidation of (poly)phenols which in turn induce lipid peroxidation.
4.4.6 The metal chelation effect

The findings of the antioxidant behavior of the 5 (poly)phenols used in this study matches the ability of these (poly)phenols to scavenge metal ions (chapter 5). Luteolin and its glucosylated forms which had the ability of chelating Cu\(^{2+}\) and Fe\(^{2+}\) showed a higher antioxidant effect compared to ECG and trolox. When ECG and trolox were compared, ECG showed a higher effect in the reduction of lipid oxidation levels obviously due to higher capacity to chelate copper ion more than trolox.

The role of metal ions involvement in lipid oxidation and the scavenging activity of (poly)phenols also play a role in the explanation of the antioxidant activity of compounds used in this work. Probably both activities are involved in the protective effect and it is difficult to distinguish the respective roles played (Ferrali et al., 1997). TBARS formation in rat brain homogenates was increased with Fe\(^{3+}\) addition (100-1000 \(\mu\)M) (Callaway et al., 1998). It is hypothesized that EDTA can control the formation of hydroxyl radicals via trapping Fe\(^{2+}\) (Matsufuji et al., 2006). The intracellular level of “labile” iron plays an essential role in the promotion of the pro-oxidant condition after UVA. Labile iron belonging to this intracellular pool can undergo redox cycling thereby generating toxic oxidants such as hydroxyl radical and lipid-derived alkoxy and peroxy radicals (Merwald et al., 2005). It has been shown in keratinocytes and fibroblasts that the immediate increase in labile iron after UVA is dose dependent and sustained for 2 h after which it return to baseline at about 6 h (Merwald et al., 2005).
4.4.7 Conclusions

In conclusion, our findings demonstrate that flavonoids are able to prevent UVA radiation induced lipid oxidation probably by scavenging oxygen free radicals generated by UVA irradiation or metal ion chelation such as iron and copper.

By comparing the effects of the 5 (poly)phenols used to reduce lipid peroxidation in HSF caused by UVA, the following can be concluded;

- ECG showed the same effect towards controls and UVA irradiated cells in reducing PV and MDA-TBARS giving a significant protection at the high dose only.

- Trolox reduced the two lipid peroxidation parameters, PV and MDA-TBARS when added to control cells without UVA irradiation. Upon irradiation trolox had no effect or minimal protective effect in terms of PV at 500 kJ/m² and even increased the MDA-TBARS production at the two irradiation doses.

- Although luteolin showed substantial prooxidant activity when incubated with control cells, it demonstrated the highest protection in terms of reducing PV and MDA-TBARS when added to irradiated cells.

- Luteolin-4’-O-glucoside decreased PV and MDA-TBARS significantly upon irradiation. In contrast, luteolin-7-O-glucoside showed the best protection effects, decreasing both peroxidation parameters in control and irradiated HSF.

Regarding the structural properties of the five (poly)phenols used in the research, the following may be concluded:
ECG lacks the 2,3 double bond and the 4 keto group; also it has the pyrogalloyl unit which renders the molecule large. Moreover, ECG contains 7-OH hydroxyl groups which makes the molecule more hydrophilic thus reducing its activity compared to luteolins. Trolox lacks all the structure properties of a flavonoid and showed the least protection as it has only one hydroxyl group. Luteolin with 4 hydroxyl groups located in the active scavenging sites, 2,3 double bond, free 3',4', hydroxyls, free 7, 5 hydroxyls, and 4 keto group showed the strongest protection activity but with a prooxidant effect when no irradiation is applied. It appears that the position of glycosylation also plays an important role in the antioxidant activity of a compound. This was obvious when comparing the antioxidant effects of luteolin-7-O-glucoside to luteolin-4'-O-glucoside.
CHAPTER 5
5. STUDY OF THE INTERACTION BETWEEN 5 (POLY)PHENOLIC COMPOUNDS AND COPPER AND IRON

5.1. Introduction

5.1.1 Metal ions

Iron in the human body is stored and transported bound to proteins. In normal human subjects, 25-27% of total body iron (800-1000 mg) is stored mostly as ferritin (Pennathur et al., 1999; Ozgová et al., 2003) and 68% of total body iron is bound to hemoglobin (Ozgová et al., 2003). Like iron, trace amounts of copper are essential for life; ceruloplasmin is the principal copper-containing plasma glycoprotein (150-200 mg/l). It consists of six copper atoms and plays a key role in iron metabolism (Aouffen et al., 2004).

Metal chelation is an important attribution in the antioxidant mechanism of some phenolic compounds in which damage to cell components can be prevented. Antioxidants interact with metal ions to produce complexes that prevent the generation of free radicals that damage target biomolecules (Fernandez et al., 2002). Iron and copper can generate the reactive •OH through Haber-Weiss reactions (Cheeseman and Slater, 1993; Hughes, 1999; Fernandez et al., 2002).

5.1.2 Ultraviolet (UV) and metal release

Skin contains significant iron levels which may be released after UV exposure stimulating ROS production (Polla et al., 2003). The epidermal skin contains 22.5 mg total iron (Trommer et al., 2003) who stated that iron concentration of 10 μM was harmful to the skin model. Iron overload caused
tumor development in mouse skin (Bhasin et al, 2003). Gutteridge and Tyrrell, (2002) found that UVA irradiation of human skin fibroblasts leads to an immediate release of LIP in the cells. UVA induce the release of harmful free iron within the cells which exacerbate the damaging effects of photoperoxidation. Reelfs et al, (2004) showed that UVA irradiation of human skin fibroblasts with 250 kJ/m² caused immediate degradation of high levels of ferritin (Ft) and increased the LIP from 1.06 μM in control cells to 2.25 μM in UVA irradiated cells. UVA produces ROS such as $^1O_2$ and $H_2O_2$ that promote biological damage in exposed tissues via iron-catalyzed oxidative reactions (Haber-Weiss reaction eq-1) (Lovaas, 1995; Reelfs et al, 2004).

$$O_2^- + H_2O_2 \rightarrow O_2 + OH^- + OH^-$$ eq-1

The concentration of LIP was reduced to 0.03 μM when the iron chelator, desferrioxamine was added. In another study, desferrioxamine (DFO) was added to UVB irradiated human cell line which reduced the iron content of the cell thus preventing oxidative damage (Kramer-Stickland et al, 1999). It was reported by Giordani et al, (2000) that UVA induced lipid peroxidation is doubled when human skin fibroblasts were treated with 5 μM iron prior to 30 kJ/m² irradiation.

5.1.3 (Poly)phenols and chelation

One feature of some (poly)phenols is their ability to interact with metal ions to produce complexes that prevent the participation of those metal ions in free radical generating reactions that damage target biomolecules (Fernandez et al, 2002). Radicals that are formed in the vicinity of the flavonoid, which surrounds the catalytically active $Fe^{2+}$, can be scavenged immediately. In this
case, the flavonoid has a double, synergistic action, making it an extremely powerful antioxidant (Van Acker et al., 1998). Iron chelation by flavonoid ingestion or by topical application of 2-furildioxime (FDO) is reported to prevent skin photo damage (Polla and Polla, 2003).

Iron contributes to UVA-mediated peroxidation of human skin fibroblasts membranes (Reelfs et al., 2004). The antioxidant effect of (poly)phenols is referred to two main effects, the ability to scavenge free radicals and to chelate metal ions such as iron and copper inhibiting metal catalyzed Fenton reaction (Van Acker et al., 1998; Sugihara et al., 1999; Shao et al., 2002; Khokhar et al., 2003). Continuous increase in body iron enhances the risk of cancer development, particularly in a population preexposed to carcinogenic agents such as UVA irradiation (Bhasin et al., 2003). The binding of iron or copper to the (poly)phenols such as luteolin-7-O-glucoside was demonstrated by the shift in the peak absorbance (Miller et al., 1996; Brown and Rice-Evans, 1998; Mira et al., 2002).

Epigallocatechin gallate (EGCG), the main catechin in green tea protects against oxidative damage by acting as both scavenger and chelating agent against UVA (Erba et al., 2003). Gallocatechins are extremely efficient chelators of free iron and form stable complexes with Fe$^{3+}$ even at very low concentrations (0.003-0.01%). It appears that Fe$^{3+}$ forms coordinate bonds with the phenolic oxygen from the gallate moiety; however, the antioxidant ability of gallocatechin is lost in the complexes (Jovanovic et al., 1998).

The mechanism of the inhibitory effect of flavonoids on the iron-dependent lipid peroxidation systems have not been clarified. Iwahashi et al,
(2004) have focused on the mechanism of luteolin-7-O-glucoside on lipid oxidation.

Several studies have proposed methods for determining the metal chelation properties of flavonoids such as electrochemical methods (Vestergaard et al, 2005), atomic absorption, fluorescence spectroscopy (Gutierrez and Gehlen, 2002) and mass spectroscopy (Fernandez et al, 2002). Most studies on flavonoids use UV-visible absorption spectroscopy to analyze the shifts of UV bands I and II that characterize the flavonoid spectra. Although UV spectroscopy measurement gives only indirect evidence for complex formation between phenolic compounds and metal ions (Fernandez et al, 2002) it is simple and provides useful information especially when combined with measuring the antioxidant activity.

In this chapter the ability of 5 (poly)phenols (ECG, trolox, luteolin, luteolin-4'-O-glucoside and luteolin-7-O-glucoside) to chelate iron and copper ions was studied by UV-visible absorption spectroscopy to elucidate the potential involvement of chelation properties to protect human skin fibroblasts against UVA oxidative damage.
5.2 Materials and methods

5.2.1 Materials for iron and copper chelation

Epicatechin gallate from green tea, 98% HPLC grade purchased from Sigma St. Louis, 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic (trolox) acid 97% purchased from Sigma Aldrich, St. Louis. Luteolin (3',4',5,7-tetrahydroxyflavone), luteolin-4'-O-glucoside, and luteolin-7-O-glucoside were purchased from Extrasynthese, Genay, France. Ethylenediaminetetraacetic acid (EDTA) was purchased from NTL; Brixworth, UK. Ferric chloride hexahydrate, double distilled water, and Dulbecco’s phosphate buffered saline (DPBS) free of Ca$^{2+}$ and Mg$^{2+}$ were obtained from Sigma, St. Louis, USA. Copper sulphate pentahydrate (CuSO$_4$.5H$_2$O) was from BDH Poole, England. Shimadzu 1201, ultraviolet-visible spectrophotometer model UV-1601PC Japan was used in all experiments.

5.2.2 Method of iron chelation

The method described by Brown and Rice-Evans (1998) was used to determine the Fe$^{3+}$ interaction with phenolic compounds. Stock solutions (1 mM) of trolox, epicatechin gallate (ECG), luteolin, luteolin-4'-O-glucoside and luteolin-7-O-glucoside were prepared in methanol. Stock solutions of each of FeCl$_3$ and EDTA (10 mM) were also freshly prepared. Each phenolic compound solution was diluted in DPBS (pH 7.4) to reach a final concentration of 25 μM and scanned between 200 nm and 600 nm in a quartz cuvette. FeCl$_3$ (final concentration of 50 μM) was then added to the flavonoid and the spectrum was
recorded after 1 min. Finally, a strong metal chelator, EDTA (final concentration 125 μM) was added and the spectrum was recorded at 1, 20, and 30 min.

5.2.3 Method of copper chelation

The metal chelation method was used to determine the copper(II)-chelating activity of the 5 phenolic compounds. Each phenolic compound was diluted in DPBS to reach a final concentration of 25 μM and scanned between 200 nm and 600 nm. CuSO₄ was added to give a final concentration of 25 μM and a shift in the spectrum was recorded after 1 min. Finally, EDTA solution (final concentration 125 μM) was added and the spectrum was recorded after 1, 10, and 20 min.
5.3 Results

5.3.1 Iron chelation

Results for chelation effects of the 5 phenolic compounds (the flavanol ECG, trolox, flavones, luteolin, luteolin-4'-O-glucoside and luteolin-7-O-glucoside) with iron studied at physiological pH 7.4 are reported. At the outset, FeCl₃ and CuSO₄ absorbance peaks were identified at 275 and 234 nm respectively. None of the (poly)phenols used showed similar spectral features indicating no interference in peaks except for ECG.

ECG absorbance peak was identified at 278 ± 0.4 nm. Binding of FeCl₃ (50 μM) to ECG caused this peak shift to 280 ± 0.3 nm (Fig. 5.1).

![Graph](image-url)

Fig. 5.1 Effect of the addition of Fe³⁺ on the absorbance spectrum of ECG. (1) ECG (25 μM) 278 nm; (2) ECG + FeCl₃ (50 μM) 280 nm; (3) ECG + FeCl₃ after addition of EDTA (125 μM) 276 nm. The figure represents a typical result of 3 separate experiments.
The addition EDTA as a strong metal chelator (Ozgova et al., 2003), caused the FeCl₃-EGC peak to shift to 276 ± 0.6 after 30 min of EDTA (125 μM) addition.

Small modifications in the absorbance spectrum of the phenolic compound trolox were observed upon the addition of Fe³⁺. The absorbance peak of trolox at 288 ± 0.3 nm underwent a hypsochromic shift to 284 ± 0.3 nm. The addition of EDTA did not cause the peak to return to the original position 283 ± 0 nm after 30 min (Fig. 5.2).

![Absorbance Spectrum](image)

Fig. 5.2 Effect of the addition of Fe³⁺ on the absorbance spectrum of trolox. (1) trolox (25 μM) 288 nm; (2) trolox + FeCl₃ (50 μM) 284 nm; (3) trolox + FeCl₃ after addition of EDTA (125 μM) 283 nm. The figure presents a typical result of 3 separate experiments.
In contrast to ECG and trolox, the peaks of luteolin and its glycosylated forms appeared in the UVA region (320-400 nm). The spectra of these 3 (poly)phenols typically consisted of two absorption maxima in the ranges 240-280 nm (band II) and 300-400 (band I) which refers to ring A and ring B + C, respectively. In the presence of Fe$^{3+}$ the luteolin peak at 364 ± 0.3 nm shifted to 375 ± 0.6 nm and returned to its original position, 365 ± 0.0 nm, after 30 min of EDTA addition.

![Absorbance vs Wavelength](image)

**Fig. 5.3** Effect of the addition of Fe$^{3+}$ on the absorbance spectrum of luteolin. (1) luteolin (25 µM) 364 nm; (2) luteolin + FeCl$_3$ (50 µM) 375 nm; (3) luteolin + FeCl$_3$ after addition of EDTA (125 µM) 365 nm. The figure presents a typical result of 3 separate experiments.
Fig. 5.4 Effect of the addition of Fe$^{3+}$ on the absorbance spectrum of luteolin-4'-O-glucoside. (1) luteolin-4'-O-glucoside (25 µM) 352 nm; (2) luteolin-4'-O-glucoside + FeCl$_3$ (50 µM) 345 nm; (3) luteolin-4'-O-glucoside + FeCl$_3$ after addition of EDTA (125 µM) 348 nm. The figure presents a typical result of 3 separate experiments.

Both luteolin-4'-O-glucoside and luteolin-7-O-glucoside presented peaks at approximately the same wavelength at 352 ± 0.6 nm and 351 ± 0.8 nm, respectively. For luteolin-4'-O-glucoside band I shifted hypsochromically to 345 ± 0.8 nm whereas for luteolin-7-O-glycoside interaction with Fe$^{3+}$ shifted band I to 375 ± 2.5 nm. Both peaks returned to approximately their starting position (348 ± 0.8 and 351 ± 0.6 nm respectively) after 30 min of EDTA addition.
Fig. 5.5 Effect of the addition of Fe$^{3+}$ on the absorbance spectrum of luteolin-7-O-glucoside. (1) luteolin-7-O-glucoside (25 µM); (2) luteolin-7-O-glucoside + FeCl$_3$ (50 µM); (3) luteolin-7-O-glucoside + FeCl$_3$ after addition of EDTA (125 µM). The figure presents a typical result of 3 separate experiments.
5.3.2 Copper chelation

The ECG absorbance peak was identified at 279 ± 0 nm. The addition of an equimolar solution of CuSO$_4$ (25 μM) caused this peak to shift to 326 ± 0.3 nm (Fig. 5.6). The addition of EDTA, caused the CuSO$_4$-ECG peak to return close to the original position (277 ± 0.3 nm) after one minute of adding EDTA.

Fig. 5.6 Effect of the addition of Cu$^{2+}$ on the absorbance spectrum of ECG (pH 7.4). (1) ECG (25 μM) 279 nm; (2) ECG (25 μM) + CuSO$_4$ (25 μM) 326 nm; (3) ECG + CuSO$_4$ after addition of EDTA (125 μM) 277 nm. The figure presents a typical result of 3 separate experiments.

The peak for trolox at 288 ± 0.3 nm was shifted by only 2 nm to 286 ± 1 nm. Adding EDTA shifted the peak to 283 ± 3 nm after one minute (Fig. 5.7).
Fig. 5.7 Effect of the addition of Cu$^{2+}$ on the absorbance spectrum of trolox (pH 7.4). (1) trolox (25 μM) 288 nm; (2) trolox (25 μM) + CuSO$_4$ (25 μM) 286 nm; (3) trolox + CuSO$_4$ after addition of EDTA (125 μM) 283 nm. The figure presents a typical result of 3 separate experiments.

Luteolin Band I peak at 362 ± 2 was shifted to 400 ± 4.6 nm. It was apparent that a longer incubation time (10 min) was required with EDTA to shift the spectrum of luteolin to its original position 362 ± 2 nm (Fig. 5.8).

Luteolin-4'-O-glucoside peak at 356 ± 0.7 nm was shifted hypsochromically, to 335 ± 1.5 nm by CuSO$_4$ addition and returned towards to the original position 341 ± 2 after 10 minutes of adding EDTA (Fig. 5.9). The luteolin-7-O-glucoside peak, 352 ± 0.3 nm, was shifted to 413 ± 1.3 nm. The addition of EDTA shifted the peak to its original position 352 ± 0.3 nm after 10 min.
Fig. 5.8 Effect of the addition of Cu$^{2+}$ on the absorbance spectrum of luteolin (pH 7.4). (1) luteolin (25 μM) 362 nm; (2) luteolin (25 μM) + CuSO$_4$ (25 μM) 400 nm; (3) luteolin + CuSO$_4$ after addition of EDTA (125 μM) 362 nm. The figure presents a typical result of 3 separate experiments.

Table (5.1) summarizes the overall change in the peaks of (poly)phenols with iron and copper ions.

Table 5.1 The overall change in the peaks ($\lambda$ max) for (poly)phenols with iron and copper ions presented as experiments ± SD.

<table>
<thead>
<tr>
<th>(poly)phenol (25 μM)</th>
<th>Peak of (poly)phenol (nm)</th>
<th>Spectral shift (nm) in the presence of FeCl$_3$ (50 μM)</th>
<th>Spectral shift (nm) in the presence of CuSO$_4$ (25 μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ECG</td>
<td>278-279</td>
<td>2.0 ± 0.0</td>
<td>47 ± 0.3</td>
</tr>
<tr>
<td>Trolox</td>
<td>288-288</td>
<td>4.0 ± 0.3*</td>
<td>2.5 ± 0.8*</td>
</tr>
<tr>
<td>Luteolin</td>
<td>364-362</td>
<td>10 ± 0.3</td>
<td>38 ± 4.0</td>
</tr>
<tr>
<td>Luteolin-4'-Oglucoside</td>
<td>351-356</td>
<td>7.0 ± 1.0*</td>
<td>21 ± 0.7*</td>
</tr>
<tr>
<td>Luteolin-7-O-glucoside</td>
<td>351-352</td>
<td>24 ± 3.0</td>
<td>61 ± 0.7</td>
</tr>
</tbody>
</table>

*Indicates hypsochromic shift.
Fig. 5.9 Effect of the addition of Cu$^{2+}$ on the absorbance spectrum of luteolin-4'\-O-glucoside (pH 7.4). (1) luteolin-4'-O-glucoside (25 μM) 356 nm; (2) luteolin-4'-O-glucoside (25 μM) + CuSO$_4$ (25 μM) 335 nm; (3) luteolin-4'-O-glucoside + CuSO$_4$ after addition of EDTA (125 μM) 341 nm. The figure presents a typical result of 3 separate experiments.
Fig. 5.10 Effect of the addition of Cu$^{2+}$ on the absorbance spectrum of luteolin-7-O-glucoside (pH 7.4). (1) luteolin-7-O-glucoside (25 μM) 352 nm; (2) luteolin-7-O-glucoside (25 μM) + CuSO$_4$ (25 μM) 413 nm; (3) luteolin-7-O-glucoside + CuSO$_4$ after addition of EDTA (125 μM) 352 nm. The figure presents a typical result of 3 separate experiments.
5.4 Discussion

All (poly)phenols studied showed a higher capacity to interact with copper ions than iron ions. This can be explained by two factors, that the standard reduction potential of copper is much lower than that for iron (Table 5.2) and to the lower solubility of iron at physiological pH 7.4 compared with copper (Mira et al., 2002; Schultz and Grundl, 2004).

Table 5.2 The standard reduction potentials of metals and metals with some (poly)phenols.

<table>
<thead>
<tr>
<th>Compound</th>
<th>E. vs. N.H.E.</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe$^{3+}$-transferrin/Fe$^{2+}$- transferrin</td>
<td>-0.4</td>
<td>Halliwell and Gutteridge, 1999</td>
</tr>
<tr>
<td>Fe$^{3+}$-ferritin/Fe$^{2+}$- ferritin</td>
<td>-0.19</td>
<td>Halliwell and Gutteridge, 1999</td>
</tr>
<tr>
<td>Fe$^{3+}$/ Fe$^{2+}$</td>
<td>+0.77</td>
<td>Jovanovic et al, 1998</td>
</tr>
<tr>
<td>Fe$^{3+}$/ bis-ECG</td>
<td>+0.325</td>
<td>Jovanovic et al, 1998</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>+0.280</td>
<td>Lotito and Fraga, 2000</td>
</tr>
<tr>
<td>ECG</td>
<td>+0.444*</td>
<td>Furuno et al, 2002</td>
</tr>
<tr>
<td>Luteolin*/Luteolin&quot;</td>
<td>+0.424*</td>
<td>Galati et al, 2002</td>
</tr>
<tr>
<td>Cu$^{2+}$/Cu$^{3+}$</td>
<td>+0.15</td>
<td>Mira et al, 2002</td>
</tr>
<tr>
<td>Fe$^{3+}$-EDTA/Fe$^{2+}$- EDTA</td>
<td>+0.12</td>
<td>Halliwell and Gutteridge, 1998</td>
</tr>
<tr>
<td>Fe$^{3+}$-citrate/Fe$^{2+}$- citrate</td>
<td>+0.1</td>
<td>Halliwell and Gutteridge, 1998</td>
</tr>
</tbody>
</table>

E. = reduction potential, N.H.E= normal hydrogen electrode.
* = 244 was added to convert Saturated Calomel Electrode (SCE) to N.H.E= natural hydrogen electrode.

The small shift in the (poly)phenols absorbance with iron may be due to low solubility of iron at pH 7.4. Also, it was found by Van Acker et al, (1998) and Khokhar et al, (2003) that iron chelation is a way of increasing the antioxidant activity of compounds with low scavenging activity, whereas the contribution of iron chelation to the antioxidant activity of good scavengers such as ECG is almost negligible. In contrast, EGCG, which has a similar structure to
ECG was described as a strong Fe$^{2+}$ and Fe$^{3+}$ chelator (Erba et al, 2003, Jovanovic et al, 1998), while small changes in the spectra were detected by Ozgova et al, (2003) with catechin and epicatechin and iron. In our study, using double the concentration of iron (50 μM) instead of (25 μM) showed (2 nm) shift in the ECG. Thus, the weak interaction between ECG and Fe may indicate that the reaction needs a higher ECG concentration. The importance of the ratio of the amount of released iron and the amount of added (poly)phenols for the prevention of lipid peroxidation was observed by Ferrali et al (1997). Also, the presence of a trihydroxyl (galloyl) group of the C ring is associated with reduced Fe-binding (Khokhar et al, 2003; Vestergaard, et al, 2005). Interestingly, Kubo et al, 2000 observed that Kaempferol reacts with Cu$^{2+}$ but the shift in the peaks was not observed with Mg$^{2+}$ and Ca$^{2+}$; this was the case with ECG when reacted with Fe$^{3+}$ and Cu$^{2+}$.

Chelators with small dimensions and high lipid solubility tend to interact with metabolic pools more rapidly than larger and more hydrophilic molecules. Thus, such reactions are concentration as well as time-dependent (Kushner et al, 2001). As indicated by Mira et al (2002) the more abundant the hydroxyl groups, the more effective the molecules are as an iron chelators. The greater numbers of OH groups results in higher negative charge density at the chelation site. In spite of this, ECG with 7-OH groups showed very weak iron chelation with high capacity to chelate Cu$^{2+}$; this suggests that flavonoids chelate iron more effectively when the metal ion is in its bivalent form, which means that the flavonoid needs to reduce Fe$^{3+}$ to Fe$^{2+}$ before association.
Trolox showed only small interactions with both copper and iron indicating poor chelating property probably due to its structure (Fig. 1.13). Trolox lacks the B ring which contains the catechol group responsible for metal chelation. It also lacks the 4-keto group on the C ring; thus the only available site for metal chelation is the sole –OH group on position 7. Further more, the position of the –OH group may be responsible for the hypsochromic shift of the band on interaction with iron. Thus, the bathochromic shift of the peak of trolox with Cu$^{2+}$ may indicate that there is no interaction of trolox with copper. In fact, trolox was ineffective in protection rat astrocytes from ROS production induced by copper; however trolox increased the toxicity of copper. Trolox probably catalyzes the reduction of Cu$^{2+}$ to Cu$^{+}$ followed by rapid reaction of Cu$^{+}$ with basal levels of H$_2$O$_2$ produced by mitochondria, leading to generation of more reactive radicals (Gyulkhandanyan et al, 2003). Addition of EDTA to trolox-Cu mixture failed to shift the peak to the original position. This may emphasize that there was no interaction between trolox and copper, and other chemical changes may take place instead as observed by Ozgova et al (2003) with coumaric acid and iron.

<table>
<thead>
<tr>
<th>(poly)phenol</th>
<th>Iron:(poly)phenol</th>
<th>Copper:(poly)phenol</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trolox</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Luteolin</td>
<td>1:2, 2:3</td>
<td>1:2, 1:3</td>
<td>Mira et al, 2002.</td>
</tr>
<tr>
<td>Luteolin-4'-O-glucoside</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Flavones meet most of the structural criteria for good chelators. They contain the 3',4'-OH groups (di-hydroxylcatechol group) on the B ring except luteolin-4'-O-glucoside, the 2,3 double bond, 4-keto group (the pyran-4-one), and 3,5-OH group (Sugihara et al, 1999). According to the above structural properties, only the flavones luteolin and luteolin-7-O-glucoside presented a high Fe$^{3+}$ chelation providing evidence for the importance of the presence of both the catechol in the B ring and the 4-keto group in the C ring with conjugation of the 2,3 double bond. This can be explained by the fact that in flavones the presence of the 2,3 double bond increased the planarity, and rigidity of the C ring and held the A and C rings in a more coplanar position, allowing the 5-hydroxyl 4-keto groups to be closer to the molecule (Mira et al, 2002).

The influence of the catechol group was obvious with the shift in the peaks of luteolin and luteolin-7-O-glucoside compared to luteolin-4'-O-glucoside in which one of the catechol groups (4' group) is occupied with a glucose preventing
this site from chelation with metals. Also, the hypsochromic shift in luteolin-4'-O-glucoside emphasized that the catechol group of the B ring is not involved in chelation of copper and iron. Instead, the 4-keto group in C ring and 2,3 double bond are possibly involved.

Similar results were obtained by Iwahashi et al (2004) who identified the luteolin-7-O-glucoside peak at 350 nm at pH 7.4. When Fe^{2+} was added, this peak shifted 32 nm to 382 nm. The increased shift of the peak compared to our results may be due to the fact that they used higher concentrations of both luteolin-7-glucoside (90 μM) and Fe^{2+} (33 μM). Moreover, Iwahashi et al (2004) began with the reduced form of iron; this may suggest that flavonoid reduced iron first before chelation. Brown and Rice-Evans (1998) compared the interaction of Cu^{2+} with luteolin and luteolin-7-O-glucoside at pH 7.4. They had similar results of 42, 65 nm shift in band II peaks respectively. The greater shift with luteolin-7-glucoside was explained by the glycosylation of the 7- position which would render the flavonoid more soluble in the aqueous environment thus increasing access to Cu^{2+} and enhancing the extent of chelation. This may explain the larger shift in peaks of luteolin-7-O-glucoside compared to luteolin with both metals. It was reported that the catechol group in luteolin-7-O-glucoside (Iwahashi et al, 2004) and luteolin (Khokhar et al, 2003) is one of the important factors for the chelation of Fe^{2+}. This illustrates why luteolin-4'-O-glucoside showed a lower capacity for iron and copper chelation compared to luteolin and luteolin-7-O-glucoside.

Iron (III) (Fe^{3+}) has six coordination sites, which need to be chelated completely if the generation of free radicals through redox cycling is to be
prevented. Chelators, which coordinate all six sites using a single molecule (hexadentate chelators) such as desferrioxamine (DFO), tend to form more stable iron-chelate complex than ligands, which require more than one molecule. Chelators that possess only two co-ordination sites (bidentate chelators) tend to dissociate from iron at low concentrations, resulting in partial co-ordination and the potential to generate free radicals under these conditions (Kushner et al, 2001).

The use of natural metal chelators, such as (poly)phenols, should be favoured against other synthetic chelators which present associated toxicity problems (Fernandez et al, 2002). These findings combined with their antioxidant activity on lipids and proteins, may be used to prevent the effect of UVA on skin and other diseases in which copper and iron are involved. Administration of iron chelating drugs is well known for treatment of many iron overload diseases. An example of such drug is DFO used in the treatment of thalassaemia (Beutler et al, 2003). A number of complications may occur from excessive treatment of DFO; these include a decreased growth rate (Ryter et al, 2000) and renal toxicity (Hershko, 1998), low efficacy and high cost of production (Samuni et al, 2001). Also, treatment of rats with the ferric chelator, nitrilotriacetate (NTA), used in detergents, was reported to produce severe nephrotoxicity and carcinogenecy (Athar and Iqbal, 1998). This metal chelator induces renal malondialdehyde (MDA) and (•OH) generation. Also, photo reduction of iron (III)-NTA to iron (II)-NTA complex with UVA-UVB light, facilitated the initiation of lipid peroxidation (Akai et al, 2004). The membrane impermeable copper chelator penicillamine is widely used in the treatment of Wilson’s disease in which copper is accumulated in brain, liver and kidney (Gyulkhandanyan et al, 2003). Also, bathocuproine as a copper chelator reduced
the oxidative damage of ceruloplasmin (Aouffen et al, 2004). Ryter, et al (2000) compared DFO, as a water soluble chelator which remain in the extracellular media, and O-phenanthroline which is a lipophillic membrane-permeable iron chelators. He found that both chelators, whether intracellular or extracellular, restrict the potential of iron from catalyzing Fenton-type reactions.

Considering human skin and its constant exposure to UV light and oxygen, combined with the increase LIP content of the exposed skin, topical application in cosmetic or addition of (poly)phenols to pharmaceutical formulations could be protective for the skin.

5.5 Conclusions

- All the tested phenolic compounds showed large interaction with iron and copper except trolox and ECG with iron.
- Luteolin and its glucosylated forms showed absorbance in the UVA region (320-400 nm) compared to 278 and 288 for ECG and trolox respectively.
- It also can be concluded that the role of iron chelation in the antioxidant activity of flavonoids largely depends on the assay and the conditions used.
CHAPTER 6
6. EFFECT OF (POLY)PHENOLIC COMPOUNDS ON LIPID PEROXIDATION AND PROTEIN CROSS-LINKING IN UVA IRRADIATED HUMAN SKIN FIBROBLASTS

6.1 Introduction

6.1.1 UVA and protein cross-linking

The ROS generated as by-products of UVA and UVB may cause modifications to amino acids of proteins in the epidermis, dermis (Sander et al., 2002) and stratum corneum (Thiele et al., 1999).

Protein cross-linking may be attributed in part to dityrosine formation (Fig. 6.2). Dityrosine is a fluorescent cross-linker of protein and is formed by various oxidation systems (Kato et al., 2001) including UV irradiation (Malencik et al., 1996), ageing, exposure to oxygen free radicals and when tyrosyl radical reacts with tyrosine (Saeed et al., 1999; Pennathur et al., 2001) (Fig 6.1). Recent studies by Saeed et al., (2006) confirmed dityrosine formation after the addition of UVB oxidized lipids to β-lactoglobulin by ESR and NMR spectroscopy. On the other hand, being a potent oxidant, free tyrosyl radicals can initiate lipid peroxidation by abstracting a hydrogen atom from fatty acids (Fig. 6.1). Dityrosine formed exhibits fluorescence at an excitation wavelength of 320-360 nm and emission of 410-460 nm, which were the wavelengths used in this study.
2 Tyrosines + H_2O_2 \rightarrow \text{Dityrosine} + 2 H_2O

Fig. 6.1 Dityrosine formation (Malencik, et al, 1996).

6.1.2 Methods used for the assessment of protein oxidation

The knowledge that some proteins have long half lives and hence are likely to accumulate oxidative hits, suggests that the formation of lesions on proteins may be a highly sensitive marker for oxidative damage in mammalian systems. The delayed use of radical-mediated protein oxidation as markers of oxidative damage \textit{in vivo} are due to two factors: the absence of data on the nature of the products formed and the lack of sensitive methods for the detection of these material in complex systems (Davies \textit{et al}, 1999).

The estimation of carbonyl yields from proteins is an excellent technique for examining the overall extent of protein damage in systems \textit{in vitro}. The carbonyls resulting from protein oxidation are measured using three major methods; these include the immunologic studies on tissue or isolated protein samples, GC/MS, and HPLC. Also \(o,o'\)-dityrosine has been analyzed in urine by a recently developed isotope dilution HPLC –atmospheric pressure chemical ionization tandem-mass spectrometry (HPLC-APCI-MS/MS) method (Orhan \textit{et al}, 2004). However, the use of these assays \textit{in vivo} face some difficulties due to
potential interference from aldehydes, and ketones generated from sugars or lipids bound to proteins. (Davies et al, 1999). Although the antibodies used in these studies can be specific for the oxidation products under study, the quantification of tissue staining/binding obtained is difficult to achieve. Both the HPLC and GC/MS methods require the isolation, purification, and hydrolysis of the proteins under study before analysis (Davies et al, 1999).

Elevated levels of \( o,o' \)-dityrosine have been detected in a wide range of disorders. Dityrosine is considered as a marker of endogenous protein oxidation in photoageing (Shen et al, 2000), red blood cells, in cataract lenses (Kato et al, 2001) and in protein hydrolysate (Malencik et al, 1996). Pennathur et al, (2001) reported increased levels of \( o,o' \)-dityrosine in diabetic aortic tissue \textit{in vivo}. Also, elevated levels of \( o,o' \)-dityrosine have been detected in inflammatory lung disease, neurodegenerative disorders and ageing (Heinecke, 2002) and in Parkinson's Disease (Pennathur et al, 1999).

\subsection*{6.1.3 Effect of UVA on collagen}

Kakehashi et al (1993) demonstrated that singlet oxygen \( (^1O_2) \) generated by white light irradiation caused the formation of extensive cross-links resulting in aggregation and insolubilization of collagen shown on SDS-PAGE. Collagen aggregation or solubilization after UVA irradiation can be mediated by electron transfer (type I) or by an indirect pathway involving singlet oxygen \( ^1O_2 \) (type II) (Scharffetter - Kochanek et al, 1993). Photosensitization followed the type I pathways producing OH\(^+\) and \( \mathrm{H}_2\mathrm{O}_2 \), resulting in liquid like gels, while the type II
process resulted in cross-linkage (Menter\textsuperscript{a} \textit{et al} 2003). Both mechanisms are described in Chapter 4 in detail.

Irradiation of HSF with low doses of UVA (0-100 kJ/m\textsuperscript{2}) revealed no significant collagen cross-linking. Furthermore, UVA irradiation (140 kJ/m\textsuperscript{2}) of human skin, three times a week for 10 weeks, revealed no significant changes in collagen fibres (Kambayashi \textit{et al}, 2001). At 300 kJ/m\textsuperscript{2}, photochemical molecular alterations occur resulting in collagen fibrils that do not have the same structure and conformation as intact collagen (Menter\textsuperscript{b} \textit{et al} 2001). At a very high dose (1400 kJ/m\textsuperscript{2}) complete collagen loss occurs by extensive chain degradation (Menter\textsuperscript{b} \textit{et al} 2001).

UVA irradiation (0-500 kJ/m\textsuperscript{2}) causes human skin ageing and skin cancer through activation of interstitial collagenase which is responsible for the degradation of collagen and is involved in tumor progression in human skin fibroblasts (Polte and Tyrrell, 2004). Also, UVA irradiation resulted in an increased synthesis of collagenases in skin fibroblast cultures (Scharffetter-Kochanek \textit{et al}, 1993) which were accompanied by collagen degradation and decreased collagen synthesis (Rittie and Fisher, 2002; Yan \textit{et al}, 2005). Such increases were reported with UVA doses of 100 kJ/m\textsuperscript{2} (Petersen \textit{et al}, 1992; Yoshiharu \textit{et al}, 1996), 150 kJ/m\textsuperscript{2} (Yan \textit{et al}, 2005), 200 kJ/m\textsuperscript{2} (Lee\textsuperscript{b} \textit{et al}, 2005), 300 kJ/m\textsuperscript{2} (Menter\textsuperscript{b} \textit{et al}, 2001) and 940 kJ/m\textsuperscript{2} (Menter\textsuperscript{a} \textit{et al}, 2003). At lower UVA doses (6, 18, 36 kJ/m\textsuperscript{2}) such stimulation did not occur (Philips \textit{et al}, 2003).

\textbf{6.1.4 Effects of antioxidants in preventing protein damage}

Literature concerning the effects of flavonoids on age related and UVA exposed collagen modification is very limited. Nevertheless, it has been reported
by Cheng et al, (2003) that urokinase, one of the hydrolases implicated in the degradation of collagen is inhibited by EGCG at physiologically high concentrations relevant to tea drinkers but the exact concentration was not reported. Bedoui et al, (2005) reported that ECG decreased MMP-2 level at 50 \( \mu \text{M} \) but not 30 \( \mu \text{M} \) while (Adcocks et al, 2002) reported that ECG inhibited collagen II breakdown at 20 \( \mu \text{M} \) when added to a rat cartilage model. The EGCG (0.1 \( \mu \text{M} \)) was more potent in decreasing MMP levels after UVA irradiation (Lee et al, 2005).

The trolox showed decrease of collagen I accumulation in myocardial fibroblasts and decreased MMP-1 production at 10 \( \mu \text{M} \). Luteolin (20 \( \mu \text{M} \)) also showed a reduction in MMP-2 at 10 \( \mu \text{M} \) (Lee et al, 2004) and 50 \( \mu \text{M} \) (Bagli et al, 2004).

This chapter describes cross-linking in proteins after UVA irradiation (250 and 500 kJ/m\(^2\)) by fluorescence microscopy; the effects of UVA irradiation on collagen I and IV after being separated by SDS-PAGE and confirmed by Western blotting; and the effects of 5 (poly)phenols (ECG, trolox, luteolin, luteolin-4'-glucoside and luteolin-7-O-glucoside) acting as antioxidant against collagen degradation and protein cross-linkage after UVA irradiation.
6.2 Materials and Methods

6.2.1 Materials

6.2.1.1 Fluorimetry

Quinine sulfate \((C_{20}H_{24}N_2O_2)_2.H_2SO_4.2H_2O\), epicatechin gallate (ECG) from green tea, (98%, HPLC grade) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) 97% were purchased from Sigma Aldrich, St. Louis, USA. Sulphuric acid (98%), was purchased from NTL Brixton, UK, chloroform from Lab-Scan Dublin, (Ireland) and methanol from Fisher Scientific, Leicestershire, UK. Luteolin (3',4',5,7-tetrahydroxyflavone), luteolin-4'-O-glucoside, and luteolin-7-O-glucoside were purchased from Extrasynthèse, Genay, France.

Equipments used included Fluorimeter and quartz micro cuvettes from Perkin Elmer (LS55 Luminescence-Spectrometer), and a refrigerated centrifuge from International Equipment Company (IECB-22M), Massachusetts, USA.

6.2.1.2 Peroxide Value (PV)

Materials as described in section 4.2.6.1.

6.2.1.3 Cell lysate buffer

Dulbecco’s phosphate buffer saline (10x) with and without \(Ca^{2+}\) and \(Mg^{2+}\) (PBS), Tris-(hydroxymethyl) methylamine buffer, sodium chloride (NaCl), sodium floride (NaF), and sodium dodecyl sulfate (SDS) were purchased from Sigma-Aldrich, Louis, USA. Ethylenediaminetetraacetic acid (EDTA) was purchased from NTL, Brixworth, UK; protease inhibitor cocktail tablets were
purchased from Roche, Germany and Triton X-100 solution from Bio-Rad, Richmond, California, USA. Centrifuge tubes (50 ml), 2 ml eppendorff tubes sterilized pipettes and cell scraper 25 cm handle, 1.8 cm blade were purchased from Falcon, Becton Dickinson Lab ware and Company, New Jersey, USA. The refrigerated centrifuge was from International Equipment Company (IECB-22M), Massachusetts, USA

6.2.1.4 Protein determination

Protein assay No. 5656 (Lowry protein determination kit) was purchased from Sigma Diagnostics, (St. Louis, USA). Glass test tubes (10 ml), visible region glass cuvettes and a spectrophotometer 1201 were purchased from Shimadzu, Japan.

6.2.1.5 Collagen separation on SDS-polyacrylamide gels

Complete vertical slab gel unit, power supply and 0.75 mm combs were obtained from Bio-Rad Richmond, (California, USA). Ammonium persulfate (APS), lauryl sulfate, sodium dodecyl sulfate, sodium salt (SDS), acrylamide/bis-acrylamide (30 % w/v solution), N,N,N',N'-tetra-methyl ethylene diamine (TEMED) and the electrophoresis reagents were purchased from Sigma-Aldrich, (St. Louis, USA). Coomassie brilliant blue R-250, glycerol, bromophenol blue, n-butanol and methanol were obtained from BDH, Poole, UK. A full range rainbow protein marker from (10-250 KDa) was purchased from Amersham Pharmacia, (Buckinghamshire, UK).
6.2.1.6 Western blotting

Polyoxethylene sorbitan monolaurate (tween 20) and monoclonal anti-collagen type IV and type I antibodies were purchased from Sigma, (St.Louis, USA). Sodium chloride, glycine, Tris-(hydroxymethyl) methylamine AR, and glacial acetic acid were from BDH, (Poole, UK). Anti-mouse IgG, horseradish peroxidase linked, whole antibody (from donkey), anti-mouse IgG, horseradish peroxidase linked, whole antibody (from sheep) and, Western blotting (ELC) kit, were purchased from Amersham Pharmacia, (Buckinghamshire UK). Kodak film developer and Kodak film fixer, was obtained from Kodak, USA; medical X-ray film, Super RX was purchased from Fuji (Europe), Germany; Whatman filter paper role (Whatman Ltd, UK), dried skimmed milk powder was purchased from Marvel, (UK).

6.2.2 Methods

6.2.2.1 Antioxidant treatment of samples

Fibroblasts were cultured in 0.75 x 10^4 cell/flask. Cells were confluent in 3 days. The (poly)phenols, ECG, trolox, luteolin, luteolin-4’-O-glucoside and luteolin-7-O-glucoside were freshly prepared and added 18 h before UVA irradiation to give a final concentration of 30 µM. Each antioxidant (100 µl) of 3 mM stock was added to 10 ml MEM in the culture plate and mixed as described in section 2.2.4.
6.2.2.2 Preparation of fibroblasts and irradiation

Prior to UVA irradiation, the cell culture medium was replaced with PBS. Confluent cells were exposed to single doses of 250 and 500 kJ/m². Cells were irradiated as described in section 2.2.

6.2.2.3 Fluorimetry

The protein cross-linking investigation was carried out based on Saeed et al. (1999) who used fluorescence to measure the cross-links between fish proteins after exposing them to oxidized lipids. In this chapter the effects of oxidized lipids on proteins in UVA irradiated fibroblasts in the presence and absence of 5 polyphenols acting as antioxidants were studied.

Control irradiated and (poly)phenol treated cells were trypsinized and collected in 15 ml pre-weighed centrifuge tubes and were counted. After centrifugation at 1500 rpm for 15 minutes at 45° C, the medium was aspirated and cell pellets were weighed carefully. The pellets were homogenized in 6 ml chloroform and methanol mixture (2:1 v/v). After mixing for 1 min, homogenates were sonicated in a water bath at 45° C for 1 min at power setting 2. Water (MilliQ) was added to the homogenate (6 ml) and tubes were vortexed for a further minute. The mixture was centrifuged at 3000 x g at 4° C. Equal volumes of the chloroform layer (2.5 ml), and the bottom layer, were pipetted into Pyrex tubes with covers, and measured at excitation wavelength 360 nm and emission wavelength 420 nm with a slit width of 2.5, open emission filter and integration time of 0.02 sec. A standard curve of quinine sulfate was constructed in the range of (0-0.4 μg/ml) in 1 M H₂SO₄. A stock solution of 10 mg/ml of quinine sulfate was prepared and further diluted to the working solution of a final...
concentration of 1 µg/ml. Of this working solution, serial dilutions with 1 M H$_2$SO$_4$ were placed in quartz cuvettes (total volume 3 ml) and measured against 3 ml of H$_2$SO$_4$ as a background which was subtracted automatically as a blank.

**Calculation**

Protein fluorescence (µg / million cells) =

(Sample absorption-background) - intercept /Slope of standard curve.

Each result was divided by the cell count to relate results to million cells.

### 6.2.2.4 Peroxide value (PV) determination

Before samples were measured for fluorimetry 100 µl, was transferred to 20 ml glass vials with covers for peroxide value measurements as described in section 4.2.

### 6.2.2.5 Protein determination and characterization by SDS-PAGE

**Cell lysis**

For each experiment 4 petri-dishes of each treatment were pooled together. Control, irradiated and (poly)phenol treated fibroblasts were washed three times with cold PBS without Ca$^{2+}$ and Mg$^{2+}$ before lysis. The PBS was removed completely and lysis buffer mixture was added (1 ml/plate). Lysis buffer was freshly prepared by dissolving EDTA (5 mM), NaCl (250 mM) and NaF (50 mM) in Tris buffer (50 mM). Triton X-100 (0.1 % w/v) was then added followed by SDS (0.1% w/v). The buffer was stirred until homogeneous. Immediately before the experiment, one tablet of protease inhibitor cocktail was dissolved in 20 ml cold lysis buffer as recommended by the manufacturer, and kept on ice.
After addition of lysis buffer, each plate was shaken for exactly 5 min. Using a cell scraper, cells were removed from plates and carefully transferred to 50 ml centrifuge tubes using sterilized pipettes. Each tube was left on ice for 30 min. The contents were mixed with a syringe (up and down) every 10 min. The cell lysate was then centrifuged (4°C) at 13 000 x g for 30 min. All the steps were carried out on ice. The supernatant was divided into Eppendorff tubes and kept at -80°C for protein determination and collagen separation on SDS-PAGE.

**Protein determination**

Proteins were measured by the Lowry spectrophotometric method (Lowry et al, 1951) based on the reaction between the alkaline cupric tartrate reagent and the peptide bonds of proteins. The resultant complex reacts with Folin and Ciocalteu phenol reagent to form a purple color which was measured at 750 nm. The protein concentration was determined from the standard curve prepared from bovine serum albumin (BSA). As recommended by the manufacturer, the Lowry reagent solution was prepared by adding 40 ml water to the Lowry reagent bottle and mixed well until dissolved without shaking to minimize foaming. Folin and Ciocalteu's phenol working solution was prepared by mixing 80 ml deionized water with 18 ml Folin and Ciocalteu's reagent.

**Standard curve for BSA**

Protein standard solution provided with the assay kit was prepared by adding 1 ml deionized water to the protein vial to give a stock solution of 400 μg/ml. From this stock protein solution the standard tubes were prepared by diluting protein solution in water to a final volume of 1 ml in protein range of (0-300 μg/ml).

**Protein determination of fibroblasts**
The cell lysate (200 µl) was prepared as described in section 6.2.2.5. This was added to 800 µl of water. To each tube of standard or samples, 1 ml of Lowry reagent was added. After 20 min at room temperature (25°C) 0.5 ml Folin & Ciocalteu's phenol reagent was added to each tube. The colour developed was measured exactly after 30 minutes at 750 nm. A blank was treated in the same way as standard tubes and samples and subtracted automatically from spectrophotometric readings.

**Calculation**

The absorbance values of the BSA standards were plotted versus their corresponding protein concentrations to prepare the standard curve. Protein concentration of each sample was determined from the calibration curve in µg/ml and multiplied by the appropriate dilution factor to obtain the original protein concentration.

\[
\text{Protein (µg/ml) = concentration from protein standard curve x dilution factor (5).}
\]

**6.2.2.6 Separation of proteins on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)**

In this study, SDS-PAGE was performed using the Laemmli (1970) method to separate fibroblast proteins under non-reducing conditions. The protein was denatured by SDS detergent to break hydrophobic bonds and swamp the proteins with a negative charge. This negative charge allows proteins to move towards the positive electrode according to their molecular weight, irrespective of the normal physiological net charge. Protein samples were separated using 4-15% gradient gels. Rainbow colored protein molecular weight markers were loaded onto the gels for comparison.
Preparation of separating and stacking gels

Polyacrylamide gels consist of two layers, an upper stacking layer, which concentrates proteins without separating them and a lower gradient (4-15%) polyacrylamide resolving layer, which separates proteins. The gel equipment (glass plates and spacers) was washed with methanol, dried, carefully assembled and placed in the casting stand.

The separating gel monomer solution was prepared by combining appropriate volumes of 30% (w/v) acrylamide, 1.5 M Tris-HCl, pH 8.8 and 0.4% (w/v) SDS except ammonium persulfate (APS) 0.4% (w/v) and TEMED. The solution was degassed under vacuum for at least 10 minutes. APS and TEMED were added to the degassed resolving buffer solution. A gradient chamber was used to mix the solution. One chamber was filled with 4% acrylamide solution and the other was filled with 15% acrylamide solution (1.8 ml mixture/chamber). Briefly, after the addition of freshly prepared APS and TEMED to the acrylamide solution in the two chambers, the ingredients were mixed and the gel was poured immediately. The solution was overlaid with iso-butanol. The gel was left to polymerise for 1 h, after which the iso-butanol was removed by washing the gel with distilled water.

The stacking gel monomer solution was prepared by combining appropriate volumes of 30% (w/v) acrylamide, 0.5 M Tris-HCl, pH 6.8 and 0.2% (w/v) SDS except APS and TEMED and degassed under vacuum at least 10 minutes. Next, a comb was placed in the gel sandwich; freshly prepared APS 0.2% (w/v) and TEMED were added to the degassed monomer stacking buffer solution. The solution was then poured between the two glass plates. The gel was left to polymerise for 30 min. After the removal of the comb, the wells were
rinsed with distilled water and the samples were loaded. The electrophoresis tank was filled with a 1:10 dilution of the electrolyte-running buffer (0.25 M Tris-HCl, pH 8.3, 0.2 M glycine, 0.1% (w/v) SDS) and electrophoresis was carried out at 80 V for the first 30 min and 200 V for the remainder of the electrophoretic separation.

**Sample preparation**

Samples were prepared by mixing proteins (5 µg/well) with 6X SDS sample buffer (6X: 0.35 M Tris-HCl (pH 6.8), 10.28% (w/v) SDS, 36% (v/v) glycerol, 0.012% (w/v) bromophenol blue) at a 1:1 ratio (v/v) and they were then heated at 95°C for 5 min before loading onto the gels.

**Gel staining**

The proteins were visualised by staining with Coomassie blue (0.2% (w/v) Coomassie Brilliant Blue R, 50% (v/v) methanol, 40% (v/v) distilled water and 10% (v/v) glacial acetic acid) for 1 hour and destained using rapid destained solution (40% (v/v) methanol, 5% (v/v) glacial acetic acid and 55% (v/v) distilled water) until clear gels were obtained.

**6.2.2.7 Western blotting**

After SDS-PAGE separation, stacking gels were discarded and separating gels were equilibrated in freshly made transfer buffer (0.15 M glycine, 20 mM Tris-base, 20% (v/v) methanol) for 15 min. The protein samples separated by SDS-PAGE were transferred to Hybond ECL nitrocellulose membranes using a Bio-Rad wet transfer system. For each gel, filter papers were cut to fit the transfer membrane cassette (Fig. 6.4 A). The sandwich components were
assembled in the following order: the grey side of the cassette/sponge/2 pieces of filter papers/nitrocellulose membrane/gel/2 pieces of filter paper/sponge/black side of the cassette. The transfer tank (Fig. 6.4 B) was filled with the transfer buffer and put on magnetic stirrer in the cold room over night at 30 V.

The membrane was blocked for 1 hour at room temperature 5-10% (w/v) skimmed milk in Tris buffer saline (TBS; 20 mM Tris-HCl, pH 7.7 containing 0.9% (w/v) NaCl) and washed briefly with TBS-Tween (TBS containing 0.1% (v/v) Tween 20) to block other sites on the membrane. The membrane was probed with the appropriate dilution of primary antibody dissolved in 1-2% (w/v) skimmed milk in TBS, and gently rocked for 1 hour at room temperature. Two types of collagen anti-bodies were used, monoclonal anti-collagen type I (mouse IgG) in dilution of (1:500). This antibody recognizes the native (helical) form of collagen I in humans. The second type was monoclonal anti-collagen IV (mouse IgG) which recognizes an epitope located on the α 1 and/or α 2 chains of human collagen type IV used in 1:500 dilution.

The membrane was washed 3 times with TBS-Tween for 15 minutes each. The membrane was probed with the secondary antibodies polyclonal anti-mouse IgG horseradish peroxidase conjugated antibodies (HRP) (1: 2000 dilution) for 1 hour at room temperature with gentle rocking. The membrane was washed again with TBS-Tween 3 times for 15 minutes and the protein bands were visualised using an enhanced chemiluminescence ECL western blotting analysis system in accordance with the manufacturer’s instructions. The membrane was incubated with detection reagents for 1 min and exposed to X-ray film. The film was left to develop in a dark room for 5 min to 1 hour depending
on the intensity of the band. The film was developed and fixed with Kodak developer and fixer. Finally, the film was washed with tap water and left until dry.

Fig. 6.2 (A) Transfer tank assembly. The stack is oriented so that the negatively charged molecules migrate towards the grey side of the cassette (anode). (B) Diagram of the assembly of an immunoblot for tank transfer.
6.3 Results

6.3.1 Protein cross-linking by fluorimetry

Protein oxidation and cross-linking was measured in human skin fibroblasts using fluorescence spectrometry. Results were calculated using quinine sulphate standard curve (Figure 6.3).

![Figure 6.3](image)

Fig. 6.3 The standard curve of calculation of fluorescence and protein cross-linking. The curve represent the average ± SD of 8 experiments each of N= 3.

The data are presented as the arithmetic mean percentages ± the standard error of mean (SEM). For significant differences between control and experimental values the p-value between groups was determined using SPSS version 11.5 one way analysis of variance (ANOVA) followed by Bonferroni test with \( p \leq 0.05 \).

6.3.1.1 Effect of UVA on protein cross-linkage

The results of the fibroblast irradiation revealed a significant increase in protein cross-linking after UVA irradiation. When cells were irradiated with 250 and 500 kJ/m², protein cross-linking increased 2 and 5.4 times respectively.
compared to control cells (control = 0.0027 ± 0.0004 μg/10^6 compared to 0.0054 ± 0.0005 μg/10^6 cells when irradiated with 250 kJ/m^2, p=0.03) and (0.0146 ± 0.001 μg/10^6 cells, p =0.00) compared to irradiation with 500 kJ/m^2 (Fig 6.4 A). Consequently, the peroxide value (PV) of the controls and UVA irradiated samples was determined and there was a significant difference between controls and 250 kJ/m^2 (control = 0.68 ± 0.06, 250 kJ/m^2 UVA irradiated = 1.4 ± 0.2, p=0.02) and at 500 kJ/m^2 (2.5 ± 0.3 at, p=0.002) (Fig. 6.4 B).

6.3.1.2 Effect of (poly)phenols on protein cross-linking

ECG is known to have inhibitory effects on lipid and protein oxidation. When ECG was added to skin fibroblasts without UVA irradiation, there was a slight decrease in fluorescence from 0.0027 ± 0.0004 of controls to 0.0020 ± 0.0002 μg/10^6 but this decrease was not significant (p=0.6) (Fig. 6.4 A). However, ECG reduced protein cross fluorescence significantly when added to 500 kJ/m^2 treated cells from 0.014 ± 0.001 to 0.004 ± 0.0007 μg/10^6 (p=0.00) but no significant difference was obtained when ECG was added to 250 kJ/m^2 (0.0054 ± 0.0005 increased to 0.007 ± 0.0008 μg/10^6 p= 0.08).

The PV results also reflected a similar trend as the protein cross-linkage studies (Fig. 6.4 B). Compared with irradiated cells, ECG lowered PV with the high UVA dose of 500 kJ/m^2 from 2.5 ± 0.06 μg/10^6 to 1.1 ± 0.3 μg/10^6, p<0.001). However, there was no significant difference in PV for 250 kJ/m^2 irradiated cells (1.4 ± 0.06 μg/10^6) compared to ECG treated cells (1.5 ± 0.2 μg/10^6, p= 0.8 at 250 kJ/m^2).
Fig. 6.4 Effect of UVA (250 and 500 kJ/m²) and ECG (30 µl) in human skin fibroblasts. (A) Protein fluorescence (µg/million cells). (B) Peroxide value (PV) (µg/million cells).

Like ECG, trolox showed a small but insignificant decrease in protein cross-linkage in untreated fibroblasts (control= 0.0027 ± 0.0004 µg/10⁶ to 0.0017 ± 0.0002 µg/10⁶) but this decrease was insignificant (p= 0.5) Fig.6.5 A. Also trolox showed no protective effect when added to 250 kJ/m² UVA irradiated cells (0.0054 ± 0.0005 µg/10⁶ to 0.0048 ± 0.0008 µg/10⁶ p= 0.6). At 500 kJ/m², trolox caused a significant decrease in protein cross linkage compared to untreated irradiated cells 0.014 ± 0.001 µg/10⁶ to 0.0066 ± 0.0009 µg/10⁶ , p= 0.00). A similar observation was also obtained with PV (Fig. 6.5 B). Trolox had no effect
on control cells (0.68 ± 0.06 µg/10⁶ compared to 0.7 ± 0.1 µg/10⁶, \( p = 0.8 \)) or those irradiated at 250 kJ/m² (1.4 ± 0.2 µg/10⁶ to 1.1 ± 0.1 µg/10⁶, \( p = 0.2 \)) respectively, but decreased the PV significantly when trolox was added to cells at 500 kJ/m² (2.5 ± 1 µg/10⁶ to 1.3 ± 0.2 µg/10⁶, \( p = 0.00 \)).

![Bar chart A](image)

![Bar chart B](image)

**Fig. 6.5** Effect of UVA (250 and 500 kJ/m²) and trolox (30 µl) in human skin fibroblasts. (A) Protein fluorescence (µg/million cells). (B) Peroxide value (PV) (µg/million cells).

The effect of luteolin and its glycosylated forms were also tested for their antioxidant activity in preventing protein cross-linkage after UVA irradiation. When luteolin was added to fibroblasts without irradiation the decrease in protein cross-linkage was insignificant (0.0027 ± 0.0004 µg/10⁶ compared to 0.0023 ±
However, luteolin showed highly significant antioxidant effects when added to 250 kJ/m² irradiated cells \(0.0054 \pm 0.0005 \mu g/10^6\) to \(0.002 \pm 0.0003 \mu g/10^6, p=0.012\) and to 500 kJ/m² \(0.014 \pm 0.001 \mu g/10^6\) to \(0.003 \pm 0.0009 \mu g/10^6, p=0.00\) (Fig 6.6 A). However luteolin did not affect the PV significantly whether in control cells \(0.68 \pm 0.06 \mu g/10^6\) compared to \(0.6 \pm 0.08 \mu g/10^6, p=0.8\) in 250 kJ/m² irradiated cells \(1.4 \pm 0.2 \mu g/10^6\) compared to \(0.82 \pm 0.1 \mu g/10^6, p=0.07\) or in 500 kJ/m² irradiated cells \(2.5 \pm 0.3 \mu g/10^6\) compared to \(2.1 \pm 0.5 \mu g/10^6, p=0.3\) (Fig. 6.6 B).

Fig. 6.6 Effect of UVA (250 and 500 kJ/m²) and luteolin (30 μl) in human skin fibroblasts. (A) Protein fluorescence (μg/million cells). (B) Peroxide value (PV) (μg/million cells).
Figure 6.7 A shows that luteolin-4'-O-glucoside caused non-significant changes when added to controls (control = 0.0027 ± 0.0004 compared to 0.003 ± 0.001 μg/10^6, (p= 0.7) or to 250 kJ/m² UVA irradiated cells (0.0054 ± 0.0005 compared to 0.004 ± 0.0005 μg/10^6, p= 0.1). For 500 kJ/m² dose, luteolin-4'-O-glucoside showed a significant decrease in protein cross-linkage reducing the value for the irradiated sample from 0.014 ± 0.001 to 0.005 ± 0.0008 μg/10^6, (p<0.00). Luteolin-4'-O-glucoside did not affect the PV significantly when added to controls (control = 0.68 ± 0.06 compared to 0.66 ± 0.08 μg/10^6, p= 0.9) or to 500 kJ/m² (2.5 ± 0.3 increased to 2.8 ± 0.4 μg/10^6, p= 0.4) (Figure 6.7 B). However, when luteolin-4'-O-glucoside was added to 250 kJ/m² treated cells, a significant increase was obtained (from 1.4 ± 0.2 to 2.6 ± 0.4 μg/10^6, p= 0.007).
Fig. 6.7 Effect of UVA (250 and 500 kJ/m²) and lut 4 (30 µl) in human skin fibroblasts. (A) Protein fluorescence (µg/million cells). (B) Peroxide value (PV) (µg/million cells). Lut4= luteolin-4'-O-glucoside.

Luteolin-7-O-glucoside caused insignificant changes when added to control fibroblasts (0.0027 ± 0.0004 compared to 0.002 ± 0.0004 µg/10⁶) (Fig. 6.8 A). Like all (poly)phenols except luteolin, luteolin-7-O-glucoside significantly reduced protein cross-linkage at the high UVA dose, 500 kJ/m² from (0.014 ± 0.001 to 0.0055 ± 0.001 µg/10⁶, p<0.00). In contrast, the reduction was insignificant at 250 kJ/m² (0.005 ± 0.0005 to 0.003 ± 0.0004 µg/10⁶, p=0.06). Luteolin-7-O-glucoside did not affect the PV significantly when added to
controls (control= 0.68 ± 0.06 compared to 0.56 ± 0.08 µg/10^6, p= 0.7) or to cells irradiated with 250 kJ/m^2 (1.4 ± 0.2 reduced to 0.7 ± 0.08 µg/10^6, p= 0.051) or 500 kJ/m^2 (2.5 ± 0.3 reduced to 2 ± 0.5 µg/10^6, p= 0.2) (Fig. 6.8 B).

![Graph A](image)

![Graph B](image)

Fig. 6.8 Effect of UVA (250 and 500 kJ/m^2) and lut 7 (30 µl) in human skin fibroblasts. (A) Protein fluorescence (µg/million cells). (B) Peroxide value (PV) (µg/million cells). Lut7 = luteolin-O-7-glucoside.
6.3.2 Results of protein determination by the Lowry method

Table 6.1 The protein content in µg/ml ± SD in different samples determined by Lowry method.

<table>
<thead>
<tr>
<th>Controls</th>
<th>Without (poly)phenols</th>
<th>ECG</th>
<th>Trolox</th>
<th>Luteolin</th>
<th>Luteolin-4'-glucoside</th>
<th>Luteolin-7'-glucoside</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>190 ± 0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UVA 250 kJ/m²</td>
<td>160 ± 0.019</td>
<td>140 ± 0.011</td>
<td>145 ± 0.011</td>
<td>130 ± 0.005</td>
<td>155 ± 0.00</td>
<td>140 ± 0.009</td>
</tr>
<tr>
<td>UVA 500 kJ/m²</td>
<td>190 ± 0.014</td>
<td>140 ± 0.002</td>
<td>145 ± 0.007</td>
<td>140 ± 0.002</td>
<td>170 ± 0.03</td>
<td>130 ± 0.003</td>
</tr>
</tbody>
</table>

6.3.3 Protein separation on SDS-PAGE gels and Western blot for collagen I and IV

Confluent HSF were used to test the effects of both UVA (250 and 500 kJ/m²) and 5 (poly)phenols on collagen I and IV. Results for SDS-PAGE for control cells, UVA irradiated cells (250 and 500 kJ/m²) and antioxidant treated cells were similar. For all experiments a range of protein bands of MW 15-250 KDa were obtained (Fig. 6.9-13 (A)). For UVA 250 and 500 kJ/m² irradiated cells (lane 3,4) the intensity of all bands decreased to a similar extent due to protein denaturation. In the presence of each of (poly)phenols tested no obvious changes were detected compared to irradiated controls.

6.3.3.1 Collagen I Western blot (WB)

Results for the control fibroblasts (Fig. 6.9-13, B lane 1) showed 3 main bands with MW 160, 105, and 53 KDa. The 160 and 105 were likely to be α1 and α2 chains of collagen I. When (poly)phenols were added to controls without UVA irradiation, no change in the gels patterns were obtained (Fig. 6.9-13 B lane 2).
With UVA treatment (250 and 500 kJ/m²) (Fig. 9-13, B) lane 3 and 4, α1 chain (160 KDa) was either very faint or had disappeared completely. Also, there was a split in the low MW band (60 KDa) band with a new band at 53 KDa particularly for UVA 500 kJ/m² treated cell proteins. Bands of collagen I were more resistant to UVA irradiation. Bands became faint (105 KDa), or disappeared (160 KDa and/or split 60 KDa), especially at the high UVA dose. In comparison to collagen I, two of the three bands of collagen IV (250 and 160 KDa) disappeared completely when HSF were irradiated.

For all samples, the addition of the 5 (poly)phenols did not protect the α1 chain (160 KDa) (Fig. 6.9-13 B lane 5 and 6).

### 6.3.3.2 Collagen IV Western blot (WB)

Results for the control fibroblasts indicated 3 main bands at 250, 160 and 105 KDa (Fig. 6.9-13 C lane 1). The same bands were observed after incubation of fibroblasts with (poly)phenols (Fig. 6.9-13 C lane 2).

For UVA irradiated samples 250 and 500 kJ/m², the band at >250 and 160 completely disappeared (Fig. 6.9-13 C lane 3 and 4). A band at 105 KDa was obtained with different treatments. Except the of 105 KDa band, none of the (poly)phenols protected collagen IV proteins against UVA effects (Fig. 6.9-13 C lane 5 and 6).
Fig. 6.9 Characterization of proteins extracted from human skin fibroblasts treated with ECG. (A) Coomassie Brilliant stained SDS polyacrylamide gel. (B) Western Blot analysis with monoclonal collagen I. (C) Western blot analysis with monoclonal collagen IV. 1 = Control fibroblasts, 2 = Control fibroblasts with 30 μM ECG for 18 h, 3 = Fibroblasts irradiated with 250 kJ/m², 4 = Fibroblasts irradiated with 500 kJ/m², 5 = Fibroblasts irradiated with 250 kJ/m² irradiated ECG as antioxidant, 6 = Fibroblasts irradiated with 500 kJ/m² irradiated with ECG as antioxidant, 7 = Rainbow protein marker.
Fig. 6.10 Characterization of proteins extracted from human skin fibroblasts treated with trolox. (A) Coomassie Brilliant stained SDS polyacrylamide gel. (B) Western blot analysis with monoclonal collagen II. (C) Western blot analysis with monoclonal collagen IV. 1 = Control fibroblasts, 2 = Control fibroblasts with 30 μM trolox for 18 h, 3 = Fibroblasts irradiated with 250 kJ/m², 4 = Fibroblasts irradiated with 500 kJ/m², 5 = Fibroblasts irradiated with 250 kJ/m² irradiated with trolox as antioxidant, 6 = Fibroblasts irradiated with 500 kJ/m² irradiated with trolox as antioxidant, 7 = Rainbow protein marker.
Fig. 6.11 Characterization of proteins extracted from human skin fibroblasts treated with luteolin. (A) Coomassie Brilliant stained SDS polyacrylamide gel. (B) Western blot analysis with monoclonal collagen I. (C) Western blot analysis with monoclonal collagen IV. 1= Control fibroblasts, 2= Control fibroblasts with 30 µM luteolin for 18 h, 3= Fibroblasts irradiated with 250 kJ/m², 4= Fibroblasts irradiated with 500 kJ/m², 5= Fibroblasts irradiated with 250 kJ/m² irradiated luteolin as antioxidant, 6= Fibroblasts irradiated with 500 kJ/m² irradiated with luteolin as antioxidant, 7= Rainbow protein marker.
Fig. 6.12 Characterization of proteins extracted from human skin fibroblasts treated with luteolin-4'-O-glycoside. (A) Coomassie Brilliant stained SDS polyacrylamide gel. (B) Western blot analysis with monoclonal collagen I. (C) Western blot analysis with monoclonal collagen IV. 1= Control fibroblasts, 2= Control fibroblasts with 30 μM luteolin-4'-O-glycoside for 18 h, 3= Fibroblasts irradiated with 250 KJ/m², 4= Fibroblasts irradiated with 500 kJ/m², 5= Fibroblasts irradiated with 250 kJ/m² irradiated luteolin-4'-O-glycoside as antioxidant, 6= Fibroblasts irradiated with 500 kJ/m² irradiated with luteolin-4'-O-glycoside as antioxidant, 7= Rainbow protein marker.
Fig. 6.13 Characterization of proteins extracted from human skin fibroblasts treated with luteolin-7-O-glycoside. (A) Coomassie Brilliant stained SDS polyacrylamide gel. (B) Western blot analysis with monoclonal collagen I. (C) Western blot analysis with monoclonal collagen IV. 1= Control fibroblasts, 2= Control fibroblasts with 30 μM luteolin-7-O-glycoside for 18h, 3= Fibroblasts irradiated with 250 kJ/m², 4= Fibroblasts irradiated with 500 kJ/m², 5= Fibroblasts irradiated with 250 kJ/m² irradiated luteolin-7-O-glycoside as antioxidant, 6= Fibroblasts irradiated with 500 kJ/m² irradiated with luteolin-7-O-glycoside as antioxidant, 7= Rainbow protein marker.
6.4 Discussion

6.4.1 Effect of UVA on protein cross linkage

Fluorimetry results indicated an increase in protein cross-linkage in HSF after UVA irradiation. The ROS generated as by-products of UVA and UVB irradiation may cause modifications of amino acids of proteins in the epidermis, dermis (Sander et al, 2002) and stratum corneum (Thiele et al, 1999). The fluorescence measured at the excitation wavelength of 360 nm and the emission wavelength of 420 nm was probably due to dityrosine cross-linkage (Saeed et al, 1999). However, different amino acids such as arginine, lysine and histidine also formed cross-links, which fluoresced at these wavelengths (Saeed et al, 1999).

Irradiation at 250 and 500 kJ/m² increased protein cross-linkage by 2 and 5.4 times respectively. This increase paralleled an increase in the lipid oxidation parameter, PV which was 2 and 3.7 times at the two doses. These results are consistent with Sander et al, (2002) who demonstrated that the exposures of keratinocytes and fibroblasts to UVB, UVA and H₂O₂ led to dose-dependant protein oxidation.

6.4.2 Effect of UVA on collagen I and IV

Irradiation of collagen is known to cause the formation of extensive cross links resulting in collagen aggregation (Kakehashi et al, 1993). The Western Blot results indicated changes in both collagen I and to a greater extent to collagen IV. Results of control HSF indicated that bands of collagen type I and
IV are considered to be $\alpha_1$ and $\alpha_2$ chains with additional band at 57 KDa in collagen type I extract.

Control samples showed 3 bands of collagen I at molecular weight of 160 and 105 KDa probably corresponding to $\alpha_1$ and $\alpha_2$ chains respectively, with an additional band at 60 KDa. The same typical pattern of bands was also obtained by Kakehashi et al., (1993) but they were at 98 and 91 KDa. The difference in molecular weight due to the reduction with 5% mercaptoethanol which may result in the broken fragments.

The present results for $\alpha_1$ (I) chain match those obtained by Lei et al, 1996 and Chen et al., 2004, who obtained $\alpha_1$ (I) collagen at 160 KDa when extracted from rat amnion and rat cardiac fibroblasts respectively. Cimpean et al (2001) detected $\alpha_2$ (I) collagen extracted from human cartilage at 110 KDa which closely matches our results at 105 KDa. Other researchers reported type I collagen $\alpha_1$ chain extracted from kangaroo tail and $\alpha_2$ chain at 97 and 91 KDa with additional band at 107 KDa (Kakehashi et al, 1993). Also Menter et al., (2003) reported bands at 200, 108, and 97 KDa of collagen I extracted from mouse skin fibroblasts without specification.

The small band obtained at 57 KDa has also been observed by other researchers. Small collagenous fragments of 50 KDa and smaller were found by Glanville et al, (1979) during the purification of $\alpha$ (IV) chains. Also, separation of collagen I indicated bands at 66 and 45 KDa (Deyl et al, 1998). Further non-specific bands corresponding to 50 KDa immunoglobulin heavy chain (IgG) were observed by Western blotting of type IV collagen (Mahmoodian and Peterkofsky, 1999). Moreover, under non-reducing conditions, such as in this study, the 50
KDa band in the basement membrane zone of human skin was separated into two bands at 46 and 50 KDa (Gayraud et al, 1997).

Collagen IV band at ≥ 250 KDa may be referred to as native collagen IV or to β chains which were composed of combined α₁ and α₂ chains without complete digestion. This band totally disappeared from controls when HSF were irradiated with both 250 and 500 kJ/m² doses. Many researchers report α₁ (IV) and α₂ (IV) chains in different locations. Grande et al (2002) used a similar technique to the one described in this study to extract collagen IV from mice renal tubular epithelial cells and obtained two bands at 185 and 170 KDa for α₁ (IV) and α₂ (IV) respectively. Similar to our results, Soder and Poshcl (2004) detected human α₂ (IV) extracted from Do4 cell line at 175 KDa, and detected α₁ (IV) chains extracted from mouse cell line PF-HR9 at 185 KDa. Lei et al (1999) reported chains at 180 and 120 KDa when extracted from rats amnion basement membranes. Also Glanville et al (1979) reported two chains at 160 and 140 KDa when extracted from human placenta. It is obvious that the discrepancies among publications may arise from differences in experimental approach and methodology as well as collagen sensitivity to enzymes which differ with different collagen origins (Yoshimura et al 2000).

Collagen IV proteins were more denatured than collagen I especially the high MW 250 and 160 KDa bands. This may be due to two factors, the location and the nature of collagen structure. The fact that collagen IV is the main constituent of the basement membrane (Mohmoodian and Peterkofsky, 1999), it may be more susceptible to UVA irradiation than collagen I which is mainly located in the extracellular matrix (Krahn et al, 2006). Also collagen I is
considered as a fibril collagen (Ottani et al. 2002) which needs 2 degradation
steps involving initial cleavage at the collagen triple helix by collagenases
followed by MMP digestion (Ohuchi et al., 1997). Also collagen I tends to resist
UV to conserve the mechanical integrity of the skin (Menter et al., 2001;
Kligman et al., 2000). In addition, degradation of collagen is controlled largely
by melting point (Collins et al., 1995); collagen IV being more susceptible to
degradation than collagen I due to a higher content of proline and hydroxyproline
(Soder and Poschl, 2004).

6.4.3 Effects of 5 (poly)phenols on protein cross-linkage

When 5 (poly)phenols were added to HSF at 30 μM for 18 h without
UVA irradiation, none of them showed antioxidant or prooxidant effects
compared to control cells. After UVA irradiation at 250 kJ/m^2 only luteolin
showed a reduction (67%) in cross-linkage but not its glycosylated forms. This
role of luteolin emphasizes the importance of the free hydroxyl group in 4' and 7
positions which is absent in the other two glycosylated forms.

Without exception each of the 5 (poly)phenols showed antioxidant
activity when added to cells treated with 500 kJ/m^2. Up to 79 % reduction in
cross-linkage was obtained by luteolin; this was parallel to its potent activity in
the protection of lipids (Chapter 4) and reduced apoptosis in HSF (Chapter 3).
ECG was second in protein protection (71.5 %) while the glycosylated forms of
luteolin were close in percentage (64 %) for luteolin-4'-O-glucoside and luteolin-
7-O-glucoside respectively. Trolox showed least protection at 53% reduction in
protein cross-linkage.
The results indicated that the antioxidant effect of ECG was greatest with the higher UVA dose (500 kJ/m²) rather than 250 kJ/m². The antioxidant activity of ECG is due to the phenolic hydroxyl groups and the galloyl moiety of ECG (Wang et al, 2003), and to the chelating effect of ECG on copper and to lesser extent on iron and free radicals scavenging. This makes ECG a powerful antioxidant. However, the greater the amount of free radicals generated (in 500 kJ/m² ECG treated cells) the more effective the antioxidant properties of ECG.

In situations where only small amounts of free radicals are generated, (as in 250 kJ/m² ECG treated cells) ECG appears to act as a prooxidant. Other researchers have also mentioned the possibility of a prooxidant property of ECG. Azam et al, (2004) reported oxidative cleavage properties of green tea catechins either alone or in the presence of copper. Furukawa et al, (2003) has reported ECG to cause DNA damage but to a lesser extent than epigallocatechin gallate (EGCG) and epigallocatechin (EGC). A possible explanation for the small effect of ECG is that the catechin concentrations in the HSF or the incubation time which may not be enough to inhibit the free radical reactions that lead to the formation of cross-linkages or degradation in collagen IV and I. The effect obtained in this study at 30 µM is consistent with results obtained by Bedoui et al, (2005) indicating that the inhibitory effect of ECG on the collagen degradation enzyme MMP-2 was concentration dependent and started at 50 µM. In agreement with our results of the effect of ECG on collagen I and IV, Song et al, (2002) also found that green tea extract (50 mg/ml) failed to inhibit free radical production which leads to the formation of carbonyl groups in the collagen of aged rat skin.
It was reported by Polte and Tyrrell, (2004) that incubating irradiated (250 kJ/m²) HSF with trolox (100 μM) for 18 h led to a 55 % inhibition of the interstitial collagenase. Interestingly the addition of trolox alone without irradiation had no effect on the enzyme activity. The same research group suggested that trolox inhibited lipid peroxidation by scavenging lipid radicals thereby preventing the propagation of the oxidation process.

Kato et al, (2001) revealed that dityrosine formation is not likely to be a product of the metal-catalyzed oxidation systems such as H₂O₂/Cu in vivo because biological systems have a high concentration of reducing agents and indicated that dityrosine is mainly produced by heme proteins. On the other hand, not only heme proteins may cause dityrosine formation, H₂O₂ and HO• radical will also generate dityrosine in vitro (Heinecke, 2002). Since UVA produces both HO• and H₂O₂, it seems that (poly)phenols showed its antioxidant activity at the high UVA dose when a threshold concentration of HO• and H₂O₂ was produced.

6.4.4 Antioxidant activity of 5 (poly)phenols on collagen I and IV

Collagen loss is a characteristic of sun damaged skin (Kligman et al, 2000). All (poly)phenols used in this study had no toxic and no protective effects on collagen I or IV. Bands of both collagens remained unchanged compared to control bands. After UVA irradiation, none of the 5 (poly)phenols used had an antioxidant effect on collagen I except luteolin at the 250 kJ/m² at the lower dose of irradiation. This matches the results of luteolin obtained in dityrosine cross-linkage. For collagen IV, all (poly)phenols, without exception, were unable to
protect the 160 KDa α1 (IV) band from degradation. Band 105 KDa was not affected by UVA or (poly)phenols protected in both collagen I and IV. It is worth noting that other antioxidants such as vitamin E had no effect on collagen protection in HSF when examined against skin aging (Robert et al, 2004).

UVA irradiation of calf skin collagen resulted in extensive chain degradation at 1400 kJ/m² and no intact collagen chains were present. UVA irradiation with 300 kJ/m² caused significant chain degradation and cross linkage (Menter et al, 2001). Both UVA and UVB induce degradative damage to collagen in the upper and mid dermis through induction of interstitial collagenase (MMP – 1) and gelatinases (MMP – 2 and 9) (Menter et al, 2003 and Menter et al, 2001 Scharffetter-Kochanek et al, 1993, Rittie and Fisher, 2002 Yan et al, 2005, Petersen et al, 1992 and Yoshiharu et al, 1996). The (poly)phenols used in this study did not have an effect on the reduction of collagen degradation by MMP or the concentration used was not enough for collagen protection. Cheng et al, (2003) found that EGCG had no effect on reduction of MMP-2 binding to collagen I even at high a concentration (100 μM).

The effect of UVA on collagen degradation cannot be compared to the results of collagen cross-linkage in term of tyrosine cross-linkage due to low content of tyrosine in collagen (2-6 amino acids/ 1000 residues) (Glanville et al, 1979; Gallop and Seifter 1963). Also, it is possible that collagen may play a direct role as an adsorbing chromophore (Menter et al, 2001).

It also noted that the protein concentration determined by the Lowry method reflected the decrease in protein content after UVA irradiation and this
could be as a result of decrease in the cell number which may be reduced by irradiation.

6.5 Conclusion

- The 5 (poly)phenols tested showed a reduction in protein cross-linkage in HSF in term of dityrosine formation only at the high UVA dose (500 kJ/m²) and not 250 kJ/m² except for luteolin which showed reduction in dityrosine cross-linkage at both doses.
- The descending order of these compounds was luteolin (4.6 times reduction in protein cross linkage) > ECG (3.5 times) > luteolin-4'-O-glucoside (2.8 times) > luteolin-7-O-glucoside (2.5 times) > Trolox (2.1 times).
- Increases in the degradation and/or cross-linkage of collagen I and IV molecules after UVA irradiation were apparent in this study.
- Regarding collagens, (poly)phenols showed no protection effect on collagen I and IV α1 chain when HSF was irradiated with both UVA.
CHAPTER 7
7. GENERAL DISCUSSION

The amount of UVA radiation (320-400 nm) penetrating the basal layer of the epidermis is approximately 30 % of the UVA dose (Vile and Tyrrell, 1995). UVA irradiation causes changes in the skin through the formation of reactive oxygen species (ROS) (Masaki et al, 1995). These ROS are responsible for DNA damage (Stray et al, 1997), alteration in the structure and function of mitochondria (Somosy, 2000), lipid oxidation (Saliou et al, 1999), protein cross-linking (Shen et al, 2000) and collagen degeneration (Philips et al, 2003). In addition, UVA causes the rapid release of harmful free iron into the cytosol (Pourzand et al, 1999). Flavonoids can scavenge ROS, thus protecting lipids (Matsushita et al, 2000), proteins and DNA (Kligman, 2000).

In the present study it was assumed that UVA irradiation of human skin fibroblasts (HSF) involves ROS generation. This assumption appears to be valid as UVA irradiation resulted in increased lipid peroxidation, reduced cell viability, increased apoptosis as well as increased protein oxidation (di-tyrosine cross-linkage) and denaturation of collagen I and IV.

Five (poly)phenols were investigated in terms of their photoprotective activity. They were added exogenously to HSF for 18 h and were subsequently exposed to UVA (250, 500 kJ/m²). These (poly)phenols (30 μM) were epicatechin gallate (ECG), trolox, luteolin, luteolin-7-O-glucoside and luteolin-4'-O-glucoside.
7.1 The effect of UVA on HSF

UVA irradiation of HSF reduced cell survival, as determined by MTT, down to 16% and 7% at 250 and 500 kJ/m², respectively. Apoptosis increased by 2.5 and 3 fold at 250 and 500 kJ/m², respectively, when compared to non-irradiated cells.

Also, UVA irradiation increased lipid oxidation products in HSF. UVA doses of 250 and 500 kJ/m² increased the production of lipid hydroperoxides 1.5 and 1.6 fold and increased the MDA-TBARS levels 5 and 6 fold at the two UVA doses respectively.

Irradiation of HSF at 250 and 500 kJ/m² increased protein cross-linkage by 2 and 5.4 fold, respectively, with parallel increases in lipid peroxidation. The results from the Western blots indicated a degradation of collagen I and IV after UVA irradiation. It was apparent in this study that collagen IV was more susceptible to denaturation than collagen I, especially in the highest molecular weight bands (250 and 160 KDa). This may be due to two factors, namely the location and the nature of the collagen structures. The fact that collagen IV is a constituent of the basement membrane with a network-like structure (Mohmoodian and Peterkofsky, 1999), may make it more susceptible to UVA irradiation than the fibril collagen I which is mainly located in the extracellular matrix (Krahn et al, 2006).

7.2 The effect of (poly)phenols on non-irradiated HSF

Many studies have indicated that (poly)phenols reduce the risk or ameliorate a variety of pathological states. These include inflammation (Tapiero et al, 2002), coronary heart disease (Majchrzak et al, 2004), cancer (Kao et al, 2000; Tang et al,
200), chemically induced skin cancer (Conney et al, 1992; Jung and Ellis, 2001) and lipoprotein oxidation (Hodgson et al, 2000).

In general, each of the (poly)phenols incubated with HSF had no cytotoxic effect as measured by the MTT assay. Their behaviour as antioxidants reducing apoptosis, lipid oxidation and protein cross-linking in HSF differed according to the oxidation parameter that was measured. ECG and luteolin-7-O-glucoside reduced both lipid oxidation parameters (PV and MDA-TBARS) in control cells but did not affect protein cross-linking. In contrast, luteolin resulted in an increase in apoptosis and lipid oxidation products but decreased protein cross-linkage in control cells while luteolin-4'-O-glucoside showed no effect in the protection of control cells against apoptosis or lipid and protein oxidation. Trolox had no effect as an antioxidant except in reducing one lipid oxidation parameter, the PV.

7.3 Effect of (poly)phenols on UVA irradiated HSF

7.3.1 Effect of (poly)phenols on apoptosis

Interestingly, only some of the (poly)phenols examined in this study reduced the number of apoptotic cells after UVA irradiation. ECG reduced apoptosis by 68 % and 63 %, at 250 and 500 kJ/m² respectively. ECG was also reported to protect HSF from ROS resulting from irradiation (Lee et al, 2005) and reduced apoptosis in cultured cells up to 50 % (Katunuma et al, 2006). Luteolin decreased apoptosis by a similar amount (62 and 64 %) and is reported to influence the downstream effectors of apoptosis (Bagli et al, 2004). It is noteworthy that the position of the glucose moiety had a large impact on the activity of the luteolin glucosides. Luteolin-7-O-glucoside reduced apoptosis by 80 and 40 % at the two UVA doses, respectively, in
contrast to luteolin-4'-O-glucoside which demonstrated no significant effect on this parameter. Although trolox was reported to suppress apoptosis in the rat brain cells (Usuki et al, 2001), in this study, trolox demonstrated no effect on UVA induced apoptosis.

7.3.2 Effect of (poly)phenols on lipid oxidation parameters

The effectiveness of each of the five (poly)phenols in reducing UVA-induced lipid peroxidation in HSF differed depending on the compound tested. Despite the pro-oxidant behaviour of luteolin in non-irradiated cells, it demonstrated the highest protection in terms of reducing PV and MDA-TBARS after UVA irradiation. Luteolin-7-O-glucoside was weaker than its corresponding aglycone but also decreased PV and MDA-TBARS significantly and was more effective than luteolin-4'-O-glucoside in reducing lipid oxidation. ECG reduced PV and MDA-TBARS at the high UVA dose only. On the other hand, trolox had no protective effect in reducing PV at 500 kJ/m² but reduced PV at 250 kJ/m² upon irradiation.

7.3.3 Effect of (poly)phenols on protein cross-linkage and collagen I and IV

Each of the (poly)phenols tested showed a reduction in protein cross-linkage in HSF in terms of dityrosine formation. This activity was observed at both the high and low UVA doses for luteolin. In contrast, the other four compounds tested, only reduced protein cross-linkage at the high UVA dose.

Consistent with the above-mentioned effect of luteolin in reducing protein cross-linkage, was the protective effect of this compound on the 160 KDa α1 chain of collagen I at 250 kJ/m². The other (poly)phenols tested were ineffective in this respect. For collagen IV, all (poly)phenols, without exception, had no ability to
protect the 160 KDa α1 (IV) band. The presence of the band at 105 KDa was intact in the presence or absence of (poly)phenols in both collagen I and IV.

7.4 Prooxidant activity of some (poly)phenols

Although (poly)phenols and flavonoids are reported to prevent photo-oxidative skin damage (Bonina et al, 1996) they may also exhibit prooxidant properties (Galati et al, 2002). Two of the 5 (poly)phenols, trolox and luteolin, showed prooxidant activities on different occasions. The prooxidant characteristic of trolox and luteolin was also reported by Massaeli, et al, (1999).

In this study, the prooxidant behaviour of trolox could not be attributed to the fact that trolox may act as a photosensitizer producing ROS by itself (Nocentini et al., 2001), since trolox does not absorb in the UVA region. In addition to the fact that trolox is an efficient radical scavenger at the water/lipid interface, it may also need the presence of a synergistic antioxidant such as ascorbic acid to be regenerated (Chen and Tappel, 1995; Iuliano et al 1999), thus producing maximum protection. The low potency found in the present study may therefore be due to the absence of ascorbic acid which has been reported to be an important cofactor for increasing trolox antioxidant activity (De Mulder et al, 1995; Iuliano et al, 1999).

The concentration of trolox may also be an important feature of its antioxidant/prooxidant effect. Trolox increased the MDA-TBARS production at both irradiation doses. In other studies, trolox reduced TBARS formation in a dose dependant manner (0-60 μM) (Santos et al, 2001). Furthermore, Callaway et al, (1998) reported that trolox completely inhibited TBARS formation at a very high concentration (1000 μM).
Luteolin showed a different pattern; it showed a prooxidant effect in the absence of UVA irradiation and increased apoptosis and lipid oxidation when added to HSF. This is in agreement with Yamashita, et al., (1999) who reported that luteolin has a dual effect of increasing oxidation but also demonstrating antioxidant effects.

This prooxidative effect may be explained by the fact that reducing agents, such as (poly)phenols, can increase HO• generation by metal ion and H₂O₂ interaction thereby increasing lipid oxidation and MDA production (Nocentini et al, 2001). However, the aroxyl radical of a (poly)phenol can also interact with oxygen, generating quinones and O₂• which may be responsible for the undesired prooxidant effects of flavonoids (Tiwari, 2001). Chelation of Fe may prevent its pro-oxidant action, but this feature may decrease its redox potential making the polyphenol more active in the conversion of O₂• to H₂O₂ (Ozgova et al, 2003). For example, the redox potential of Fe/bis-ECG (+0.325) is lower than that of Fe³⁺/Fe²⁺ (+0.77).

7.5 Metal ion chelation

The present results suggest that the polyphenols used are able to prevent apoptosis and oxidative damage to lipids or proteins induced in HSF as a result of UVA irradiation by scavenging ROS or metal chelation. In fact, it is difficult to distinguish between the two mechanisms of the inhibiting action of (poly)phenols, scavenging active free radicals and chelating transition metals (Rogensky, 2003). It is probable that both activities are involved in their protective activities.

All the tested phenolic compounds showed large interactions with iron and copper except for trolox and ECG with iron. Luteolin and its glucosylated forms
showed absorbance in the UVA region (320-400 nm) compared to 278 and 288 for ECG and trolox, respectively, which may also be an important attribute.

ECG and each of the flavones meet most of the structural criteria for effective chelators. They contain the 3', 4'-dihydroxy group (except for luteolin-4'-O-glucoside), the 4-oxo group in the C ring (except for ECG) and the 3, 5-hydroxy groups on the B and C rings, respectively (Sugihara et al, 1999). In addition to the hydroxyl groups in the flavone structure, ECG contains a gallic acid moiety on the 3 position.

The weak interaction of trolox with metal ions may be explained by its structure. Trolox lacks the catechol group present in some of the other phenols tested, which is responsible for metal chelation. It also lacks the 4-oxo and 5-OH group arrangement; thus the only potential site for metal chelation is the sole OH group.

Often strong free radical scavenging activity is accompanied by moderate metal chelation capacity (Wu et al, 2006); in this study luteolin showed strong free radical scavenging activity (80 %) and moderate Fe²⁺-chelating activity (60 %) at 0.5 mg/ml. Also, the substitution in the B-ring reduced the activity of luteolin glucosides compared to luteolin.

Flavonoids acting as natural metal chelators may have advantages over synthetic chelators. Synthetic iron chelators are cytotoxic to dividing cells at concentrations of 1-10 μM depending on the cell type involved and the duration of exposure (Kushner et al, 2001). Thus, considering human skin and its constant exposure to UV light and oxygen, combined with the increase LIP content of the
exposed skin, topical application in cosmetic or addition of (poly)phenols to pharmaceutical formulations could be protective for the skin.

7.6 Structural Properties of (poly)phenols

Although the relationship between chemical structure and suppressing effect of flavonoids is not clear at present (Lu et al., 2002), the structural properties of the five (poly)phenols used in this research clearly affected their antioxidant activity. The number and arrangement of the hydroxyl groups on the A and B rings, as well as glucosylation of these groups and the 4-keto group present on the C ring are important features for their antioxidant activity (Rice-Evans et al, 1996; Mustada et al, 2002; Meeteren et al 2004).

ECG, which has a total of 7 hydroxyl groups (4-OH groups on A and B rings plus 3-OH groups on gallic acid), was more effective than trolox in reducing lipid oxidation. On the other hand, trolox, which lacks all the structure features of a flavonoid, showed the least protection. The low antioxidant activity of ECG compared to each of the flavones may due to the fact that ECG lacks the 2,3 double bond and the 4 keto group in the C ring. The lack of the 2,3 double bond renders the molecule less able to lose a hydrogen atom as easily. Moreover, the 7-OH hydroxyl groups, including those on gallic acid, make ECG more hydrophilic. This reduces its ability to interact in lipophilic environments in which the luteolin-based compounds may be more active.

In comparing the structure of luteolin to its glucosylated forms, it is noted that luteolin with 4 hydroxyl groups (including a 3', 4' dihydroxy group arrangement in the B ring), a 2, 3 double bond and a 4 keto group in the C ring showed the
strongest antioxidant activity although a prooxidant effect was observed when no irradiation was applied. It has been observed by others that the B ring dihydroxy structure is essential in the prevention of free radical induced cell damage (Miura *et al.*, 2003; Meeteren *et al.*, 2004). Furthermore, the presence of the glucose moiety in the luteolin structure reduces this activity. The positioning of this moiety within this polyphenol was also found to be important. This was clear when comparing the effects of luteolin-7-O-glucoside and luteolin-4'-O-glucoside. Luteolin-4'-O-glucoside reduced apoptosis by 25 % and 10 % which was less active than luteolin-7-O-glucoside, which reduced apoptosis by 80 and 40 %. This highlights the importance of the 4'-hydroxyl group.

In addition to differences in structure and hydroxy group arrangement, the partition coefficients of the phenolic compounds investigated is also relevant. Hydrogen bonding is also an important parameter that determines the antioxidant activity of a compound (Schwarz *et al.*, 2000). The lipid bilayer is the first barrier that external agents encounter when coming into contact with cells. Hence, to have a protective effect, an antioxidant has to reside in close proximity to the membrane interior where lipid peroxidation chain propagation occurs.

Partition coefficients and the ease of diffusion into membranes may be important when considering the efficacy of luteolin as an antioxidant compared with its glucosylated forms. The partition coefficient of glucosylated (poly)phenols is less than the partition coefficient of the parent aglycon. The greater effectiveness of luteolin compared with luteolin-7-O-glucoside in inhibiting lipid oxidation may be explained by the fact that luteolin is more hydrophobic, with a partition coefficient of 22.2. The glucosylation at the 7-position would render the flavonoid more soluble in
the aqueous environment (Brown and Rice-Evans, 1998) and less able to interact with propagating peroxyl radicals.

Damiani et al, (2002) highlighted that the UVA protective effect of a compound is related to the capability of a compound to absorb in the UVA region. The solubility of a (poly)phenol may also contribute greatly to its antioxidant activity. Free radicals and ROS can be generated in both lipid and aqueous phases (Chen and Tappel, 1995). Thus combining both lipids and water soluble antioxidants is generally more effective in protecting against lipid or protein oxidation. Water soluble metal chelators such as EDTA may be unable to access iron bound within the lipid layers, thus this antioxidant is often less active in UVA irradiated HSF (Vile and Tyrrell, 1995).

Trolox has moderate water solubility and can be incorporated in both water and lipid compartments of cells (Poljsak et al, 2006). Thus the increase of UVA-induced DNA damage suggests that in normal HSF, trolox, upon irradiation, facilitates the formation of ROS which are more reactive, or in greater quantity than those normally produced by UVA alone.
7.7 Conclusions

- UVA irradiation of HSF resulted in an increase in lipid oxidation and protein cross-linking as well as cell apoptosis.
- All (poly)phenols used showed antioxidant effects in reducing some parameters but not others.
- When the (poly)phenols were added to irradiated cells, they showed a reduction in apoptotic cell number, except for trolox which had no significant effect on cell viability or apoptosis.
- ECG showed the same effect towards controls and UVA irradiated cells in reducing PV and MDA-TBARS giving a significant protection at the high dose only.
- Trolox reduced the two lipid peroxidation parameters, PV and MDA-TBARS when added to control cells without UVA irradiation. Upon irradiation trolox had no effect or a minimal protective effect in terms of PV at 500 kJ/m$^2$ and even increased MDA-TBARS production at the two irradiation doses.
- Although luteolin showed substantial prooxidant activity when incubated with control cells, it demonstrated the highest protection in terms of reducing PV and MDA-TBARS when added to irradiated cells.
- Luteolin-4'-O-glucoside decreased PV and MDA-TBARS significantly upon irradiation. In contrast, luteolin-7-O-glucoside showed the best protection effects, decreasing both peroxidation parameters in control and irradiated HSF due to its structural features.
- (Poly)phenols tested showed reduction in protein cross linkage in HSF in term of di-tyrosine formation at the high UVA dose except luteolin which showed reduction in protein cross-linkage at both doses.
• The 5 (poly)phenols selected showed no protection for collagen I and IV in irradiated HSF.

• All the tested phenolic compounds showed large interactions with iron and copper except trolox and ECG with iron. These metal chelation properties increased protection against lipid peroxidation and UVA apoptosis in HSF.

• The concentration of a potent antioxidant such as luteolin, which showed prooxidant activity without UVA irradiation, may be reduced. The prooxidant effect of luteolin may be of great potential in reducing cancer by induction of apoptosis in cancer cells.

• The weak effect of trolox as an antioxidant may be increased by increasing its concentration or introducing a synergetic antioxidant such ascorbic acid.

• The green tea (poly)phenol, ECG, was less effective in its antioxidant activity compared to luteolin and its glucosides which may be due to the structural and chelation properties of this compound.

• Luteolin and its glucosylated forms were more potent antioxidants in reducing UVA-induced lipid oxidation products compared with ECG and Trolox. They also may be able to penetrate deeper into skin layers. Thus, they may be useful candidates as successful protective agents in UVA irradiated HSF. On the other hand, the glucosylation of flavonoids reduces their activity when compared to the corresponding aglycon this was confirmed in that luteolin was stronger than its glucosylated forms.
7.8 Future work

- To study the metabolism of selected (poly)phenols after 18 h incubation to investigate the actual concentration of compounds taken up by HSF and the specific form in which the (poly)phenols exert their action on the skin cells.

- To undertake Raman microscopy studies of HSF to monitor changes in the lipids and proteins of the cell membrane as well as changes in the nucleus with and without UVA irradiation and to study the effect of those (poly)phenols in preventing such changes.

- To measure the amount of free iron present in HSF before irradiation and the amount released by UVA irradiation; and to evaluate the effect of the 5 selected (poly)phenols on reducing this free iron pool.

- To measure the hydroxyproline content of HSF before and after UVA irradiation in order to monitor the degradation of cellular collagens and to study the antioxidant activity of these (poly)phenols in reducing collagen degradation.


Ahmad, S, Control of skin infections by a combined action of ultraviolet A (from sun or UVA lamp) and hydrogen peroxide (HUVA therapy), with special emphasis on leprosy. Medical Hypotheses, 57(4), 484-486, 2001.


De Mulder, C, Madabushi, H, and Tappel, A, Protection by vitamin E, selenium, trolox, ascorbic acid palmitate, acetylcysteine, coenzyme Q, β-carotene, and (+)-catechin against oxidative damage to rat liver and heart tissue slices measured by oxidized heme proteins. Nutritional Biochemistry, 6, 452-458, 1995.


Diffey, B, School in Photodermatology Section Editor: Rik Roelandts What is Light?. Photodermatology, Photoimmunology & Photomedicine, 18(2), 68-74, 2002.


Dominici, S, Pieri, L, Comporti, M, and Pomella, A, Possible role of membrane gamma-glutamyltransferase activity in the facilitation of transferrin-dependent


Gurr, J, Wang, A, Chen, C, and Jan, Kun, Ultrafine titanium dioxide particles in the absence of photoactivation can induce oxidative damage to human bronchial epithelial cells. Toxicology, 213,66-73, 2005.


Halliwell, B, Why and how should we measure oxidative DNA damage in nutritional studies? How far have we come?. American Journal of Clinical Nutrition, 72(5), 1082-1087, 2000.


Iwahashi, H, Akata, K, Sunaga, A, Tone, Y, Yamada, N, and Iijima, K, The Inhibitory Effect of Luteolin-7-O-glucoside on the Formation of Pentyl and 7-


Nakagawa, T, and Yokozawa, T, Direct scavenging of nitric oxide and superoxide by green tea. *Food and Chemical Toxicology*, 1745-1750, 2002.


Usuki, F, Yasutake, A, Umehara, F, Tokunaga, H, Matsumoto, M, Eto, K, Ishiura, S, and Higuchi, I, *In vivo* protection of a water-soluble derivative of vitamin E, Trolox,


