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**INVESTIGATION OF THE ANTIOXIDANT ACTIVITY  
OF PHENOLIC COMPOUNDS IN CELL CULTURE  
SYSTEMS**

**By**

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in the Department of Chemical Engineering  
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## DECLARATION

I, **Eunice Nonhlanhla Zwane**, hereby declare that the work on which this dissertation is based is my original work (except where acknowledgments indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university. I empower the university to reproduce for the purpose of research either the whole or any portion of the contents in any matter whatsoever.

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## Abstract

This study focuses on newly synthesized polyphenolic compounds which were expected to be antioxidants *in vivo*. Antioxidants are compounds which at low concentrations can prevent or reduce the deleterious effects caused by reactive oxygen species (ROS) induced by UVA exposure of the skin. This study focuses on developing methods required to measure the effects of these synthesized compounds in ultraviolet radiation-exposed cultured skin cells. Biocatalytic synthesis of polyphenolic compounds was carried out using isolated enzymes, tyrosinase from *Agaricus biosporus*, and laccase from *Trametes pubescens* to produce oxidized oligomeric products. Oxidation of tyrosol by tyrosinase yielded hydroxytyrosol (compound **33**). Oxidation of hydroxytyrosol and ferulic acid (compound **29**) using laccase as a biocatalyst yielded polymers, products **35** and **30** with a mass-to-charge of 451 and 589 respectively. Results from the 2, 2-Diphenyl-picrylhydrazyl antioxidant assay (DPPH) showed that products **30** and **35** had better antioxidant activity compared to substrates **29** (ferulic acid) and **33** *in vitro*. In the ferric reducing antioxidant power assay (FRAP), products **30** and **35** showed the highest antioxidant activity, with antioxidant activity equivalent to 213 mg/L and 363 mg/L of ascorbic acid compared to substrates **29** and **33**, with antioxidant activity equivalent to 126 mg/L and 124 mg/L respectively. Results from the low density lipoprotein assay (LDL) antioxidant assay showed that compounds **29**, **33** and product **35** showed higher inhibition of LDL oxidation as compared with the standard antioxidant, ascorbic acid. Product **30** showed the lowest antioxidant activity. Flow cytometry measurements showed that H<sub>2</sub>O<sub>2</sub> production in the cells was inhibited by compound **33** and products **30** and **35** in a concentration- manner. The decrease in H<sub>2</sub>O<sub>2</sub> production was observed in the following order product **30**> product **35**> compound **33**. Compound **33** and products **30** and **35** affected gene expression by resulting in the over-expression of the anti-apoptotic Bcl-2 protein, and the decreased expression of the pro-apoptotic Bax and AIF proteins in a concentration-dependent manner. This study provides the first evidence that compound **33** and products **30** and **35** are potent protective agents for keratinocytes after UVA exposure. This has implications that compound **33** and products **30** and **35** can be used in skin care products, in health and nutraceuticals.

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The difference between a successful person and others is not a lack of strength, not a lack of knowledge, but rather a lack in will.

**Vince Lombardi**

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# Chapter 1

## Introduction

Reactive oxygen species (ROS) and their proven involvement in some human physiopathologies have drawn significant interest from the health sector over the last few decades. Oxidative stress has been associated with many multifactorial diseases, chiefly cancers, cardiovascular diseases and inflammatory disorders. The development of these pathologies is attributed to the oxidative alteration, by ROS of physiologically important molecules. ROS affects molecules which include proteins, lipids, carbohydrates and nucleic acids, and cause modulation of gene expression and the inflammatory response.

The human body has developed its own defence system to deal with this oxidative stress involving enzymatic systems, such as superoxide dismutase, catalases, glutathion peroxidase and thioredoxin, which are thought to be highly efficient in ROS detoxification. The body also has non-enzymatic antioxidants including glutathion, bilirubin, estrogenic sex hormones, uric acid, coenzyme Q, melanin, melatonin, and lipoic acid. Certain external factors, such as over-exposure of skin to ultraviolet light radiation (UVR), may result in elevated cellular ROS concentrations, overwhelming the body's antioxidant system and leading to disease. To neutralize this effect, exogenic antioxidants, particularly polyphenols, which are mainly found in food sources, may be helpful for counteracting ROS. Antioxidants are compounds which can prevent the oxidation caused by ROS. They function at tissue level through a scavenging action, using hydrogen transfer or electron transfer mechanisms, to render the ROS inactive.

Previously, polyphenolic compounds have been successfully synthesized in our laboratory through biocatalytic reactions using phenol oxidases, to produce dimeric and oligomeric phenols which were suggested to have higher antioxidant activity than the starting compounds (Ncanana, 2007; Murefu, 2007). These compounds can be considered to be natural compounds as they have been synthesized using biological agents, and this is significant for the success of such products in the world market, since consumers prefer natural products to products synthesized through chemical processes.

### 1.1 Structure of thesis

This thesis reports the synthesis of putative polyphenolic antioxidants from their parent compounds using oxidase enzymes in biocatalytic reactions. The structures of the putative

antioxidants were elucidated using mass spectroscopy and nuclear magnetic resonance spectroscopy. *In vitro* antioxidant assays were conducted to investigate the type of antioxidant activity of the putative antioxidants. Given that skin is a major target of ROS damage due to daily exposure to the atmosphere and UVR, cultured skin cells were used to test the antioxidant activity of the putative antioxidants *ex vivo*. This investigation would give an indication of the type of antioxidant action that the putative antioxidants might have *in vivo*, and provide evidence for the use of these putative antioxidants in health, nutraceutical or cosmetic industry.

This thesis comprises of five chapters. Chapter 1 (Introduction) describes the project background of this study. Chapter 2 (Literature Review) summarizes important information regarding oxidase enzymes and their use as biocatalysts, the significance of polyphenols as effective antioxidants, and methods used to test the efficacy of putative antioxidants *in vivo* and *ex vivo*. Chapter 3 (Materials and Methods) describes the extraction of the oxidase enzymes used, their application as biocatalyst in producing polyphenolic putative antioxidants, and *in vitro* and *ex vivo* methods of testing the efficacy of these putative antioxidants. Chapter 4 (Results and Discussion) presents the results of the investigations performed in the laboratory. The experimental findings are discussed and compared with results found in literature. Chapter 5 (Conclusion) outlines the significance of the study and its findings and the objectives achieved.

# Chapter 2

## Literature Review

This thesis reports an investigation into the synthesis of organic compounds derived from selected phenolic compounds, and their antioxidant activity against ROS (reactive oxygen species) damage of cells. Thus, this literature review comprehensively covers the characteristics of ROS, phenolic and polyphenolic antioxidants, and describes the use of tissue culture as a means to determine activity antioxidants *in vitro*.

### 2.1 Reactive oxygen species (ROS)

Reactive oxygen species have been known to chemists since the beginning of the 20<sup>th</sup> century. In the 1950's, the pioneering work of Gilbert and Gersham (1981) suggested that radicals were important participants in biological environments and were responsible for deleterious effects in the mammalian cell. Following this, Harman (1981) showed that the ROS did have deleterious effects on cell metabolism, and further suggested that they played an essential role in the aging process. This hypothesis, namely the free-radical causes of aging, inspired many studies and research efforts, and contributed significantly to the currently available information on ROS (Allen and Tresini, 2000; Beckman and Ames, 1998; Davies, 1999; Droge, 2000; Halliwell, 1990).

ROS have an unpaired electron in their outer orbital which may significantly increases the reactive properties of the ROS (Halliwell and Gutteridge, 1999). ROS tend towards achievement of stable states by reacting with suitable substrates, and will therefore abstract electrons from co-reactants such as antioxidants thus rendering them inactive and unable to cause physiological damage (Laguette *et al.*, 2007).

#### 2.1.1 Sources of ROS

##### 2.1.1.1 Endogenous sources of ROS

ROS are continually produced in mammalian and plant cells and are required for certain metabolic functions (Vallyathan and Shi, 1997). They are produced through various processes within mammalian cells which involve microsomes, enzymes, phagocytes and metal ions, but the most prolific formation of ROS occurs in the mitochondria (Vallyathan and Shi, 1997). The formation of ROS is important in that they function in microbial killing, blood pressure control,

endothelial function, cell signalling, apoptosis, cell division, and gene transcription (Clement and Pervaiz, 2001; Dalton *et al.*, 1999; Halliwell and Gutteridge, 1999; Suzuki *et al.*, 1997)

Mitochondria are a major source of ROS, generating them in normal metabolic processes, including the electron leakage of ROS during their passage along the respiratory chain (Turrens, 2003; Laguerre *et al.*, 2007). Other enzymatic processes in the mitochondria also result in the formation of ROS (Vallyathan and Shi, 1997).

### **2.1.1.2 Exogenous sources of ROS**

ROS from exogenous sources can result in damage in biological systems such as the oxidation of protein, lipids and DNA. The skin serves as an external barrier for the human body but it is constantly at risk of exposure to these exogenous sources. Some of these sources are asbestos (Craighead *et al.*, 1982), crystalline silica (Craighead *et al.*, 1988), coal (Green, 1988), diesel (Cheng *et al.*, 1984), chromium (De Flora, 1990), ozone (Frank, 1988), and ultraviolet light radiation (Cleary, 1987; Laguerre *et al.*, 2007), the latter being particularly significant for this study.

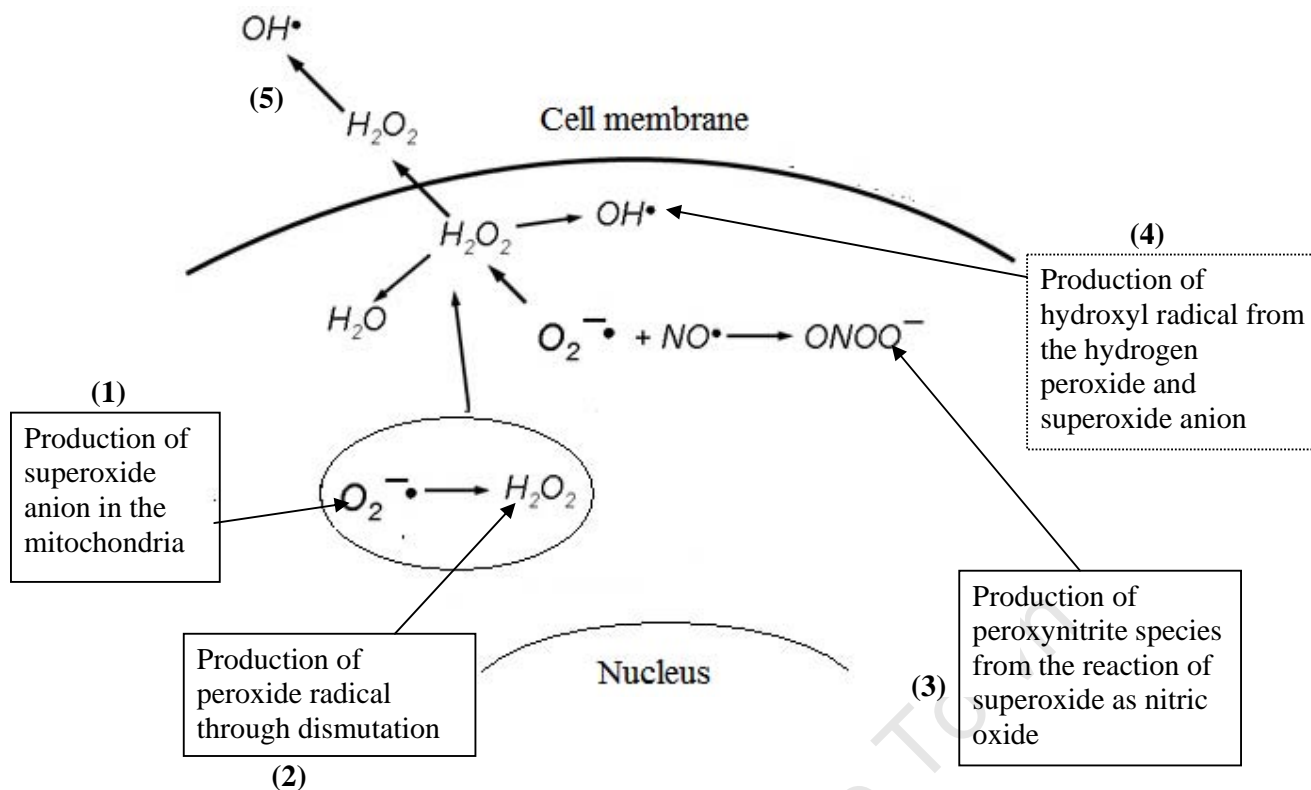
UVR consists of ultraviolet A (UVA) (320-400 nm), ultraviolet B (UVB) (280-320 nm) and ultraviolet C (UVC) (200-280 nm). Exposure to both UVA and UVB causes wavelength-dependent damage in human skin while UVC is absorbed by the atmospheric ozone layer (Peak and Peak, 1993; de Gruijl, 2000). The skin acts as a barrier against the harmful effects of UVR but it is susceptible to damage due to UVR over-exposure. Of most interest for this study is UVA which is solar radiation that reaches the earth and is considered to be the aging ray of the sun. UVA can penetrate deep into the epidermis and dermis of the skin (Black *et al.*, 1997). Once absorbed, the radiation induces direct molecular disruption of chromophores resulting in the producing free radicals and ions (Vallyathan and Shi, 1997). Intense exposure of skin to UVA can result in a burning effect on sensitive skin, and prolonged exposure can damage underlying structures and eventually result in premature photoaging of the skin (Svobodova *et al.*, 2003; Urbach, 1997; Carona *et al.*, 2001; Laguerre *et al.*, 2007).

Cells contain photosensitive molecules called chromophores which, upon receiving photons of UV radiation, raise electrons to higher energy states. The chromophores may then pass their excitation energy to other molecules and thus cause a chain reaction (Batista *et al.*, 2009; Lotenen and Laiho, 2005). Bash and Haseltine (1982) and Freeman (1988), concurred that skin exposure to ultraviolet light causes an increase in the melanin pigmentation of skin; this melanin can absorb most of the radiation, acting as a chromophore. Hochberg *et al.*, (2006) also reported

that since pyrimidines in DNA have an absorbance spectrum that overlaps with the UVB (280-320) range, the pyrimidine bases themselves can serve as chromophores which can lead to extensive DNA damage.

### **2.1.2 The production of ROS *in vivo***

ROS are formed in the body by various physiological processes, as well as arising due to external influences such as UVR. The superoxide anion is a key oxidant within mammalian mitochondria as shown in Figure 2.1 (1). A superoxide radical is the starter molecule for the production of other ROS, including hydrogen peroxide and the hydroxyl radical (Halliwell and Gutteridge, 1999; Benzie, 2000; McCord, 2000; Laguerre *et al.*, 2007). In this system, superoxide radicals are unable to migrate far from their site of formation owing to lipophilic membrane barriers. However, the endogenous enzyme superoxide dismutase (SOD) converts the superoxide into hydrogen peroxide [Figure 2.1 (2)], which readily diffuses in and out of the cells [Figure 2.1 (5)]. Hydrogen peroxide is not highly reactive but may accumulate at high levels, resulting in a reaction with reduced metal ions, hydroxyl radicals [Figure 2.1 (4)]. The hydroxyl radicals produced from this reaction are highly reactive and will interact with any substrate they encounter. Superoxide anions can also react with nitric oxide to form the highly reactive peroxynitrite species (ONOO<sup>-</sup>) [Figure 1 (3)] which can lead to DNA, protein, and lipid damage.

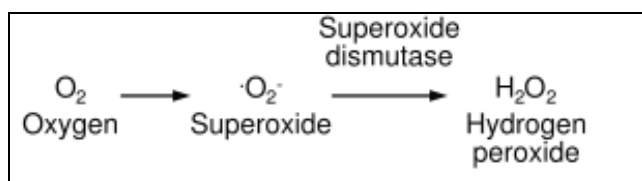


**Figure 2.1.** Formation of intracellular ROS and nitrogen species in mammalian mitochondria (Kelly and Havrilla, 1998).

## 2.1.3 Chemical properties of some key ROS

### 2.1.3.1 Properties of superoxide ion radicals

Superoxide ion radicals have different properties depending on their chemical environment. They exist in the form of  $O_2^{\cdot-}$  at neutral pH, but at low pH they exist as hydroperoxyl ( $HO_2$ ), and the latter form can penetrate biological materials. In a hydrophilic environment both forms can act as reducing agents capable of reducing metal ions such as ferric and ferrous ions (Kohen and Nyska, 2002). The most significant reaction is that of dismutation (Figure 2.2), in which a reaction between superoxide anions and oxygen produces hydrogen peroxide. The function of this reaction is to detoxify the reactive oxygen species (Bielski *et al.*, 1985; Laguerre *et al.*, 2007) thus keeping ROS at levels that are not harmful to the cells.



**Figure 2.2.** Enzymatic pathway of detoxification of reactive oxygen species (Zelko *et al.*, 2002).

### **2.1.3.2 Properties of hydrogen peroxide ( $H_2O_2$ )**

Hydrogen peroxide is a product of dismutation of superoxide radicals. It is able to dissolve freely in aqueous solutions and in biological membranes. Although hydrogen peroxide is considered a reactive oxygen metabolite, it is not a radical by definition and can cause relatively little damage to biological compounds. However, it can oxidize biological substrates when in high concentrations and it is a source of highly reactive hydroxyl radicals (Halliwell and Gutteridge, 1999; Halliwell *et al.*, 2000; Laguerre *et al.*, 2007).

### **2.1.3.3 Properties of hydroxyl radicals**

The reactivity of hydroxyl radicals is extremely high (Bielski and Cabelli, 1995). They are short lived species with a very high affinity for other molecules. Their strong oxidizing properties can be very damaging to DNA, protein, lipids, amino acids, sugars and metals. Reactions involving hydrogen radicals include hydrogen abstraction, addition, and electron transfer (Bielski and Cabelli, 1995).

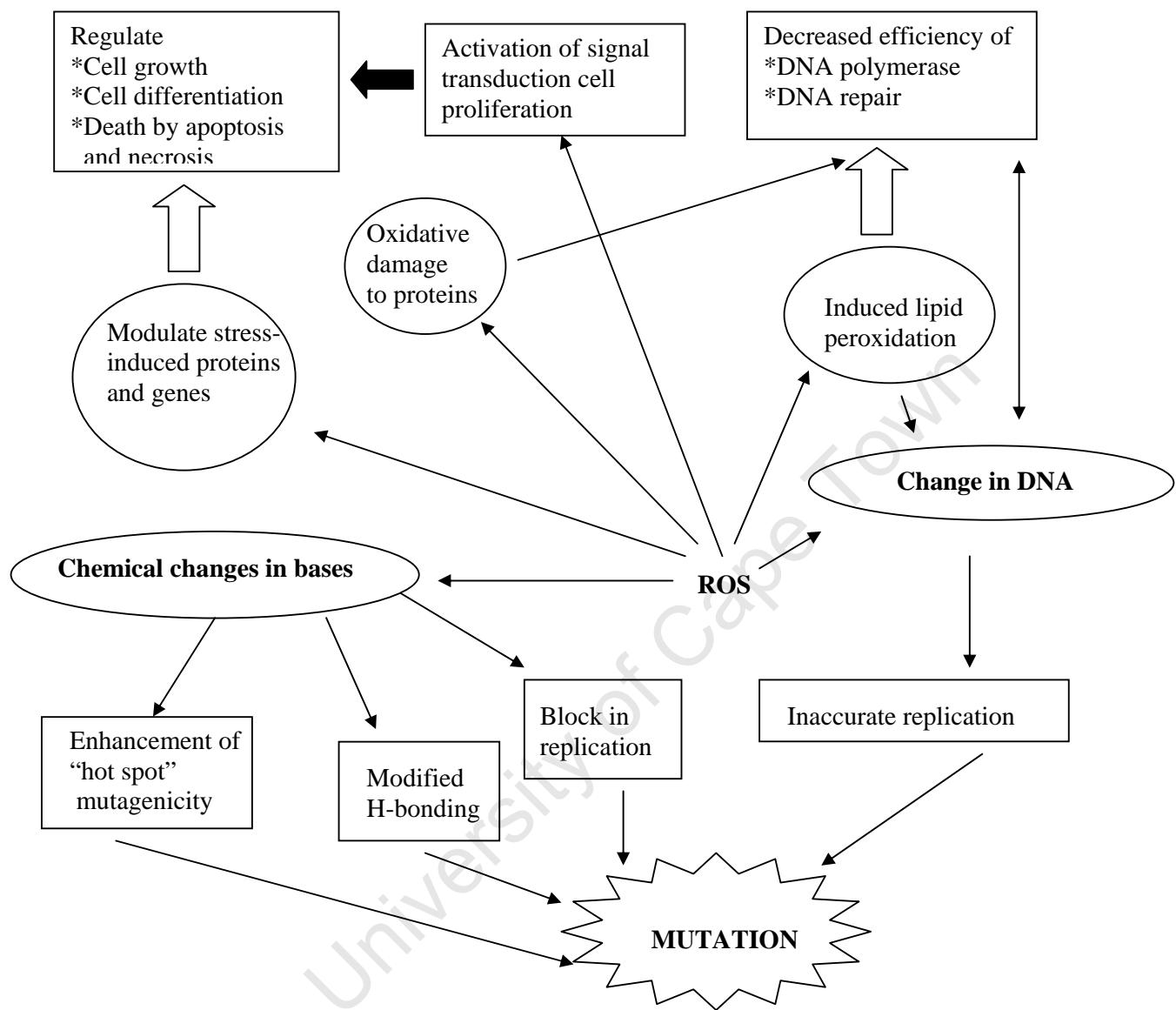
### **2.1.3.4 Properties of the nitric oxide radical**

Nitric oxide ( $NO\cdot$ ) is produced when there is an oxidation of one of the terminal guanido nitrogen atoms of L-arginine. This reaction requires a group of enzymes called nitric oxide synthase complex (NOS), where L-arginine is converted to nitric oxide and L-citrulline. The nitric oxide can further react with a variety of other radicals and substances. One of the most significant reactions is its reaction with superoxide under physiological conditions to yield peroxynitrite. This reaction is beneficial as it helps to reduce superoxide radicals and other ROS and is also important in redox regulation. Peroxynitrite in its protonated form is a powerful oxidizing agent which may cause oxidative damage of DNA and protein (Kohen and Nyska, 2002).

## **2.1.4 Damage of biological substrates by the presence of ROS**

A number of authors have investigated and reported the detrimental effects due to ROS in biological systems. The review by Comparti (1989) reported that the constant efflux of ROS from both endogenous and exogenous sources results in the accumulation of oxidative damage to cellular components and alters cellular function. Other authors have reported the oxidative damage due to ROS in proteins (Levine and Stadtman, 2001; Laguerre *et al.*, 2007), DNA

(Laguerre *et al.*, 2007) and lipids (Beckman and Ames, 1997; Laguerre *et al.*, 2007) (Figure 2.3). The damage caused to these groups of biomolecules is described in more detail in the following sections.

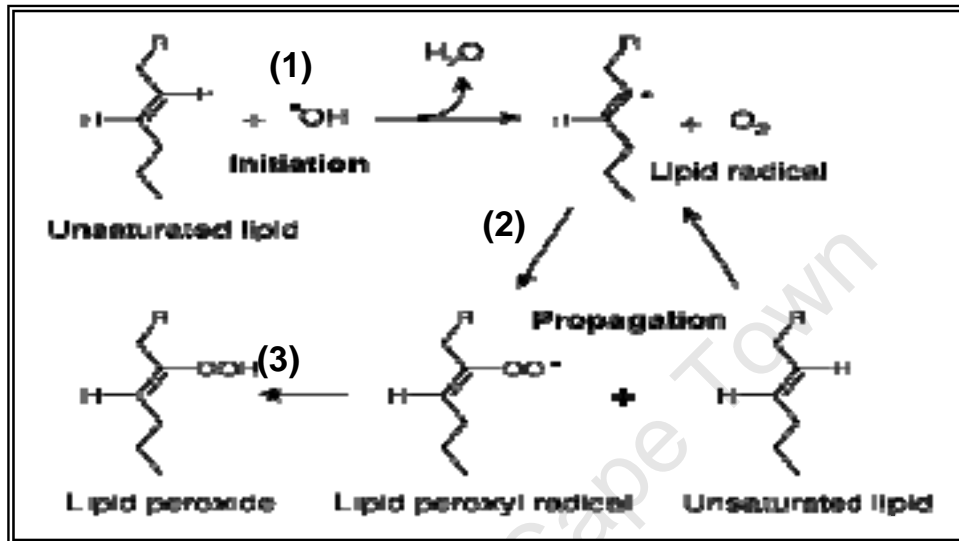


**Figure 2.3.** Different effects and roles of ROS in biological systems (Meral *et al.*, 2000).

#### 2.1.4.1 Oxidative damage to lipids caused by ROS

High concentrations of unsaturated fatty acids make all cellular membranes very vulnerable to oxidation by ROS (Halliwell and Gutteridge, 1999). It has been shown that the damage to lipids occurs in three stages (Laguerre *et al.*, 2007; Meral *et al.*, 2000). The initial stage [Figure 2.4 (1)], called initiation, involves ROS attack by abstraction of a methyl group in the lipid. The resulting fatty acid radical [Figure 2.4 (2)], retains one electron and is stabilized by the rearrangement of the molecular structure to form a conjugated diene. When oxygen is present

in sufficient concentrations, the fatty acid radical can react with it to form a peroxy radical (ROO<sup>•</sup>) capable of abstracting another hydrogen atom from a neighbouring fatty acid molecule [Figure 2.4(3)]. The fatty acid radical formed can also undergo the same process as the initial fatty acid resulting in a chain reaction that can lead to the peroxidation of all lipids within the entire membrane. An antioxidant that can bring this reaction to a halt is defined as a chain-breaking antioxidant (Meral *et al.*, 2000).



**Figure 2.4.** The free radical mechanism of lipid peroxidation (Halliwell, 1995).

#### 2.1.4.2 Oxidative damage to proteins caused by ROS

The ROS which result in oxidative damage to proteins are OH<sup>•</sup>, ROO<sup>•</sup>, nitrogen-reactive radicals and H<sub>2</sub>O<sub>2</sub> (Droge, 2002). Hydrogen peroxide itself, at physiological concentrations is considered to exert only weak effects on proteins (Grune *et al.*, 1997). The different types of damage to proteins resulting from the availability of ROS include peroxidation, damage to specific amino acids residues, changes in their protein tertiary structure, degradation, and fragmentation. Such damage leads to loss of enzyme activity, altered cellular function such as energy production, interference with the creation of cellular membrane potentials, and changes in the levels of some cellular proteins (Laguerre *et al.*, 2007). The products formed as a result of oxidative stress are usually aldehyde, keto compounds and carbonyls which can be used as biomarkers of oxidation (Levine and Stadtman, 2001; Stadtman, 1986).

#### **2.1.4.3 Oxidative damage to DNA caused by ROS**

Under normal circumstances, DNA is a very stable, well protected molecule. Even so, ROS can interact with it to cause serious oxidative damage. The most significant reactive species with regard to damaging DNA is the hydroxyl radical, and the damage that can result from this interaction includes the modification of DNA bases, single and double-DNA breaks, loss of purines and damage to the DNA repair system (Ames, 1999; Beckman and Ames, 1997; Dizdaroglu *et al.*, 2002; Halliwell and Gutteridge, 1999; Hellbock *et al.*, 1999; Halliwell, 2000; Laguerre *et al.*, 2007). The consequences of these reactions can lead to mutations, some of which can ultimately result in cancer.

The interaction of the hydroxyl radical with DNA is usually identified by certain biomarkers which are produced during the reaction. An example is the formation of 8-hydroxydeoxyguanosine (8-OHdG) after an attack by the hydroxyl radical at the C-8 position of guanine (Hellbock *et al.*, 1999). The hydroxyl radical can also attack other bases, such as adenine, to yield 8-hydroxyadenine. Other less significant ROS, such as  $O^{\cdot-}$  and  $H_2O_2$ , lead to less serious damage (Halliwell and Gutteridge, 1999; Laguerre *et al.*, 2007).

#### **2.1.5 Antioxidant protection against ROS damage**

Initially, the term antioxidant was used to refer specifically to a chemical that prevented the consumption of oxygen. Currently the term is used to refer to any substance which, in small quantities, is able to prevent oxidation of easily oxidisable substrates, such as lipids, protein and DNA (Laguerre *et al.*, 2007).

Oxidation can be described as a redox chemical reaction involving the transfer of electrons from a particular substrate to an oxidizing agent. The process of oxidation can have detrimental effects, resulting in the formation of free radicals which can disrupt biological systems. Antioxidants can terminate these reactions by the removal of radical intermediates or by being oxidized themselves. Thus, antioxidants are often reducing agents, as is the case for phenols (Laguerre *et al.*, 2007).

The continuous exposure of cells to various types of oxidative stress from a number of sources has led to the evolution of various mechanisms of protection against ROS, such as the DNA repair system. Amongst these various defence mechanisms, antioxidant mechanisms are the most significant, because antioxidants can directly remove oxidative compounds such as free

radicals, thereby ensuring maximum protection for biological sites (Kohen and Nyska, 2002; Laguerre *et al.*, 2007).

Antioxidants can be classified into two broad groups, depending mainly on their solubility in water. In general, water-soluble antioxidants react with oxidants in the cell cytoplasm and the blood plasma, while lipid-soluble antioxidants protect cell membranes from lipid peroxidation (Sies, 1997). These compounds are synthesized by the body or can be obtained from dietary sources (Gonzalez *et al.*, 2008).

Various antioxidants can co-exist in cells, and interactions can take place between them. The interaction between the antioxidants is a complex process, with the various metabolites and enzyme systems having synergistic and interdependent effects on one another (Chaudiere and Ferrari-Iliou, 2001; Sies, 1993). Further, the amount of protection provided by any one antioxidant depends on concentration and its reactivity towards a particular ROS (Vertuani *et al.*, 2004).

#### **2.1.5.1 Endogenous antioxidants and their role in protecting cells against ROS damage**

Endogenous antioxidants are made up of low mass-to-charge antioxidants (LMWA) which include several compounds capable of preventing oxidative damage through direct and indirect interaction with ROS (Kohen, 1999; Kohen and Gati, 2000). The indirect mechanism involves the chelation of transition metals, preventing the metals from participating in the metal-mediated Haber-Weiss reaction (Haber and Weiss, 1934; Sen, 1998). The direct mechanism of antioxidant action involves donation of electrons to oxygen radicals so that the radicals are stabilized and cannot damage biological sites. This group of antioxidants provides many advantages for cells over enzymatic antioxidants. Their small size enables them to penetrate the cellular membranes and be localized at the sites of biological targets. The cell is able to regulate their concentration and they can also be regenerated with the cell. LMWA possess a wide spectrum of activities toward a large variety of ROS (Chance *et al.*, 1979; Gonzalez *et al.*, 2008; Kohen and Gati, 2000; Laguerre *et al.*, 2007).

The scavenging action of LMWA antioxidants turns them into radicals themselves, but not necessarily reactive ones; the scavenger radicals may undergo further oxidation or are regenerated to their reduced form by another scavenger.

The human antioxidant defence system is effective but not infallible, and break down can result in oxidative damage, accumulating with age at key biological sites, and contributing to senescence and age-related diseases (Ames *et al.*, 1993; Beckman and Ames, 1998; Halliwell and Gutteridge, 1999; Finkel and Holbrook, 2000; Laguerre *et al.*, 2007). This is due to an imbalance in the antioxidant: oxidant ratio, which tends to favour oxidation.

If oxidative damage leads to aging, cellular dysfunction and disease, then preventing it is expected to decrease the occurrence of these life-threatening processes (Beckman and Ames, 1998). Interestingly, it was noted by Cutler (1984), that the maximum lifespan potential (MLSP) of different mammalian species correlates directly with their plasma levels of uric acid which is an endogenous antioxidant.

Currently it is not certain that antioxidants can defend the body against oxidative damage due to high concentrations of ROS. Reports by Cutler (1984) and Laguerre *et al.*, (2007) show that increased melanin in skin, formed in response to exposure to ultraviolet light, leads to increased local defence. These observations may reflect a physiological attempt to reinstate balance and thus reduce oxidative stress. A more preventative and direct strategy, however, may be to increase intake of a variety of dietary antioxidants (Grey, 1998; McCall and Frei, 1999; Ames, 2001; Gonzalez *et al.*, 2008).

#### **2.1.5.2 Antioxidants from food sources**

In plants, the photosynthetic system is exposed to high levels of oxygen, and singlet oxygen can be formed by the transfer of photons of energy from chlorophyll, which acts as a photosensitizer within the chloroplast (Halliwell and Gutteridge, 1999). This singlet oxygen is very toxic to plants, and therefore plants have had to evolve strategies to eliminate this toxin (Fridovich, 1998; Benzie, 2000). Thus, photosynthetic plants also produce antioxidants to quench singlet oxygen, and to break chains of oxidation. Plants produce an array of antioxidants which include carotenoids, flavonoids, cinnamic acids, benzoic acids, folic acids, ascorbic acid, tocopherols, and tocotrienols, and these are localized and concentrated at the oxidation-prone sites of the plant (Strain and Benzie, 1999; Hollman, 2001; Gonzalez *et al.*, 2008).

Examples of antioxidants are found in various food sources include (Kandall, 2000):

- **Vitamin C:** This water-soluble vitamin is found in body fluids, and forms one of the body's first lines of defence. It is present in various important food sources which include citrus fruits, green leafy vegetables and strawberries, amongst others.

- **Vitamin E:** This lipid-soluble vitamin is thought to be important in delaying aging and it also plays a role in healing sunburn. Important sources for this antioxidant include wheat germ, nuts, seeds, whole grains, and vegetable oil.
- **Beta-carotene:** This antioxidant protects dark green, yellow and orange vegetables from solar radiation damage. Important sources include carrots, squash, broccoli, sweet potatoes, tomatoes, kale, collards, peaches and apricots.
- **Selenium:** It is suggested that this mineral helps fight cell damage caused by oxygen-derived compounds and thus may facilitate protection against cancer. The most important sources for selenium include fish, shellfish, red meat, grains, eggs, chicken and garlic.
- **Polyphenols:** Polyphenols are the most abundant antioxidants in food. The most important sources of polyphenols are fruit juices, tea, coffee, red wine, vegetables, cereals, chocolate and dry legumes (Scarbert *et al.*, 2005).

Food has been suggested to have protective effects against oxidative damage in cells. For example, in a study to examine the antioxidant activity of bran extracts growing in three locations in Colorado, it was found that wheat was able to significantly reduce lipid peroxidation *in vitro*. From the results obtained it was suggested that wheat-based products show great potential in suppressing oxidation of biological substrates *in vivo* (Yu *et al.*, 2005). Also, a study on green tea polyphenolic antioxidants, using both chronic oral feeding and topical application of green tea polyphenols, resulted in significant protection against UVR-induced cutaneous edema and erythema, lipid peroxidation and depletion of epidermal antioxidants defence enzyme system (Katiyar and Elmets, 2001; Svobodova *et al.*, 2003; Wei *et al.*, 1999).

### 2.1.6 Pro-oxidant activity of antioxidants

Pro-oxidant activity is a process whereby the antioxidant compounds at high concentrations oxidize biological substrates, which can result in lipid, DNA and protein oxidation (Yoshino *et al.*, 1999). Pro-oxidant activity is thought to be directly proportional to the total number of hydroxyl groups on an aromatic compound and the concentration of the compound (Cao *et al.*, 1997). Thus, while the antioxidant properties of certain antioxidants such as flavonoids, provide a positive means of protecting the body against oxidative damage, flavonoids and other phenolic antioxidants may also have pro-oxidant activity. Concentrated extracts of flavonoid rich plants such as propolis, pine bark, green tea leaves, soy isoflavones and grape seed are widely used as nutraceuticals for cardiovascular disease, cancer and chronic inflammatory conditions. Reports, (such as those by Yoshino *et al.*, 1999 and Yamashita and Tanemura, 1999) relating flavonoids to mutagenicity have raised obvious concerns and this needs to be thoroughly investigated. It is

important to investigate the correct concentration of any antioxidant to use, to avoid adverse effects such as prooxidant activity. This can be done by cytotoxicity studies, where cells are exposed to increasing concentrations of the antioxidant to determine the concentration range that does not result in adverse effects.

### **2.1.7 Phenolic compounds as antioxidants**

Phenolic compounds are currently considered to be very significant as antioxidant compounds. The antioxidant activity of phenolic compounds varies according to their chemical structure (Moure *et al.*, 2001), and the relationship between structure and activity of phenolic compounds has been used as a theoretical method of predicting antioxidant activity. Both the configuration as well as the total number of hydroxyl groups influence the activity of various phenolic antioxidants (Heim *et al.*, 2002). The activity of an antioxidant is determined by (Gonzalez *et al.*, 2008; Rice-Evans *et al.*, 1997; Rietjens *et al.*, 2006):

- Its reactivity as a hydrogen or electron-donating agent, which relates to its reduction potential;
- The fate of the resulting antioxidant-derived radical, which is governed by its ability to stabilize and delocalize the unpaired electron;
- Its reactivity with other antioxidants;
- Its transition metal-chelating potential.

Phenolic compounds are divided into various structural groups, namely phenolic acids and derivatives, flavonoids, stilbenes, lignans.

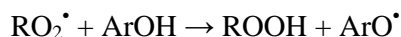
The ROS scavenging ability of phenols is primarily attributed to the high reactivities of their hydroxyl components. The hydroxyl groups on the ring donate hydrogen or electron to ROS, stabilizing them and giving rise to a relatively stable phenolic radical (Heim *et al.*, 2002). Polymeric polyphenols have been found to be more potent than their monomers. This suggestion was also emphasized by Yamaguchi *et al.* (1999), who observed that the higher the degree of polymerization of flavonols, the stronger the superoxide-scavenging activity.

#### **2.1.7.1 Reaction mechanisms of phenolic antioxidants**

Phenolic compounds can stabilize ROS using two major mechanisms: hydrogen atom transfer (HAT) and single electron transfer (SET). The end result, whichever mechanism is employed, is the same (Heim *et al.*, 2002). They are explained below:

#### 2.1.7.1.1 Free radical quenching via single electron transfer (SET)

In this mechanism, the phenolic compound quenches the reactive oxygen species by donating an electron to it, which results in spontaneous and reversible deprotonation in solution (Wright *et al.*, 2001; Gonzalez *et al.*, 2008). An example of such a reaction is represented below:



#### 2.1.7.1.2 Free radical quenching via hydrogen electron transfer (HAT)

The HAT reaction involves phenolic compounds quenching ROS by a hydrogen electron transfer reaction. By definition, phenolic compounds have at least one hydroxyl group on an aromatic ring (ArOH). The relative weakness of the OH bond in the phenol determines how fast the phenolic antioxidant will react with the ROS. To be effective, a phenolic antioxidant should react slowly with the non-radical substrates, but rapidly with ROS. In principle, the weaker the OH bond in ArOH, the faster it reacts with free radicals and hence the greater its antioxidant activity (Wright *et al.*, 2001).

### 2.1.8 Measuring antioxidant activity *in vitro*

A wide variety of antioxidant assays can be used to assess the radical scavenging ability of antioxidants *in vitro*. In general, ET-based assays entail two components in the reaction mixture, the test antioxidant and the oxidant (ROS). The antioxidant donates an electron to the oxidant resulting in a colour change to the oxidant. The degree of the colour change is directly proportional to the concentration of the antioxidant.

#### 2.1.8.1 The 2, 2-Diphenyl-picrylhydrazyl (DPPH) assay of antioxidant activity

The DPPH radical is one of the few available stable organic nitrogen radicals, and it has a characteristic deep purple colour. The assay is based on measuring the ability of antioxidants to reduce the colour of the DPPH radical. The reaction can be observed spectrophotometrically and the percentage of the DPPH radical remaining is directly proportional to the antioxidant activity (Brand-Williams *et al.*, 1995; Bondet *et al.*, 1997).

### **2.1.8.2 Ferric Reducing Antioxidant Power assay (FRAP)**

In the FRAP assay, the  $\text{Fe}^{3+}$  complex of tripyridyltriazine  $\text{Fe}(\text{TPTZ})^{3+}$  is reduced to an intensely blue coloured  $\text{Fe}^{2+}$  complex  $\text{Fe}(\text{TPTZ})^{2+}$  by antioxidants in an acidic medium. The redox potential is equivalent to that of  $\text{ABTS}^{\cdot-}$  (Huang *et al.*, 2005). The results are obtained as absorbance increases at 593 nm and can be expressed either as micromolar  $\text{Fe}^{2+}$ , or relative to an antioxidant standard (Antolovich *et al.*, 2001).

### **2.1.8.3 Low density lipoprotein assay (LDL)**

The LDL assay is used to measure LDL oxidation. It is one of the most commonly used methods for monitoring LDL oxidation *in vitro*, and thus used to measure antioxidant activity. Antioxidant activity is measured by measurement of conjugated dienes formed due to LDL oxidation. LDL oxidation involves the oxidation of polyunsaturated fatty acid (PUFA) side chains of LDL using copper in the presence of an antioxidant, by abstracting a hydrogen from a double bond in PUFA, which is accompanied by the formation of dienes that can be measured at 234 nm. Oxidized LDL remains fully soluble in buffer resulting in an increase of diene absorption (234 nm) which can be measured directly in solution (Jialal and Devaraj, 1996). Antioxidant activity is measured by the decrease in absorbance, indicating a decrease in LDL formed.

### **2.1.9 Biocatalysis in the production of polyphenolic antioxidants**

The term biocatalysis refers to the use of enzymes or living cells to catalyse organic reactions. This provides a simple, yet effective way of producing chemical products with biological activity, especially those that resemble naturally occurring products (Betancor and Luckarift, 2008). The chemical synthesis of polyphenolic antioxidants has proved difficult because of their complex structures and their synthesis requires precise site specific substitution steps (Faber, 2000). With the use of biocatalysts, it is possible to overcome these problems, and this approach is well recognized for its potential in providing such specificity. Enzymes are able to catalyze stereo- and regio-selective reactions (Patel, 2000; Betancor and Luckarift, 2008).

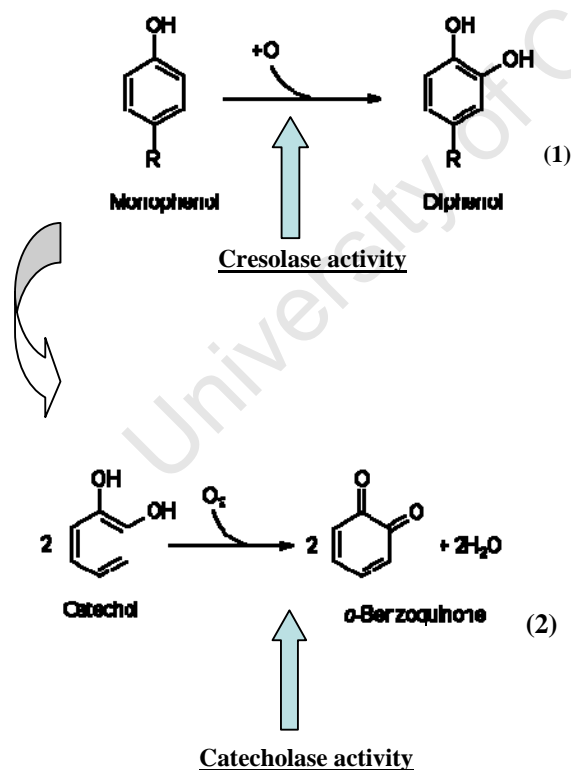
In this study, oxidase enzymes (laccase and tyrosinase) were used to catalyze the synthesis of polyphenolic compounds from simple phenolic compounds.

## 2.1.9.1 Phenol oxidases as biocatalysts for the production of polyphenols

### 2.1.9.1.1 Polyphenol oxidase (Tyrosinase) (EC 1.14.18.1)

The enzyme tyrosinase is widely distributed in bacteria, mammals and higher plants. Melanins are high mass-to-charge polymers that are found free or conjugated with proteins in pigmented tissues. These compounds result in the pigmentation of skin and the enzymatic browning at open surfaces of fruits such as bananas and the browning of mushrooms (Kendall, 2000).

Tyrosinase contains a coupled binuclear copper complex (Kendall, 2000; Duran *et al.*, 2002). In the reaction involving tyrosinase as a catalyst, both phenols and catechols [(Figure 2.5 (1)] serve as substrates, and both reactions result in the production of *o*-quinone as a product. The reaction of *ortho*-hydroxylation [(Figure 2.5 (2)] is called cresolase or phenolase activity, while the oxidation reaction of catechol is referred to as diphenolase activity (Burton, 1994; Muñoz-Muñoz *et al.*, 2009). The *o*-quinone resulting from the oxidation reaction of tyrosinase goes through a series of non-enzymatic chemical changes which ultimately yield melanins.

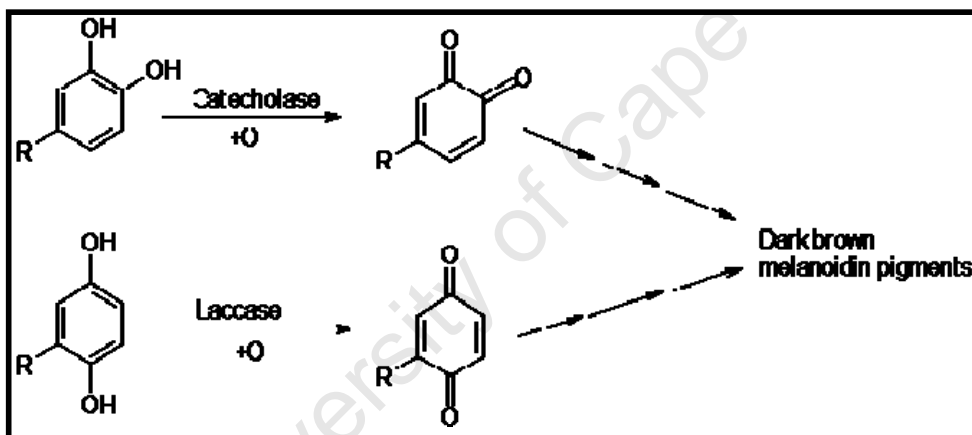


**Figure 2.5.** Hydroxylation of monophenol to diphenol (cresolase activity) with the subsequent oxidation of the diphenol to benzoquinone using molecular oxygen (Marshall *et al.*, 2000).

### 2.1.9.1.2 Laccases (EC 1.10.3.2)

Laccases have the unique ability to oxidize *p*-diphenols, which allows them to be distinguished from *o*-diphenol oxidases such as catechol oxidase [Figure 2.6]. In its pure state, laccases may be blue in colour due to the presence of Cu (II) ions. Several substrates, including polyphenols, methoxy-substituted phenols, diamines and a considerable range of other compounds serve as substrates for laccase (Reinhammar and Malmstrom 1981; Ma *et al.*, 2008).

The physiological function of these biocatalysts varies from organisms to organism, but they all catalyze polymerization or depolymerisation processes. In insects, laccase is involved in cuticle sclerotization and in the assembly of UV-resistant spores in *Bacillus* species. Laccase is involved in cell wall formation in plants and can protect fungal pathogens from toxic phytoalexins and tannins (Riva, 2006).



**Figure 2.6.** Comparison of reactions catalysed by catecholase and laccase (Marshall *et al.*, 2000).

### 2.1.9.1.3 Application of tyrosinase and laccase in relevant biocatalysis reactions

As biocatalysts, laccase and tyrosinase have been used in useful biotransformation reactions, for converting a broad range of starting compounds such as steroids and antibiotics to more useful compounds (Burton, 2003).

Laccases have been used to oxidize a range of organic compounds which includes methoxyphenols, phenols, *o*-, and *p*-diphenols, aminophenols, polyphenols, polyamines, and lignin-related molecules. They have also been used to catalyze the demethylation of lignin,

methoxyphenol acids and methoxyaromatics, the polymerization of lignin model compounds or monomers, and the simultaneous polymerisation of lignin with phenols and acrylamide. Laccases are able to catalyse the oxidation of a variety of compounds which have very high redox potentials such as aromatic alcohols in the presence of a mediator. Laccases have also been used to synthesize compounds with pharmaceutical significance (Burton, 2003).

Tyrosinases have been used in biocatalysis reactions to produce substituted catechols since the ortho-hydroxylation of phenols is not easily achieved by chemical synthesis. Reactions involving tyrosinases offer routes to a range of phenolic polymers. Some of these phenolic polymers have pharmaceutical significance; some can bind proteins and in that form are able to act as anti-inflammatories. The polypeptide polymers produced by using tyrosinase have been said to possess high tensile strength, comparable to that of mussel glue found in nature (Burton, 2003). In biocatalytic application, PPO can be used for the oxidation of synthetic polyhydroxystyrene and grafting on the polymer on to chitosan giving a novel amine-linked biopolymer. In nature, flavonoid phloridzin found in some fruits can be converted to 3-hydrox-derivative using PPO, resulting in a potent antioxidant (Burton, 2003).

In this study laccase and tyrosinase were used to catalyze the oxidation of monophenols to polyphenols.

#### **2.1.10 Tissue culturing as a method to evaluate antioxidant activity of putative antioxidants**

Tissue culture as a technique was first used almost 100 years ago to elucidate some of the most basic questions in developmental biology. Ross Harrison at the Rockefeller Institute, in an attempt to observe living, developing nerve fiber, cultured from embryo tissue in plasma clots for 1-4 weeks. He was able to observe the development and outgrowth of nerve fibers in these cultures (Harrison, 1907; Hughes, 1955). Tissue culturing is a method of growing cell lines, for example keratinocytes, melanocytes and fibroblasts, in the laboratory. Tissue culture is used in the laboratory for *ex vivo* experiments which include vaccine research and UVR cell damage studies. Currently, tissue culturing offers the opportunity to assess antioxidant action at the cellular level. Testing antioxidants using tissue culture gives an indication of the types of interactions that the antioxidant will encounter *in vivo* (Ichihashi and Ueda, 2003).

Cells that are cultured directly from animals or humans are known as primary cells. With the exception of some primary cells derived from tumours, most primary cells have a limited

lifespan. The doubling of cells after a certain period undergo a process of senescence and stop dividing, while generally retaining viability. Primary culture is recently removed from the *in vivo* situation and might therefore be expected to more closely resemble the function of that cell or tissue *in vivo* (Mather, 2002). Primary cells derived from tumours have an unlimited lifespan and are called immortalised cell lines, since they have acquired the ability to proliferate indefinitely, either through random mutation or deliberate modification, such as artificial expression of the telomerase gene (Manna and Galletti, 1997).

In cell culture practice, the culture conditions used vary significantly for each cell type, and variations of conditions for a particular cell type can result in different phenotypes being expressed. The most important factor in culture systems is the growth medium, which can vary in pH, glucose concentration, addition of growth factors, and the presence of other nutrient components. Growth factors used to supplement media are often derived from animal blood, such as calf serum (Berking, 2004).

#### **2.1.10.1 Keratinocyte cell lines (HaCaT)**

Since the main focus of this study was to investigate the antioxidant activity of phenolic compounds against the adverse effects of ultraviolet light, and the skin is the first site of UVR exposure, a cell line of keratinocytes (HaCaT) were employed for this study. The cells used in this study were originally established from a primary keratinocyte cell culture which, upon reduction of  $\text{Ca}^{2+}$  concentration, spontaneously immortalized to produce HaCaT cell lines (Boukamp *et al.*, 1988). The HaCaT cell line was established through spontaneous transformation of human keratinocytes from adult skin, and it maintains full epidermal differentiation capacity. In spite of the cell line's unlimited growth potential, HaCaTs remain very similar to normal keratinocytes by retaining an orderly structured and differentiated epidermal tissue. The HaCaT line is the first permanent epithelial cell line from adult human skin that shows normal differentiation and thus, provides a promising tool for scientific study (Boukamp *et al.*, 1988). This cell line has over a 140 passages, it does not have an altered phenotype in culture thus why one can use it for *in vivo* simulation.

#### **2.1.10.2 Uptake and metabolism of polyphenols in different cell types**

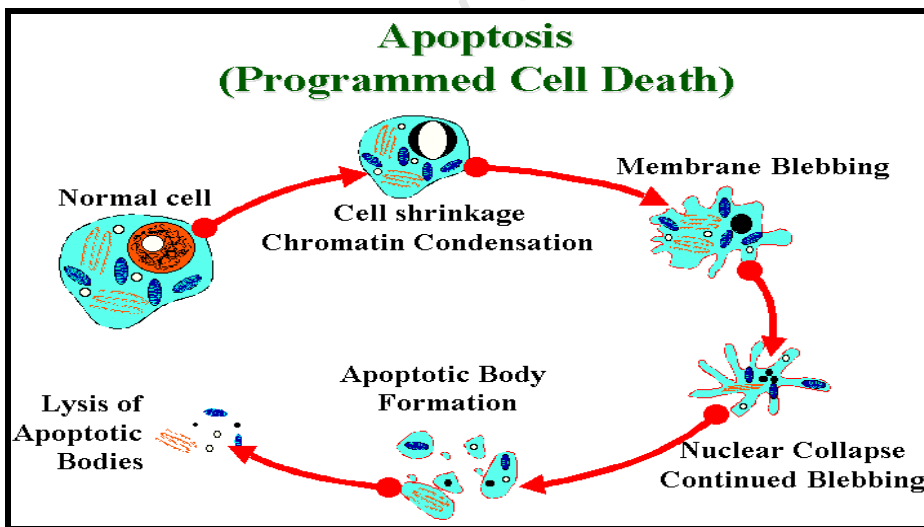
Polyphenolic compounds are absorbed by cells and have beneficial effects against many diseases, such as cancer and cardiovascular disease. However, it has become apparent that the bioactive forms of antioxidants *in vivo* are not necessarily the natural phytochemical forms, but instead, may be conjugates and metabolites arising from these, after absorption (Spencer *et al.*,

2004). There is a paucity of information with regard to the absorption and metabolism of polyphenols *in vivo* (Rice-Evans *et al.*, 1997; Gonzalez *et al.*, 2008).

Phenolics and polyphenols are all metabolized to *o*-glucuronides, sulphate esters and *O*-methy ethers (D'Angelo *et al.*, 2001). Studies such as those by D'Angelo *et al* (2001) have shown that phenolics such as hydroxytyrosol are rapidly metabolized. The metabolism of antioxidant compounds *in vivo* results in significant alteration in their redox potentials and a reduction in their antioxidant nature. In addition, studies by various authors have indicated that the reduction of the antioxidant activity of polyphenols *in vivo* is relative to the type of metabolism they have undergone (Terao *et al.*, 2001; Shirai *et al.*, 2001). This raises a question as to whether their metabolites still exhibit the variety of biological effects exerted by the initial molecule *in vitro*.

### 2.1.10.3 Induction of apoptosis by UVR

Apoptosis is a highly regulated form of cell death that is required for the removal of superfluous, infected, damaged or transformed cells in various biological systems (Assefa *et al.*, 2005; Liu *et al.*, 2010). It is characterized by cell contraction, condensation of chromatin and cytoplasm, fragmentation of nucleus, membrane blebbing, and cell fragmentation into apoptotic bodies that are phagocytised by neighbouring cells as showed in Figure 2.7 (Mammone *et al.*, 2000).

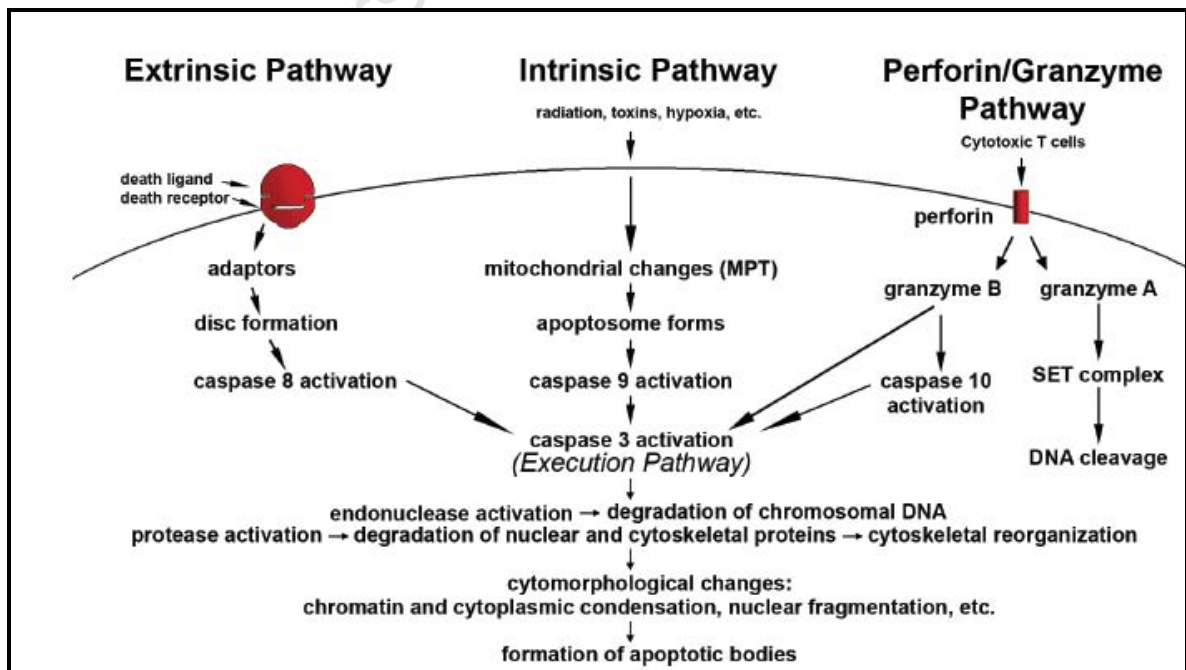


**Figure 2.7.** Apoptosis - the programmed death of a cell\*

(<http://www.microbiologybytes.com/virology/kalmakoff/baculo/baculohostinteract.html>)

Studies on apoptosis have identified the various systems that contribute to the control of UV-induced apoptosis in keratinocytes. These systems include death receptor pathways (extrinsic

pathway), the mitochondrial pathway (intrinsic pathway) (Batista *et al.*, 2009; Yau, 2004; Zhuang *et al.*, 2000). The extrinsic pathway initiates apoptosis by transmembrane receptor-mediated interactions that involve death receptors which are members of the tumor necrosis factor (TNF). The intrinsic pathway of apoptosis is initiated by intracellular signals that act directly on targets within cell and mitochondrial-initiated events (Elmore, 2007; Scorrano, 2009). This study focused on the mitochondrial pathway (intrinsic pathway) of apoptosis (Figure 2.8) which is discussed below. The intrinsic pathway is triggered by cellular stress, specifically mitochondrial stress caused by factors such as DNA damage. The stress signal resulting from cell damage or over production of ROS resulting in damage, results in the proapoptotic Bcl-2 family of proteins (Bax and BID) binding to the outer membrane of the mitochondria, stimulating the release of the internal content. However, the signal due to Bax and BID is not sufficient to elicit the full release. Another proapoptotic protein, BAK, resides within the mitochondria and is used to fully promote the release of cytochrome c and the intramembrane content from the mitochondria. The release of cytochrome c results in the formation of a complex in the cytoplasm with ATP (adenosine triphosphate) which is an energy molecule, and Apaf-1, an enzyme. The complex activates caspase-9, an initiator protein which works together with the cytochrome c complex, ATP and Apaf-1, to form an apoptosome. The apoptosome activates caspase-3, the effector protein that initiates degradation. In addition to the release of cytochrome c from the intramembrane space, the intramembrane content released contains apoptosis inducing factor (AIF) to facilitate DNA fragmentation, and Smac/Diablo proteins to inhibit the inhibitor of apoptosis (IAP) (Figure 2.8) (Yau, 2004; Elmore, 2007).



**Figure 2.8.** The intrinsic and extrinsic pathways leading to apoptosis (Elmore, 2007).

Exposure of skin to UVR can result in elevated levels of ROS in cells which can lead to apoptosis. In the skin, chromophores that instigate the absorption of UVR, which can result in cellular damage, include DNA, protein and LDL (de Bruin, 2008; Young, 2006). This absorption of UVR results in the production of ROS, which can at elevated levels, overwhelms the antioxidant and cell repair system. Cell death can occur through a process of apoptosis as a consequence (Basu-Modak *et al.*, 2003; Young, 2006).

In this study, UVA radiation was used to produce levels of ROS sufficiently elevated to cause apoptosis, in the presence and absence of the putative antioxidants. This was done to evaluate the antioxidant activity of our putative antioxidants against ROS over-production. This information would help to determine the antioxidant activity of the putative antioxidant both in being able to reduce ROS to normal physiological levels, and in inducing the over-production of protein markers that have a protective effect to cells.

#### **2.1.10.4 Measuring antioxidant activity *ex vivo* (in cells)**

The antioxidant activity of the putative antioxidant can be measured in cells (*ex vivo*), giving an indication of the antioxidant action of the putative antioxidants *in vivo*, and information regarding toxicity levels of the putative antioxidants.

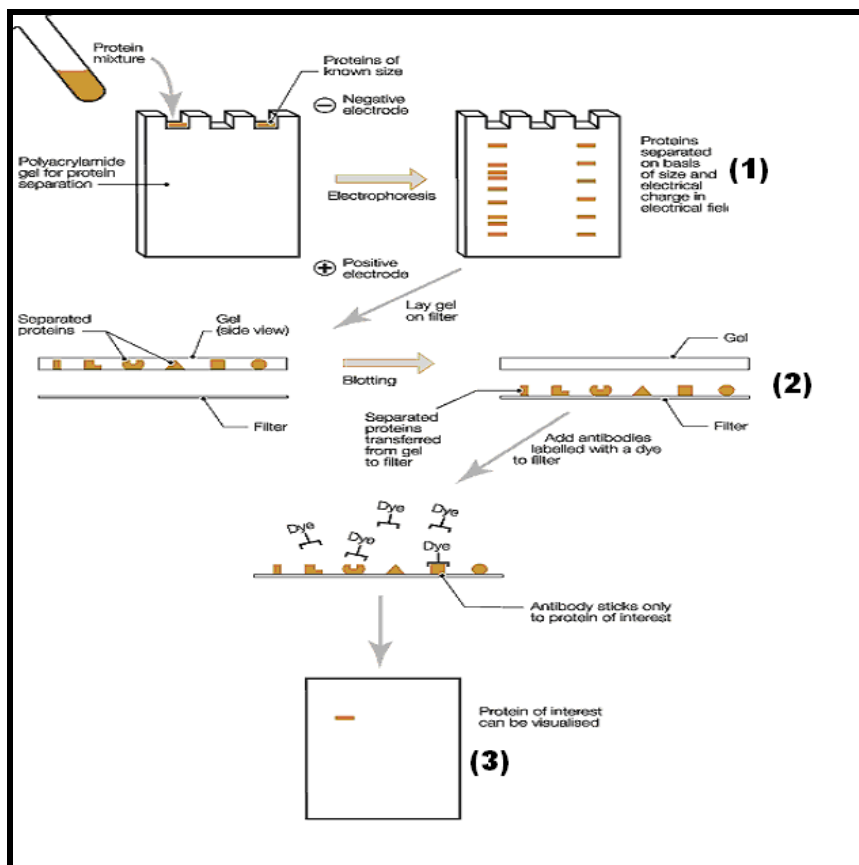
##### **2.1.10.4.1 ROS assay to evaluate the antioxidant activity of putative antioxidants *ex vivo***

UVA irradiation of cultured human skin cells results in the generation of H<sub>2</sub>O<sub>2</sub> and other ROS, with H<sub>2</sub>O<sub>2</sub> being the predominant species (Vile and Tyrell 1995). The H<sub>2</sub>O<sub>2</sub> induces oxidative stress in skin cells (Petersen *et al.*, 2000). In this assay, the DHR 123 (Dihydrorhodamine) dye is used as a detector of the production of ROS. DHR 123 has been shown to react with H<sub>2</sub>O<sub>2</sub> in the presence of peroxidase and is extensively used as a probe for the detection of intracellular H<sub>2</sub>O<sub>2</sub> (Katiyar *et al.*, 2001, Katiyar *et al.*, 2001 and Yang *et al.*, 2000). The mean fluorescence (MF) read on a FACS (Fluorescence-activated cell sorting) instrument (at a 488nm excitation wavelength) indicates intracellular H<sub>2</sub>O<sub>2</sub> production. In this study, the ROS assay was used to measure the ability of the putative antioxidants to reduce ROS levels due to UVA exposure in cells.

#### **2.1.10.5 Western blot analysis of proteins extracted following UVR exposure of HaCaTs in the presence and absence of the putative antioxidants**

Western blotting or immunoblotting allows investigators to determine, with a specific primary antibody, the relative amounts of a specific protein present in different samples. In this study, the method of western blotting was used to detect specific proteins in a given sample of tissue homogenate or extract. For the purpose of this research, western blotting was used to determine whether or not apoptotic products (proteins) were formed due to UVR exposure, giving an indication of whether or not the putative antioxidants had a protective effect against the deleterious effects of UVR. Figure 2.9 shows all the steps involved in the western blotting technique.

The method involves use of samples prepared from tissues or cells which are homogenized in a buffer that protects the protein of interest from degradation. SDS-PAGE [Figure 2.9(1)] is used to separate the sample which is then transferred to a membrane [Figure 2.9(2)] for detection using enhanced chemiluminescence (ECL). The membrane is incubated with a generic non-specific protein, such as milk proteins, to bind to any remaining sticky places on the membrane. A primary antibody is then added to the solution, to bind to the specific protein. A secondary antibody-enzyme conjugate, which recognizes the primary antibody, is then added to detect locations where the primary antibody bound Figure 2.9(3). (Renart *et al.*, 1979; Towbin *et al.*, 1979; Burnette, 1981).



**Figure 2.9.** The process of western blotting

([http://www.bseinquiry.gov.uk/report/volume2/fig1\\_8.htm](http://www.bseinquiry.gov.uk/report/volume2/fig1_8.htm))

### 2.1.11 Project hypothesis and objectives

The aim of this study was to generate new knowledge about the antioxidant capacity of selected phenolic compounds and to develop methodologies to measure their antioxidant effect in living cells, thus allowing an assessment of the protective role of the compounds as antioxidants, at a metabolic level.

#### 2.1.11.1 Hypothesis:

Phenolic compounds can reduce ROS-induced oxidative damage activity in UVA exposed cultured skin cells by promoting the over-expression of the anti-apoptotic protein Bcl-2 and the down-regulation of the pro-apoptotic proteins Bax and AIF. This is achieved by reducing ROS induced by UVA to normal physiological levels.

#### 2.1.11.2 Specific objectives:

- To produce putative antioxidant compounds in quantities sufficient for cell culture system study.
- To test the antioxidant activity of the putative antioxidants using *in vitro* antioxidant assays.
- To develop methods to measure antioxidant activity in living cells after exposure to UVA, thus allowing the assessment of the putative antioxidant's role at a metabolic level.
- To generate new knowledge about the antioxidant capacity of the synthesized phenolic group at cellular level.

University of Cape Town

# Chapter 3

## Materials and Methods`

This chapter describes the materials and methods used in investigating the antioxidant activity of the biocatalyzed putative antioxidants which include the synthesis of the putative antioxidants, *in vitro* antioxidant assays and *ex vivo* antioxidant assays.

### 3.1 Extraction of oxidase enzymes for biocatalytic reactions

#### 3.1.1 Extraction of tyrosinase from *Agaricus bisporus*

The extraction of tyrosinase was performed according to the method of Burton (1994). Fresh mushrooms (*Agaricus bisporus*) (1 kg) (obtained from a local supermarket) were frozen and then homogenized in cold acetone (2.5 L) using a blender. The resulting slurry was filtered rapidly on a Buchner funnel, and the residual pulp was air dried briefly before being frozen with liquid nitrogen. The pulp was then stirred in cold water (500 ml), and the mixture allowed to stand overnight at 4°C. The paste was filtered through cheesecloth, and the filtrate was cooled in ice while nitrogen gas was bubbled gently through the solution for 3h to remove the residual acetone. The aqueous extract was freeze dried to yield a pale brown powder (9.5 g) which was kept frozen until required. The protein content of the filtrate was determined using the Bradford method (Section 3.1.4). The activity of the tyrosinase was determined the L-DOPA assay (Section 3.1.3).

The filtrate was partially purified using ammonium sulphate precipitation. Thus, 54.2 g of ammonium sulphate was added to 200 ml of the filtrate to achieve 40% saturation. The solution was allowed to stand overnight at 4°C. The precipitate was separated by centrifugation (10000 g, 10min). The supernatant was mixed with ammonium sulphate (46.3 g in 100 ml) to bring it to 52% saturation. The solution was again allowed to stand overnight and centrifuged as above. The pellet was resuspended in 0.1 M sodium acetate buffer (pH 5) and was dialysed against water at 4°C for 18hrs. L-DOPA activity assays were performed (Section 3.1.2).

#### 3.1.2 Measuring the tyrosinase activity (Burton *et al.*, 1993)

The activity of tyrosinase was determined by monitoring the production of dopachrome at 475 nm in 3 ml 10 mM L-DOPA in potassium phosphate buffer (50 mM, pH 7). One unit of enzyme was defined as the amount of biocatalyst that catalyses the formation of dopachrome from L-

DOPA at a rate of  $1\mu\text{mol}\cdot\text{min}^{-1}$  where the extinction coefficient is  $3600\text{ M}^{-1}\cdot\text{min}^{-1}$ . A Unicam Merck UV/VIS spectrophotometer was used for all spectrophotometric assays.

### **3.1.3 Protein quantification of enzyme samples**

The protein concentrations in enzyme extracts were determined using the method of Bradford (Bradford, 1976). The analysis gives a linear response from 0.2 mg/ml to 1.0 mg/ml protein using bovine serum albumin (BSA) as a standard protein. 3ml volumes of Bradford reagent were added to 100  $\mu\text{l}$  of protein solution. The absorbance was read at 595 nm using the Unicam UV-VIS spectrophotometer after 5 min of incubation at room temperature. The enzyme protein concentration was determined graphically.

## **3.2. Extraction of laccase from *Trametes pubescens***

### **3.2.1 Strain preservation of *Trametes pubescens***

A culture of *T. pubescens* (CBS 696. 94) was obtained from the Boku Institute in Austria. This culture was maintained on 2% malt extract agar slants and subcultured every 60 days.

### **3.2.2 Growth of *T. Pubescens* in agar plates (Ncanana, 2007)**

*T. pubescens* was grown on agar containing 50 g/l malt extract agar, supplemented with a laccase inducer [1% phenol mixture (phenol: 82.8 mM, *p*-cresol: 25.8 mM, *o*-cresol: 77.03 mM)]. *T. pubescens* mycelial blocks were aseptically inoculated on the plates and incubated at 28°C for 6 days.

### **3.2.3 Growth of *T. pubescens* in flasks for laccase production**

Two portions of 200 ml of sterilized TDM medium were inoculated aseptically with homogenized (Sorvall benchtop homogenizer) mycelial plugs taken from a solid Petri dish preculture. Cultures were incubated in 1000 ml Erlenmeyer flasks at 28°C with agitation at 180 rpm, for 9 days (Ncanana, 2007).

### **3.2.4 Large scale production of laccase in airlift bioreactors**

Large scale production of laccase was performed according to the method of Ryan *et al.* (2005). *T. pubescens* was grown in a 4 l airlift bioreactor. A 400 ml production flask culture (9 days old) was used to inoculate two 3.6 l TDM in the airlift bioreactors each containing TDM

supplemented with 0.03% antifoam. The laccase activity was then monitored daily; 2 ml samples were taken from the bioreactor and centrifuged at 5000 rpm for 5 min. The supernatant was assayed for laccase activity. Five days post incubation, the medium in the bioreactor was supplemented with 30 ml phenol inducing mixture ((phenol: 82.8 mM, *p*-cresol: 24.99 mM, *m*-cresol: 25.8 mM, *o*-cresol: 77.03 mM) and 1g glucose was added daily to the medium. After 13 days, the medium, containing laccase with activity of 2-3 U/ml, was harvested by centrifugation at 10 000 rpm for 10 min.

### **3.2.5 Isolation of the laccase enzyme**

The laccase in the culture medium was precipitated using acetone or ammonium sulphate. For acetone precipitation, 300 ml of cold acetone was added to 300 ml sample (culture medium containing laccase). The mixture was then kept at -20°C for 30 min. The precipitated protein was recovered by centrifugation at 10 000rpm for 15 min at 4°C. The pellet was resuspended in 0.1 M sodium acetate buffer, pH 5 kept at -20°C or 4°C. For ammonium sulphate precipitation, 400 ml of 100% saturated ammonium sulphate was added to 100ml sample to give 80% ammonium sulphate. The mixture was kept at 4°C overnight. The precipitated proteins were recovered by centrifugation as above. The pellet was resuspended in 0.1 M sodium buffer, pH 5 and kept at -20°C or 4°C. The protein solution was then dialyzed against the 0.1 M sodium acetate buffer (pH 5) for 2 days. The dialyzed protein solution was aliquoted into Eppendorf tubes or freeze dried and then stored at -20°C or 4°C.

### **3.2.6 Laccase enzyme activity assay**

Laccase activity was determined with 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulfonate) (ABTS) (Roche) as the substrate (Wolfenden and Willson, 1982). The assay mixture contained 0.33 ml of 5 mM ABTS, 2.5 ml of 0.1 M sodium acetate buffer (pH 5), and 0.17 ml aliquots of culture supernatant or enzyme solution. Oxidation of ABTS was monitored by following the increase in absorbance at 420 nm using a Unicam UV-vis spectrophotometer. One unit (U) of laccase activity was defined as the amount of enzyme required to oxidize 1µmol of ABTS per min at 25°C.

### 3.3 Production of polyphenols using oxidase enzymes as the biocatalysts

#### 3.3.1 Oxidation of ferulic acid by laccase to yield a polymer

Laccase (prepared as described in Section 3.2.4) (100 U) was added to sodium acetate buffer (pH 5, 0.1M, 200ml) (Chigorimbo-Murefu, 2007). Ferulic acid (50 Mm; 0.39) was added to the buffer, after being dissolved in 5ml methanol (2.5 % v/v of the buffer). ABTS (2.5 mM; 0.055g) was added to the reaction to serve as the mediator for the enzyme reaction. The reaction was shaken at 180 rpm at 30<sup>0</sup>C and samples were periodically taken and analyzed with the HPLC. For HPLC analysis, The mobile phase was methanol: acetic acid: H<sub>2</sub>O (300: 20: 680) with a flow rate 1ml/min, using a C18 Waters (250 mm x 4.6 nm) reverse phase column and UV detection at 280 nm. Peaks were analyzed using HPLC Manager, Merck Hitachi model D 700 data software. The percentage conversion was obtained by comparing the peak area of reaction sample with that of a control (substrate). The products of the reaction were monitored by TLC monitored with eluent benzene: dioxane: acetic acid (5: 4: 1).

An equal volume of ethyl acetate to the reaction mixture was used to recover the organic product from the reaction mixture. The mixture was shaken and then allowed to separate. The organic phase was recovered, and dried using the rotor evaporator. The sample was dissolved in *d*-methanol for <sup>1</sup>H-NMR analysis. <sup>1</sup>H-NMR was conducted with *d*-chloroform at 300 MHz.

#### 3.3.2 Oxidation of tyrosol by tyrosinase to yield hydroxytyrosol (Ncanana, 2007)

Tyrosinase (80 U) was added to potassium phosphate buffer (pH 7, 500 mM, 240 ml). Tyrosol (0.30 g) and ascorbic acid (2.04 g) were added to the buffer. The reaction was shaken at 180 rpm, at 30<sup>0</sup>C, and samples were periodically taken and analyzed by HPLC. The mobile phase used for HPLC analysis was methanol: acetic acid: H<sub>2</sub>O (20: 2.5: 80) with a flow rate 1ml/min, using a C18 Waters (250 mm x 4.6 nm) reverse phase column and UV detection at 280 nm. Peaks were analyzed HPLC Manager, Merck Hitachi model D 700 data software. The percentage conversion was obtained by comparing the peak area of reaction sample with that of a control. The products of the reaction were monitored by TLC analysis with eluent toluene: ethyl acetate: formic acid solution (5: 4: 1).

An equal volume of ethyl acetate to the reaction mixture was used to recover the organic product from the reaction mixture. The mixture was shaken and then allowed to separate. The organic phase was recovered, and dried using the rotor evaporator. The sample was dissolved in *d*-methanol for <sup>1</sup>H-NMR analysis. <sup>1</sup>H-NMR was conducted with *d*-methanol at 300 MHz.

### 3.3.3 Oxidation of hydroxytyrosol by laccase to yield polymer

Laccase (80 U) and hydroxytyrosol (1 g) were added to sodium acetate buffer (pH 5, 0.1 M, 200 ml) and methanol (200 ml). The reaction was covered with foil and shaken at 180 rpm; 30°C. Samples were periodically taken and analyzed with the HPLC. The mobile phase used for HPLC analysis was methanol: acetic acid: H<sub>2</sub>O (20: 2.5: 80) with a flow rate 1ml/min, using a C18 Waters (250 mm x 4.6 mm) reverse phase column and UV detection at 280 nm. Peaks were analyzed using HPLC Manager, Merck Hitachi model D 700 data software. The percentage conversion was obtained by comparing the peak area of reaction sample with that of a control. The products of the reaction were monitored by TLC analysis with eluent toluene: ethyl acetate: formic acid solution (5: 4: 1).

An equal amount of ethyl acetate to the reaction mixture was used to recover the organic product from the reaction mixture. The mixture was shaken vigorously, following that the mixtures were allowed to separate. The organic part was recovered, and dried using the rotor evaporator and then resuspended in methanol. The sample was purified using flash chromatography (eluent, toluene: ethyl acetate: formic acid solution (5: 4: 1). The purified product was again recovered using the rotary evaporator and a sample was dissolved in *d*-chloroform for <sup>1</sup>H-NMR analysis. <sup>1</sup>H-NMR was conducted with *d*-chloroform at 300 MHz.

### 3.3.4 Liquid chromatography-mass spectrometry (LC-MS) analysis of products

Full Scan Liquid Chromatography-Electron Spray Mass Spectrophotometry (LC-ESMS) was performed in the negative mode between 0-1000 mV. The mobile phase was a mixture of formic acid (0.1 % v/v) (solvent A) and acetonitrile (solvent B) with a flow rate 0.7 ml/min, and using a Luna 5 µm C18 Waters (250 mm x 4.6 mm) reverse phase column and UV detection at 280 nm. The gradient elution program was: 95 % A and 5 % B (0-2 min), 20 % A and 80 % B (2-25 min), 100 % B (25-30 min), and 95 % A (30-40 min) and 5 % B (30-40 min).

### **3.4 Antioxidant assays to measure the antioxidant activity of biosynthesized phenolic products (DPPH, FRAP and LDL assays)**

#### **3.4.1 DPPH radical assay of antioxidant activity (Villano *et al.*, 2007)**

0.003 mmol of the respective sample was added to 3.9 ml DPPH solution (25 mg/ L in methanol). The decrease in absorbance at 515 nm was monitored using a Unicam UV-visible spectrophotometer, until the reaction reached steady state.

**Percentage Radical Scavenging Activity of the product calculated as follows:**

$$\frac{\text{Initial absorbance} - \text{final absorbance}}{\text{Initial absorbance}} \times 100 \quad (\text{Chandrasekar } et al., 2006)$$

#### **3.4.2 Ferric reducing antioxidant power assay of antioxidant activity**

0.003 mmol of the respective sample was added to 2.5 ml of potassium phosphate buffer (pH 7, 500 mM) 2.5 ml potassium ferricyanide was added. This was followed by incubation at 50<sup>0</sup>C for 20 min. 10% (v/v) trichloroacetic acid was added to stop the reaction. 2.5 ml of water was then added to 2.5 ml of the reaction. Iron chloride (0.5 ml) was added. The reaction was allowed to stand for 30 min after which the absorbance was read at 700 nm.

#### **3.4.3 Low density lipoprotein assay of antioxidant activity**

Oxidation of low density lipoprotein (LDL) was performed according to the method developed by Nardini *et al.*, (1995). LDL was dialyzed in a 200 fold volume of PBS of pH 7 in the dark for 18hrs. 100 µg/ml LDL, determined with the Bradford method, was oxidized with 5 µM CuCl<sub>2</sub> for 4hrs at 37<sup>0</sup>C in the presence and absence of 50 µM test antioxidant. Conjugated diene formation was measured spectroscopically at 234 nm using a Unicam UV-visible spectrophotometer. All samples were analysed in triplicate.

### **3.5 Cell culture experiments to determine the antioxidant activity of the putative antioxidants at tissue level**

#### **3.5.1 Preparation of culture medium and growing of HaCaTs cells**

All cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), and 1% penicillin/streptomycin to prevent bacterial contamination, and the pH was maintained at 7.4. Proliferation of epidermal cells was allowed at 37<sup>0</sup>C in humidified air and 5% CO<sub>2</sub>.

For experiments, cells (HaCaTs, UCT Medical School) were grown in 10cm<sup>2</sup> plates. Upon confluency, these cells were trypsinized by trypsin/ EDTA solution, collected and centrifuged at 3000 rpm for 5 min. Cells were then counted on a haemocytometer and seeded in 96 well plates at a density of 5000 cells/well. The cells were tested on a regular basis using a mycoplasma kit (UCT Medical School) and only used if they were mycoplasma-free.

#### **3.5.2 Cell viability assay**

The Cell Titer XTT proliferation assay (Roche, USA) was used to investigate the cell viability of HaCaTs following treatment with the various concentrations of the putative antioxidants. This assay is based on the ability of the viable cells to reduce yellow XTT tetrazolium salts to an orange formazan which was then read at an absorbance of 450 nm.

HaCaT cells, following culturing, were seeded into a 96 well plate and left overnight to adhere. The cells were then treated with different concentrations (for the product **33**, 200-3000 μM; for **35** and **30** 200-1000 μM) of the putative antioxidants and incubated for 24 h to investigate cytotoxic levels of the putative antioxidants if any. The XTT solution was then added and cells were incubated for 4h at 37<sup>0</sup>C. The plate was then read at an absorbance of 450 nm using a multi-well reader (VERSAmax tunable microplate reader, Labotec Molecular, USA) with associated SOFTmaxPRO 4.3.1 software. To investigate further the long term effect of the concentrations that showed non-cytotoxicity, the same experiment was repeated and cells were exposed to the putative antioxidants over a period of 3 days. Absorbance readings were converted into percentage of the untreated control values. Results are reported as mean ± SD of three separate experiments.

### **3.5.3 UVA irradiation of keratinocytes**

HaCaT cells were grown to 80% confluency and then incubated for 18h with the different concentrations of the putative antioxidant diluted in DMEM. At the end of the incubation, the medium was removed, cells rinsed with phosphate buffered saline and covered with a thin layer of PBS (3 ml) to prevent the cells from drying out during irradiation. The cells were then exposed to UVA, performed using a calibrated UVA light source (315 to 400 nm) (Walman, USA) at a dose of  $22.3 \text{ J/cm}^2$ . Subsequent to irradiation, the cells were allowed to recover for 2 h at  $37^\circ\text{C}$  in DMEM, FCS and P/S solution.

### **3.5.4 Protein extraction following UVA exposure**

Following the recovery period, the media was removed and centrifuged to recover cells that had lifted due to irradiation. Complete extraction buffer (Appendix A) ( $50 \mu\text{l}$ ) was added to the pellet. Subsequent to removing the media, the cells were washed with PBS, and  $150 \mu\text{l}$  of the complete extraction buffer was added while the Petri dish was placed on ice to prevent endogenous protease activity. A rubber policeman was then employed to scrape all the cells from the dish into an eppendorf and vortexed for 20 seconds together with the pellet from the media. The extract was placed at  $4^\circ\text{C}$  overnight, and then centrifuged ( $4^\circ\text{C}$ , 12 000 rpm, 20 min). The supernatant containing proteins was stored at  $-80^\circ\text{C}$ . Protein quantification was conducted using the Bicinchoninic Acid (BCA) protein assay.

### **3.5.5 Protein quantification of protein extracts using the Bicinchoninic Acid (BCA) protein assay (Smith, 1985)**

The amount of protein from the protein extracts was quantified by the Micro BCA method.  $200 \mu\text{l}$  of the working reagent was added to  $25 \mu\text{l}$  of each sample or unknown sample replicate into a microplate well. The mixture was thoroughly mixed on a plate shaker for about 30 seconds. The plate was then covered and incubated at  $37^\circ\text{C}$  for 30 minutes. The plate was then cooled to room temperature and the absorbance read at 562 nm using a multi-well reader (VERSAmax tunable microplate reader, Labotec Molecular, USA) with associated SOFTmaxPRO 4.3.1 software. A protein standard (BSA) curve was done to determine the concentrations of the different protein samples.

### **3.5.6 Western blot analysis**

The cells (HaCaTs) were lysed in ice-cold extraction buffer. The lysates were heated to 100<sup>0</sup>C for 5 min. The denatured samples were loaded onto SDS-polyacrylamide gel (Appendix A). After electrophoresis the proteins were electrotransferred (Appendix A) onto a Nitrocellulose membrane. Residual binding sites on the membrane were blocked with 10% milk in TBS-T (Tris-Buffered Saline Tween-20) (Appendix A), and probed with the primary antibody (1:200) of interest, made up in 5% milk in TBS-T and left overnight on a shaker at 4<sup>0</sup>C. The next day, the blot was washed twice in TBS-T for 10min. The blot was probed with monoclonal secondary antibody, goat anti-mouse (1:1500) made up in 5% milk in TBS-T for an hour, and then washed twice in TBS-T for 10min. After rinsing intensely in TBS-T, the blots were placed in ECL (enhanced chemiluminescence) western blotting reagent for 1 min at room temperature. The blots were then removed from the working solution and exposed to film (Appendix A). The percentage change in expression was calculated relative to the control, to determine how much of the protein of interest was expressed relative to the control.

Relative % expression = (protein expression in test compound/ protein expression in control x 100)

### **3.6 Flow cytometric analysis: ROS assay**

The HaCaTs cells were treated with various concentrations of the putative antioxidants for 24 hours. The cells were UVA irradiated followed by the addition of DHR 123 (Dihydrorhodamine) (Roche) and incubated at 37<sup>0</sup>C for 30 minutes. The cells were then washed once with PBS and trypsinized. The pellet was washed twice with PBS and centrifuged at 3500 rpm for 5 minutes. The cells were resuspended in PBS and analysed on a FACSCalibur flow cytometer (Becton Dickinson, USA) with the associated software CELLQuest software (Becton Dickinson, USA). Populations of cells used for the assay always consisted of 10 000 events. A 488 nm excitation wavelength was used.

### **3.7 Statistics**

Values are means  $\pm$  SE. Differences were statistically analyzed by the Student's t-test. \*P < 0.05 was considered statistically significant.

# Chapter 4

## Results and Discussion

### Biocatalytic reactions using oxidase enzymes to produce phenolic polymers

This study was conducted to investigate and characterize the products of the reactions between substrates tyrosol, ferulic acid and hydroxytyrosol and the oxidase enzymes, tyrosinase and laccase. The putative antioxidants were acquired from simple phenolic compounds using laccase and tyrosinase as biocatalysts. The products were characterized using Mass Spectrometry and NMR. The acquired products were then studied for antioxidants properties *in vitro* and in cell culture systems (*ex vivo*).

#### 4.1 Production, purification and characterization of the laccase from *T. pubescens*

Laccase was produced by *T. pubescens* and purified to be used as a biocatalyst for the production of putative antioxidants from simple phenolic compounds. To produce the laccase required as a biocatalyst for the bioconversion of tyrosol, a concentric draught-tube internal loop airlift bioreactor was used to grow *T. pubescens*, according to the method established by Ryan *et al.*, (2005). *T. pubescens* was chosen as it is a good laccase producer (Osma *et al.*, 2007). Since laccase is an extracellular enzyme it was secreted into the medium and was therefore readily extracted. As shown by studies of Pazarlioglu *et al.*, (2004) and Xavier *et al.*, (2007), laccase production can be induced by the addition of phenolic compounds and thus, for this study a phenolic inducer was added for enhanced laccase production (Section 3.2.4). The inducer was added from day 6 on a daily basis (30 ml and 1g glucose) to the medium and monitored periodically as described in Section 3.2.4. Laccase was harvested on the 13th day as the activity started decreasing after 12 days of fermentation. Following the biomass culture, laccase was partially purified by precipitation of the proteins to form a pellet, which was then collected and resuspended in buffer. This method of protein isolation exploits the nature of different proteins, which precipitate out of solution at different concentrations of the precipitating agent. The protein concentration of the collected supernatant was determined by the Bradford method as described in Section 3.2.7. The specific activity of laccase in the supernatant was found to be 2.27 U/mg following purification, as compared to the 1.9 U/mg from the crude extract (Table 4.1). These methods used to obtain the enzyme were established methods (Ncanana, 2007).

**Table 4.1** The purification table for laccase extracted from *T. pubescens*

<b>Fraction</b>	<b>Volume (ml)</b>	<b>Protein (mg/ml)</b>	<b>Activity (U/ml)</b>	<b>Specific Activity (U/mg)</b>	<b>Total Activity (U)</b>	<b>Total Protein (mg)</b>
<b>Crude</b>	500	5	6.8	<b>1.36</b>	3400	2500
<b>40 % pellet</b>	200	1.8	2.5	<b>1.39</b>	500	360
<b>52 % pellet</b>	100	3.6	6.1	<b>1.70</b>	610	360

#### **4.2 Production, purification and characterization of the enzyme tyrosinase from *A. bisporus* (mushrooms)**

Tyrosinase was obtained from *A. bisporus* using the method established by Burton (1994). Fresh mushrooms were homogenized in acetone to remove lipids and the homogenate was filtered through a Buchner funnel. The liquid nitrogen-dried residue was mixed with water to form a slurry from which tyrosinase was extracted with water. The extract, containing the tyrosinase activity, was collected by centrifugation. Ammonium sulphate fractionation was used to purify the enzyme which resulted in an enzyme extract pure enough to carry out biocatalysis (Burton, 1994). The activity and concentration of the protein in the crude and purified sample were determined by the L-DOPA and Bradford assays as described in Section 3.1.2. The maximum tyrosinase activity was detected in the 40-52% ammonium sulphate saturated pellet (Table 4.2). Extraction of the enzyme was repeated to obtain sufficient enzyme for biocatalytic reactions.

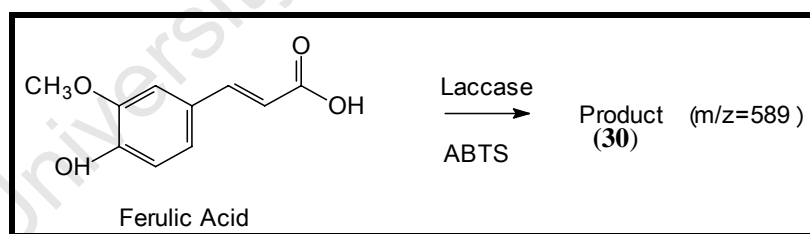
**Table 4.2** The purification table for tyrosinase extracted from *A. bisporus*

<b>Fraction</b>	<b>Volume (ml)</b>	<b>Protein (mg/ml)</b>	<b>Activity (U/ml)</b>	<b>Specific Activity (U/mg)</b>	<b>Total Activity (U)</b>	<b>Total Protein (mg)</b>
<b>Crude</b>	500	6	11.66	<b>1.9</b>	5830	3000
<b>40 % pellet</b>	200	2.16	4.3	<b>1.99</b>	860	432
<b>52 % pellet</b>	100	4.88	11.1	<b>2.27</b>	1110	488

### 4.3 Oxidation of ferulic acid by laccase

This section describes the oxidation of ferulic acid catalysed by laccase from *T. pubescens*, conducted in aqueous medium (Figure 4.1). A ratio of 2 U of laccase activity to 1mmole ferulic acid was used based on a previous study by Chigorimbo-Murefu (2007).

In the present study, the isolated laccase, and ferulic acid and ABTS (50 mM; 2.5 mM) were added to sodium acetate buffer (pH 5). The reaction was shaken at 180 rpm at 30°C and samples were taken periodically for analysis by HPLC. The reaction took place rapidly and it was observed that the solution turned a deep green colour, which was an indication of the ABTS being oxidized (Marjasvaara *et al.*, 2008). HPLC analysis was used to monitor the reaction and a 90% conversion was achieved. A single major product was observed (Figure 4.2) and this product (**30**) was observed to be more polar than ferulic acid, based on its retention time ( $R_t = 5.47$  min), which was shorter than that of ferulic acid (Chigorimbo-Murefu, 2007). Product **30** was then further analysed with LC-MS (Figure 4.3), H-NMR and TLC. Only this major product was investigated further as the other minor products were present in low concentrations only, precluding their identification by LC-MS and NMR. ABTS is well known laccase mediator, and has been shown to increase the catalytic ability of laccases to include a wider substrate range (Thurston, 1994). This work describes the effect of the presence of the laccase mediator ABTS on the reaction of laccase with ferulic acid in aqueous medium.



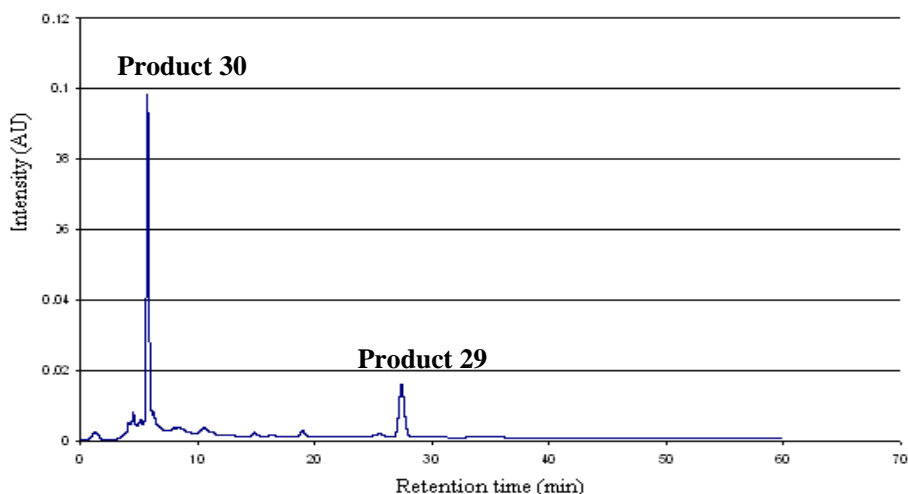
**Figure 4.1.** Oxidation of ferulic acid by laccase in the presence of ABTS

Product **30** of the reaction between ferulic acid and laccase was found to have a mass-to-charge of 589 m/z which is in agreement with studies conducted by Chigorimbo-Murefu (2007). The mass spectral analysis suggested a polymeric product with a base peak of 113 and at least 7 subunits of 68 mass units (Table 4.3). TLC analysis showed a product with an  $R_f$  value of (0.88).

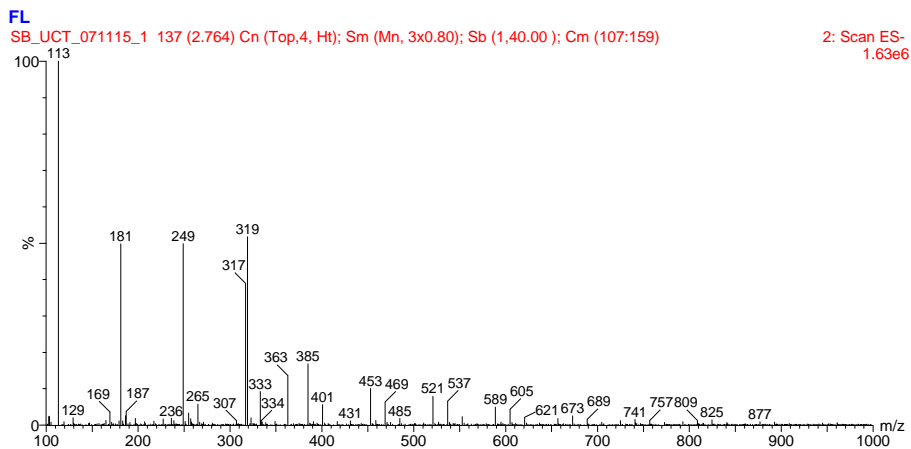
The catalytic ability of laccase results in the formation of phenoxy radicals, and the oxidation of ferulic acid using laccase as a biocatalyst would result in four possible intermediate radicals as shown in Figure 4.3, which illustrates the mechanism of polymerisation (Kupriyanovich *et al.*, 2007). These intermediate radicals are  $M_b$ ,  $M_5$ ,  $M_0$  and  $M_4$  and as a result there are 10 possible

dimeric arrangements namely  $\beta$ - $\beta$ ,  $\beta$ -5,  $\beta$ -O, 5-5 and O-O (Figure 4.4) (Kupriyanovich *et al.*, 2007; Youn *et al.*, 2006). From each of these 10 possible combinations, 10 others are possible in the formation of the tetramer, giving a total of 55 possible tetrameric products.

The fragmentation pattern of product **30** ( $m/z=589$ ) was not consistent with the fragmentation pattern of ferulic acid polymers as it consisted of 7 repeats of 68 mass units which could not be identified. In this reaction, the phenoxy radicals are suggested to have coupled with ABTS instead of coupling with each other. This phenoxy radical-ABTS coupling reaction explains the formation of a product that is not characteristic of a ferulic acid and laccase reaction but could have antioxidant activity. Thus, it can be concluded that the product formed from the oxidation of ferulic acid using laccase as a biocatalyst in the presence of ABTS resulted in a product that is not a simple ferulic acid polymer (Bourbonnais *et al.*, 1995). In a study by Chigorimbo-Murefu (2007) where ferulic acid was biocatalysed with laccase in the absence of ABTS, different products were observed, namely three different polyphenolic products as opposed to the reaction with ABTS that resulted in one major product. Thus, it can be concluded that ABTS had an effect on product formation. The elucidation of the structure the product from this study is currently underway but was beyond the scope of the current study. Nevertheless, the product was utilised in antioxidant studies for the sake of completeness.



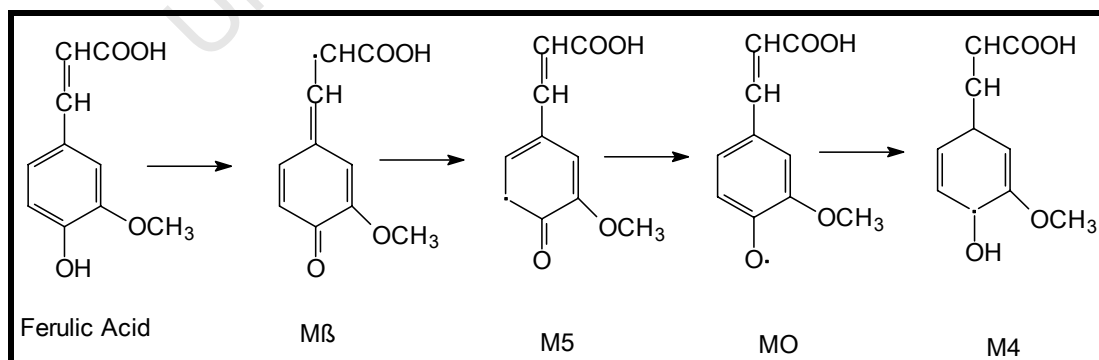
**Figure 4.2.** HPLC profile showing bioconversion of ferulic acid by laccase obtained from *T. pubescens*. The product (**30**) identified by LC-MS is a polymeric product with a retention time of 5.47 min. Ferulic acid (**29**) is represented by the peak with a retention time of 27.1 min



**Figure 4.3.** LC-MS profile of product (30), a polymer with retention time 5.47 min, from bioconversion of ferulic acid by laccase

**Table 4.3** Masses of significant product peaks detected from the mass spectrum of the product 30 of ferulic acid reaction with laccase in the presence of mediator.

Mass of significant peak detected	Difference in mass
113	68
181	68
249	68
317	68
385	68
453	68
521	68
589	68

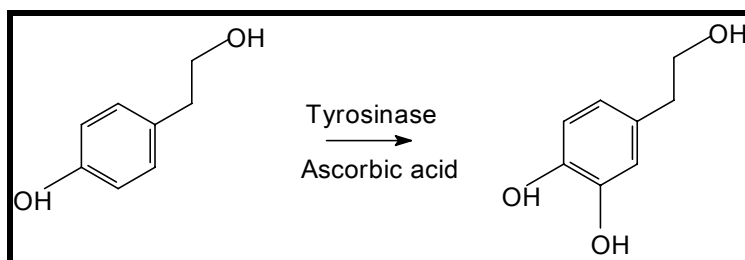


**Figure 4.4.** Formation of ferulic acid radicals M<sub>B</sub>, M<sub>5</sub>, M<sub>O</sub> and M<sub>4</sub> in the presence of laccase (Kupriyanovich *et al.*, 2007)

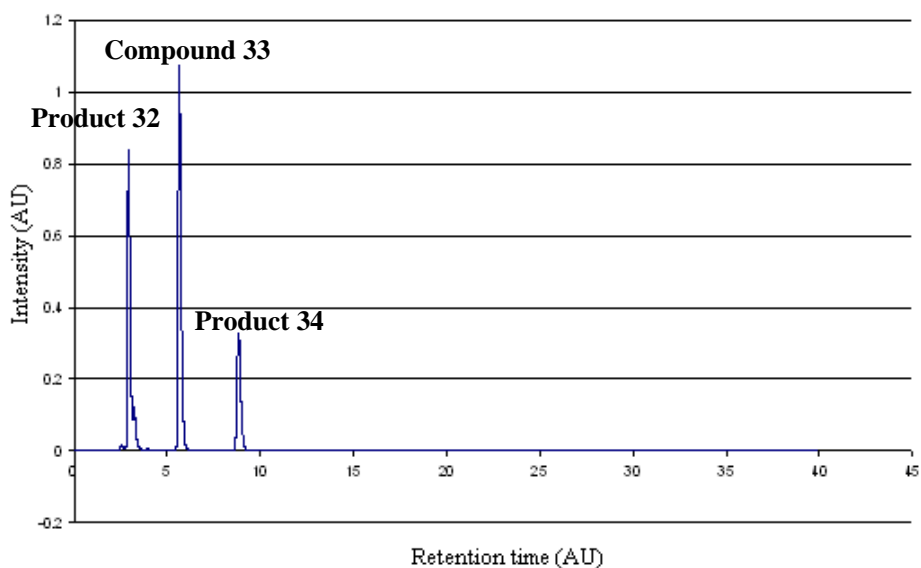
#### 4.4 Oxidation of tyrosol by tyrosinase to yield hydroxytyrosol

This section describes the biocatalytic conversion of tyrosol, catalysed by tyrosinase in aqueous medium (Figure 4.5). The method used here for the oxidation of tyrosol to hydroxytyrosol using tyrosinase as a biocatalyst, has several advantages over chemical synthesis. The chemical synthesis of hydroxytyrosol involves toxic reagents and the substrate of the biocatalytic reaction, tyrosol, is cheaper than the precursors needed for chemical synthesis of hydroxytyrosol (Espin *et al.*, 2001). The product formation during the chemical synthesis is usually rapid, but the compound obtained has to be purified further using chromatography (Bai *et al.*, 1998 and Capasso *et al.*, 1999). The biocatalytic route is an environmentally benign way of producing hydroxytyrosol, compared with chemical synthesis. Thus, it can be concluded that the enzymatic synthesis of hydroxytyrosol can be used as an alternative procedure to obtain hydroxytyrosol. The re-utilization of the enzyme in an industrial process could be achieved by means of a bioreactor and a nanofiltration system (Espin *et al.*, 2001). The hydroxytyrosol produced by this method was then used as a substrate for laccase, as described in the next section.

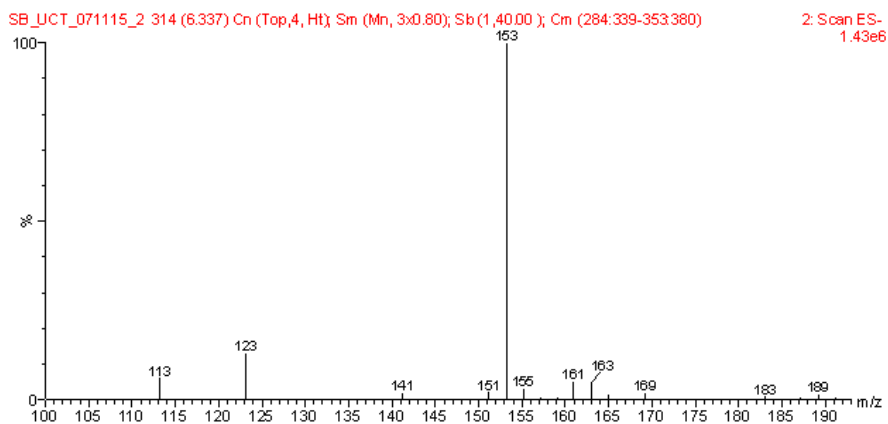
Mushroom tyrosinase catalyzed the hydroxylation of the tyrosol to give hydroxytyrosol. Hydroxytyrosol, under these conditions, may react to yield the *o*-quinone (brown pigments). To prevent this, ascorbic acid (2.04 g) was added to reduce the *o*-quinone to hydroxytyrosol (Espin *et al.*, 2001; Ncanana, 2007). For this reaction, we were interested in obtaining hydroxytyrosol as the final product. HPLC analysis was used to monitor and analyze the reaction by taking sample aliquots, withdrawn periodically during the reaction. The product, hydroxytyrosol, gave the largest peak and was found to have a retention time of 5.67 min (Figure 4.6). The reaction was also analysed by TLC and the product was found to have an R<sub>f</sub> value of 0.41. Further analysis of this product with LC-MS showed that the product had a mass-to-charge of 153 m/z (Figure 4.7). The reaction resulted in a 80% percentage yield of the product. The structure was confirmed using H-NMR. These results were in agreement with Cappaso *et al.*, (1999); Espin *et al.*, (2001); Ncanana, (2008) and thus the compound was confirmed to be hydroxytyrosol.



**Figure 4.5.** Oxidation of tyrosol by tyrosinase to yield hydroxytyrosol (Product 33)



**Figure 4.6.** HPLC profile showing bioconversion of tyrosol by tyrosinase obtained from mushrooms, after 4 hours reaction time. The reaction mixture contained ascorbic acid (**32**) which is represented by the first peak, with a retention time of 2.95 min. The second peak represents the newly formed product hydroxytyrosol (**33**) with a retention time of 5.67 min. The third peak represents the substrate tyrosol (**34**), with a retention time of 8.86 min.

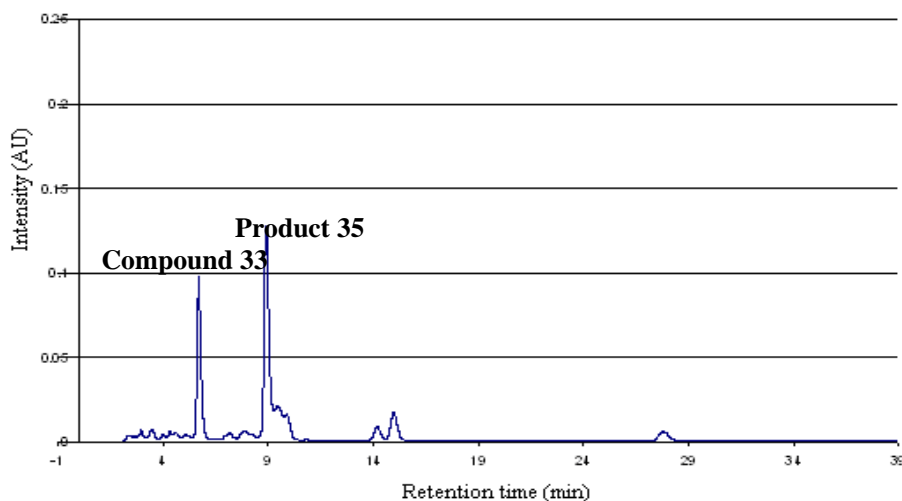


**Figure 4.7.** LC-MS profile of product **33** (hydroxytyrosol) synthesized using tyrosinase in buffer medium containing ascorbic acid and tyrosol as the substrate

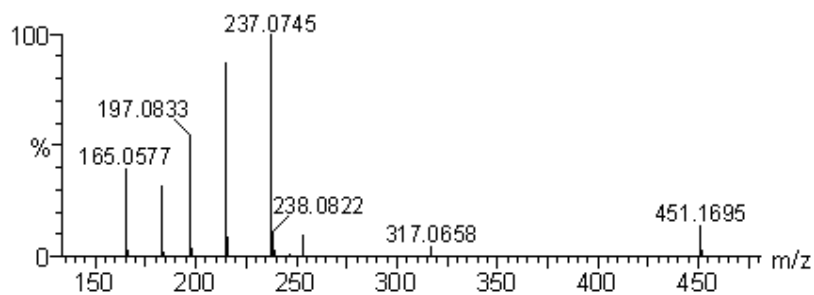
## 4.5 Oxidation of hydroxytyrosol by laccase

This section describes the bioconversion of hydroxytyrosol, catalyzed by the enzyme laccase, in an aqueous-organic medium. Hydroxytyrosol, previously synthesized using tyrosinase as a biocatalyst, was oxidized using the laccase from *T. pubescens* in sodium acetate buffer containing 50% methanol (reaction shaken at 180 rpm, 30°C) (Section 3.3.3). This ratio of methanol (50%) to buffer was chosen as it was shown in a study by Ncanana (2007) that this system resulted in one major polymeric product (Figure 4.8). By changing the percentage of the organic solvent in the reaction mixture, it had been shown to be possible to obtain different polymeric products, with different mass-to-charges and different antioxidant properties (Ncanana, 2007). Increasing the percentage of the organic solvent in the reaction medium resulted in a decrease in the number of different polymers formed, suggested to be due to the fact that organic solvents decrease the hydrophobic interaction of molecules in solution (Ncanana, 2007).

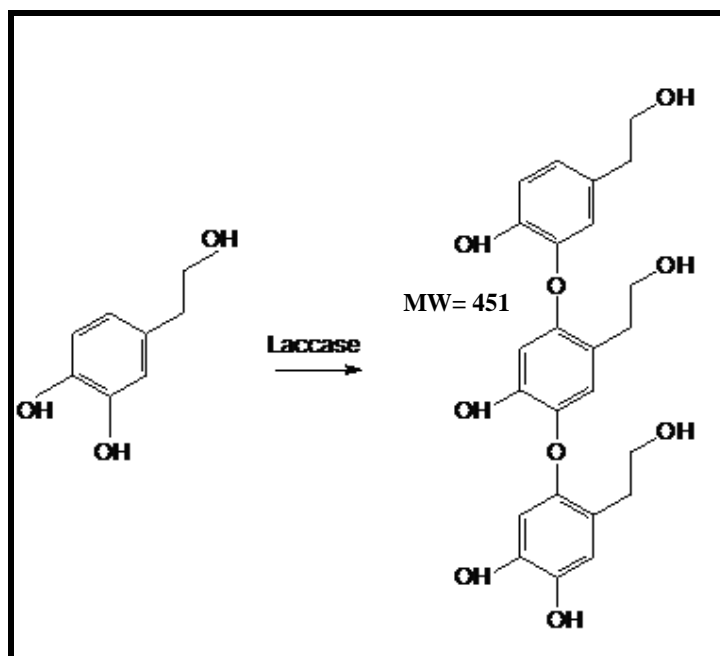
In the present study, the product obtained from the bioconversion of hydroxytyrosol using laccase as a biocatalyst, in 50% methanol, was found to be a trimer with HPLC retention time of 8.9 min (Figure 4.8), an  $R_f$  value of 0.3 on TLC and a mass-to-charge of 451 m/z by MS (Figure 4.9). The proposed possible structures are shown in Figure 4.10.



**Figure 4.8.** HPLC profile showing bioconversion of hydroxytyrosol by laccase from *T. pubescens*, after 4 hours reaction time. The first major peak represents the substrate hydroxytyrosol (**33**), with a retention time of 5.67 min. The second peak (**35**) represents the product with a retention time of 8.9 min.



**Figure 4.9.** LC-MS profile of product (35), a trimer with retention time 8.9 min, obtained by bioconversion of hydroxytyrosol by laccase in reaction medium containing 50 % methanol.



**Figure 4.10.** Suggested structures of product 35, obtained from the hydroxytyrosol-laccase reaction: shows C-O linkages (Ncanana, 2007)

#### 4.6 Antioxidant assays to measure the antioxidant activity of phenolic products

The antioxidant activity of putative antioxidants can be measured by using *in vitro* antioxidant assays (Frankel and Meyer, 2000; Antolovich *et al.*, 2001). It is useful to apply more than one type of assay, using both hydrophilic and hydrophobic systems, as occur *in vivo*, to elucidate the different types of antioxidant mechanisms (Frankel and Meyer, 2000; Antolovich *et al.*, 2001). In this study, three different antioxidant assays were used to give an indication of the putative antioxidants' activity *in vitro* namely, the DPPH, FRAP, and the LDL assays. The DPPH and

FRAP assays are hydrophilic antioxidant assays and both demonstrate the ability of a putative antioxidant to donate electrons. The DPPH assay demonstrates the hydrogen donating ability of the putative antioxidants to stabilize ROS, the FRAP assay demonstrates the iron (iii) reduction ability of putative antioxidants. The LDL assay is a hydrophobic antioxidant assay which demonstrates the ability of the putative antioxidant to inhibit lipid peroxidation caused by ROS. These methods are described in detail in sections 3.4.1, 3.4.2 and 3.4.3.

#### **4.6.1 Using the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical to assess the antioxidant ability of putative antioxidants**

The DPPH assay is based on the change in absorbance at 515 nm, upon reduction of the DPPH radical, by an antioxidant. The antioxidant activity of a putative antioxidant is measured by monitoring the decrease in absorbance when the colour of the solution fades. In this assay, the reaction between the test antioxidant and DPPH<sup>•</sup> radical is allowed to continue until it reaches a steady state. The amount of the radical quenched when steady state is reached dependent on the antioxidant capacity and the concentration of the antioxidant.

% Radical Scavenging Activity of the product is calculated as follows (Villano *et al.*, 2007):

$$\% \text{ RSA} = \frac{\text{Initial absorbance} - \text{final absorbance}}{\text{Initial absorbance}} \times 100$$

In this study, an aliquot of each respective antioxidant sample (0.003 mmol) was added to the DPPH solution. The decrease in absorbance at 515 nm was monitored, until the reaction reached steady state. From the results (Table 4.4) compounds **29**, **30**, **33** and **35** were all found to be effective radical scavengers against the DPPH radical, with radical scavenging activities of 31.3%, 96%, 33.2% and 86.7% respectively, relative to the standard ascorbic acid (100%).

**Table 4.4** Results of the measurement of hydrogen donating ability of putative antioxidants obtained using the DPPH assay, relative to the ascorbic acid standard

Name of product	Radical Scavenging Activity (%)	Final time of reaction (min)
Ascorbic acid (Std) (0.5 mg; 0.003 mmol)	100	4
Ferulic acid (Compound <b>29</b> ) (0.6 mg; 0.003 mmol)	31.3	5
Product <b>30</b> (Ferulic acid laccase reaction) (1.7 mg; 0.003 mmol)	96	76
Hydroxytyrosol (Compound <b>33</b> ) (0.5 mg; 0.003 mmol)	33.2	5
Product <b>35</b> (Hydroxytyrosol laccase reaction) (1.4 mg; 0.003 mmol)	86.7	7

The kinetic behaviour of the putative antioxidants was classified as follows: <5 minutes (rapid), 5-30 minutes (intermediate), >30 minutes (slow), based on a report by Huang *et al.* (2005). Under the standard assay conditions used (Section 3.4.1) compound **29** (ferulic acid) showed intermediate kinetic behaviour when compared to product **30** (from the Ferulic acid/laccase reaction) which had a slow kinetic behaviour. Product **35** (Hydroxytyrosol/laccase reaction) and Compound **33** (hydroxytyrosol) showed intermediate behaviour at 7min and 5min respectively.

Reactions of antioxidants found to have slow kinetic behaviour, such as product **30**, were suggested to undergo complex reactions with DPPH (Bondet *et al.*, 1997). During the DPPH reaction, the DPPH radical extracts a hydrogen atom from the putative antioxidant, and this newly formed radical can then react with a molecule of DPPH radical, resulting in the formation of an adduct. The adducts formed can retain their antioxidant activity, and hence continue to react, resulting to adducts which results in the slow kinetic behaviour of polyphenols as observed with product **30** (Osman *et al.*, 2006). This process results in the scavenging of a greater number

of radicals in total, although is a slow process. An example was given by Bondet *et al.*, (1997) as follows: 1 mole of BHT reduced 3mole of the DPPH radical over 5 hrs, while 1 mol of isougenol reduced 1mole DPPH radical and reached steady state after 0.5 min. Thus, BHT showed better antioxidant activity as it was able to quench more DPPH radicals in total.

From the results (Table 4.4) it is evident that slow kinetic behaviour does not necessarily translate to a putative antioxidant having low antioxidant activity. Thus, while product **30** showed slow kinetic behaviour (reaching steady state in 76 minutes), it gave a total radical scavenging activity of 96%, some 3x higher than the precursor **29**, on a molar basis.

It can also be observed that the products **30** and **35** showed higher antioxidant activity compared to the substrates (**29** and **33**) and standard ascorbic acid. Thus, polymerization resulted in products with increased antioxidant activity. Polymerization also results in an increase in the number of hydroxyl groups per molecule. This increased radical-scavenging activity suggests that the laccase catalyzed conversion was successful as it resulted in better antioxidant activity (de Pinedo *et al.*, 2006).

#### 4.6.2 Using the FRAP assay to assess the antioxidant activity of putative antioxidants

The FRAP assay measures the ability of an antioxidant to reduce a ferroin analog, the Fe complex of tripyridyltriazine, Fe (TPTZ)<sup>3+</sup>, to the intensely blue coloured Fe<sup>2+</sup> in an acidic medium (Section 3.4.2). Results are obtained as absorbance increases at 593 nm and can be expressed relative to an antioxidant standard, namely ascorbic acid in this study (Antolovich *et al.*, 2001). To obtain the FRAP values for the putative antioxidants, a concentration range of ascorbic acid was used and the absorbance values obtained were used to construct a standard curve (An increase in the concentration of the ascorbic acid, showed an increase in the absorbance). Thus, the standard curve (Appendix) was used to determine the concentration of the ascorbic acid equivalent to the activity of the putative antioxidant. The molar concentrations of the compounds were determined using their mass-to-charges.

Products **30** and **35** showed the highest antioxidant activity, with antioxidant activity equivalent to 213 mg/L and 363 mg/L of ascorbic acid compared to substrates **29** and **33** with antioxidant activity equivalent to 126 mg/L and 124 mg/L respectively. The reducing properties of these putative antioxidants are associated with their free radical chain breaking properties, initiated by their donating a hydrogen or electron atom (Othman, *et al.*, 2007).

These results suggest that products **30** and **35** gave the highest FRAP assay values, and thus had the most potent antioxidant activity. Thus, the ferric reducing power increased with an increase in the mass-to-charge of the putative antioxidants (Table 4.5), and the polymers (product **30** and **35**) both showed higher antioxidant activity than their substrate precursor **29** and **33** respectively. Thus it is again apparent that polymerization resulted in products with increased antioxidant activity. Products **30** and **35** can be said to be strong electron-donors which terminate oxidation chain reactions by reducing the Fe complex of tripyridyltriazine Fe (TPTZ)<sup>3+</sup> to the ferrous form (Saeed *et al.*, 2007).

**Table 4.5** Results of the ferric reducing ability of putative antioxidants obtained using the FRAP assay relative to the ascorbic acid standard

Sample name	Concentration of compounds (mg; mmol)	Ascorbic acid equivalence (mg/L)
Compound <b>33</b> (Hydroxytyrosol) Substrate	0.5 mg; 0.003 mmol	124
Product <b>35</b> (Hydroxytyrosol laccase reaction) Product	1.4 mg; 0.003 mmol	363
Product <b>30</b> (Ferulic acid laccase reaction) Product	1.8 mg; 0.003 mmol	213
Compound <b>29</b> (Ferulic acid) Substrate	0.6 mg; 0.003 mmol	126

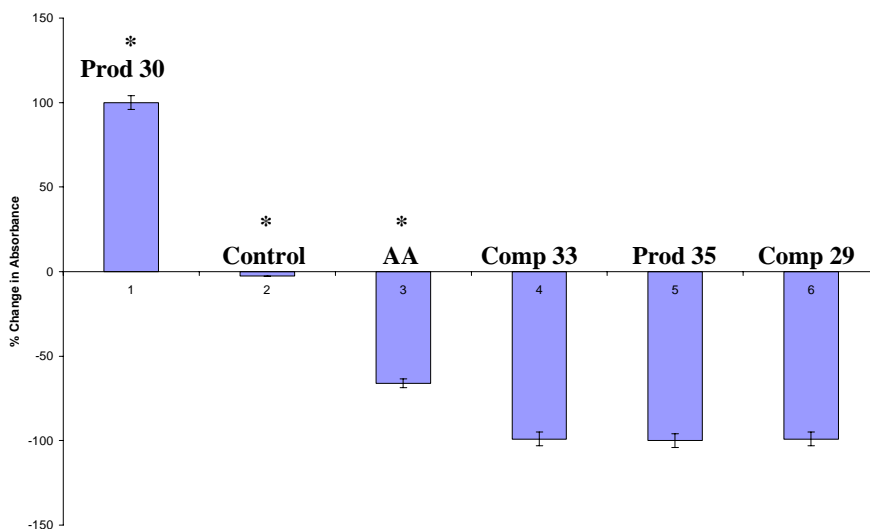
#### 4.6.3 Using the (LDL) assay to assess the antioxidant activity of antioxidants

The antioxidant ability of the putative antioxidants to inhibit LDL oxidation was assessed by adding them to a reaction mixture containing LDL previously treated with copper to initiate the oxidation, as described in section 3.4.3. The antioxidant ability of the compounds was measured based on the increase in absorbance due to diene conjugation at 234 nm.

All of the compounds tested showed an absorbance-decreasing effect and thus were able to prevent diene conjugation due to lipid peroxidation, to varying degrees relative to the standard, ascorbic acid (Figure 4.12). Of the compounds tested, compounds **29** (ferulic acid), **33** (hydroxytyrosol) and product **35** (from the hydroxytyrosol/laccase reaction) were found to show high inhibition of LDL oxidation as compared with the standard antioxidant, ascorbic acid. Product **30** (from the ferulic acid/laccase reaction) showed the lowest antioxidant activity relative to the compounds **29**, **33**, **35** and ascorbic acid.

Lipid peroxidation results in the formation diene conjugation through a chain reaction of peroxidation. Thus, the compounds **29**, **33** and **35** were able to prevent this chain reaction and can be said to be chain breaking antioxidants (Rice-Evans *et al.*, 1996).

The polar theory (Moure *et al.*, 2001) may explain the low reactivity observed with product **30**: this compound is very lipophilic (insoluble in water) and only dissolves in hydrophobic organic solvents. The polar paradox postulates that hydrophilic antioxidants are more effective than lipophilic antioxidants in non-polar media, whereas lipophilic antioxidants show greater activity in emulsions (Moure *et al.*, 2001; Porter *et al.*, 1989). The LDL particles in which compound **30** was to exert its antioxidant activity are also lipophilic (Moure *et al.*, 2001) and as a result, it is suggested that product **30** would enter the lipophilic core of the LDL particle, where it is prevented from having a high protective effect. A similar trend was also observed in a study where lipophilic  $\alpha$ -tocopherol and ascorbyl palmitate were shown to have better antioxidant activity in emulsions than in non-polar media, while the inverse was found for trolox and ascorbic acid (Samotyja and Malecka, 2006).



**Figure 4.12.** Changes in absorbance due to the  $\text{Cu}^{2+}$  induced formation of conjugated diene in LDL samples. An increase in absorbance signified the formation of conjugated diene and a negative change signified an inhibition of the formation of conjugated diene, by the respective putative antioxidants. All experiments were done in triplicate (\* $P < 0.05$ )

#### 4.7 Effect of various concentrations of the putative antioxidants on HaCaTs' cell viability

Ideally, to test the efficacy of putative antioxidants, they need to be applied in a biological model involving cells. In this study, keratinocytes (HaCaTs) were chosen, as they are targets for most solar radiation-induced skin cancers and are exposed to numerous oxidants derived from normal metabolism, patho-physiological processes and extra cellular sources (Gautam *et al.*, 2004). To test the putative antioxidant activity of the putative antioxidant compounds in this study, cells were exposed to UVA in the presence and absence of the putative antioxidants, and effects due to UVA irradiation (ie: elevated ROS production, apoptotic proteins) were measured. The first step involved determining the appropriate concentrations for each of the putative antioxidant compounds, based on toxicity limits.

In order to evaluate the toxicity of the putative antioxidants under study, the XTT assay was conducted. This assay is based on the ability of viable cells to reduce yellow XTT tetrazolium salts to an orange formazan which was then measured at an absorbance of 450 nm. The HaCaTs were incubated overnight with increasing concentrations of each antioxidant compound. Cell viability was then evaluated by adding the XTT solution, incubating for 4hrs at  $37^{\circ}\text{C}$ , and then

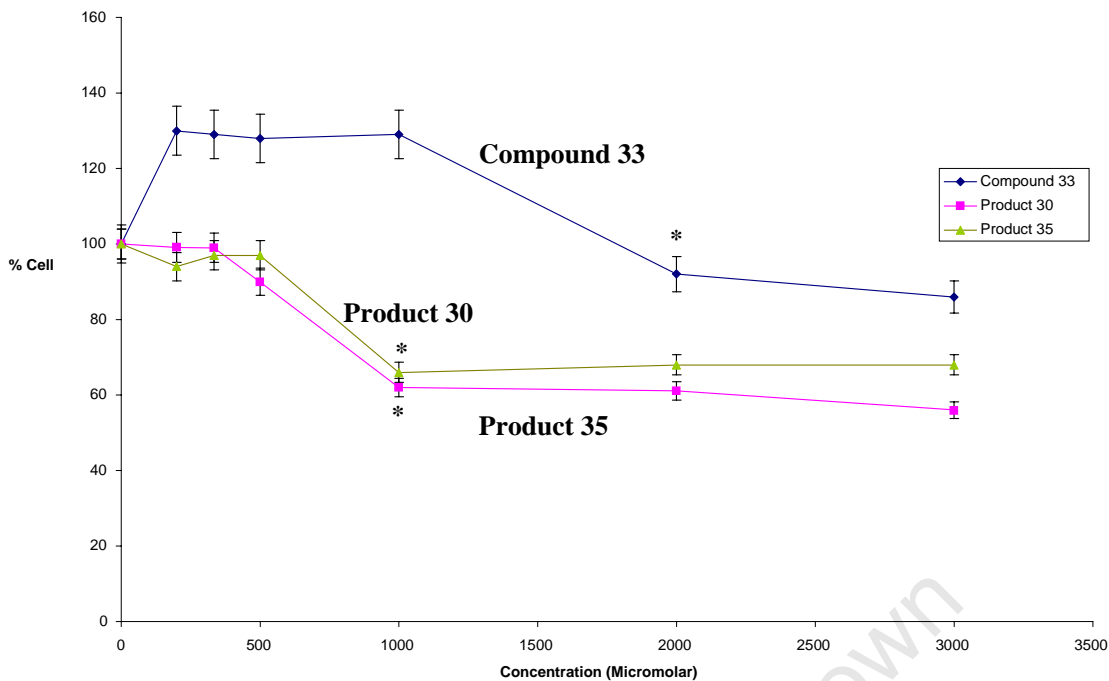
reading the absorbance at 450 nm. In this assay, the absorbance increased with an increase in the number of viable cells.

To investigate further the long term effect of the concentrations that showed non-cytotoxicity, the same experiment was repeated and cells were exposed to the putative antioxidants over a period of 3 days. Absorbance readings were converted into percentage of the untreated control values.

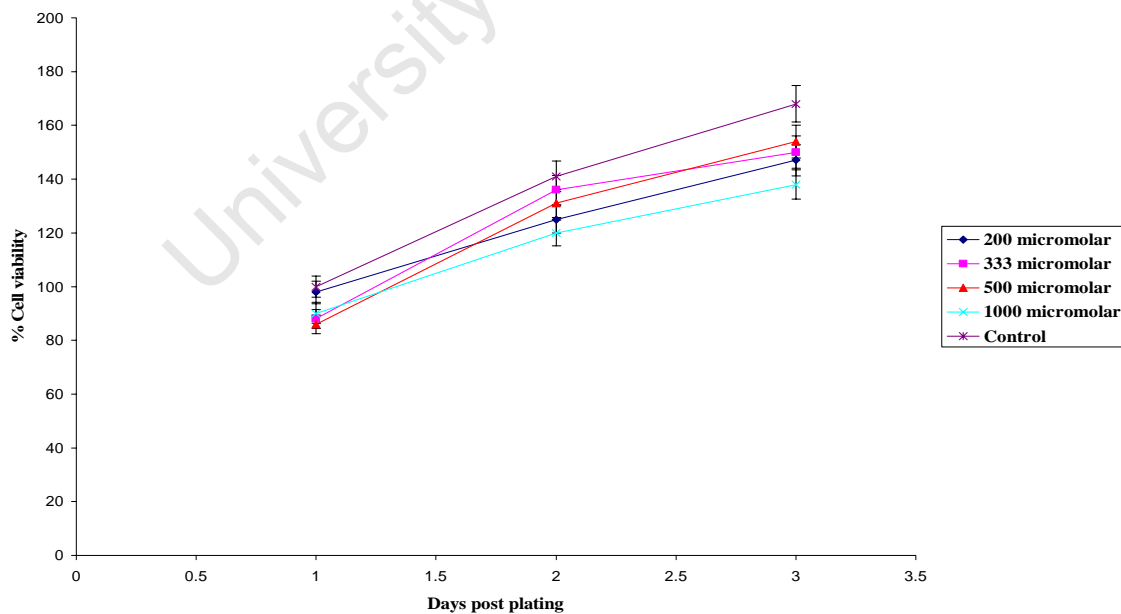
From the results (Figure 4.13) it is evident that product **33** did not cause any significant negative effect on cell viability at concentrations of 200-1000  $\mu\text{M}$ , over a 24hr period ( $P > 0.05$ ). Concentrations higher than 1000  $\mu\text{M}$  caused a marked decrease in cell viability ( $P < 0.05$ ). Products **35** and **30** showed no negative effect on cell viability at concentrations of 200-500  $\mu\text{M}$  ( $P > 0.05$ ), but concentrations higher than 500  $\mu\text{M}$  resulted in marked decreases in cell viability.

It should be noted, with regard to compound cytotoxicity, that this is strictly dependent on cell type. For example, compound **33** has been found to completely inhibit HL60 (Human promyelocytic leukemia) cell proliferation at 100  $\mu\text{M}$ , and leading to more than 50% loss of cell viability either in human prostate cancer cells or in immortalized renal proximal tubule cells (Rietjens *et al.*, 2006). In this study, using HaCaTs, the presence of compound **33** only lead to a loss of cell viability at concentrations higher than 1000  $\mu\text{M}$ . Of most interest, compound **33** had a much higher proliferative effect on cells which is interesting in that antioxidants in low concentrations often have this effect on cells in culture.

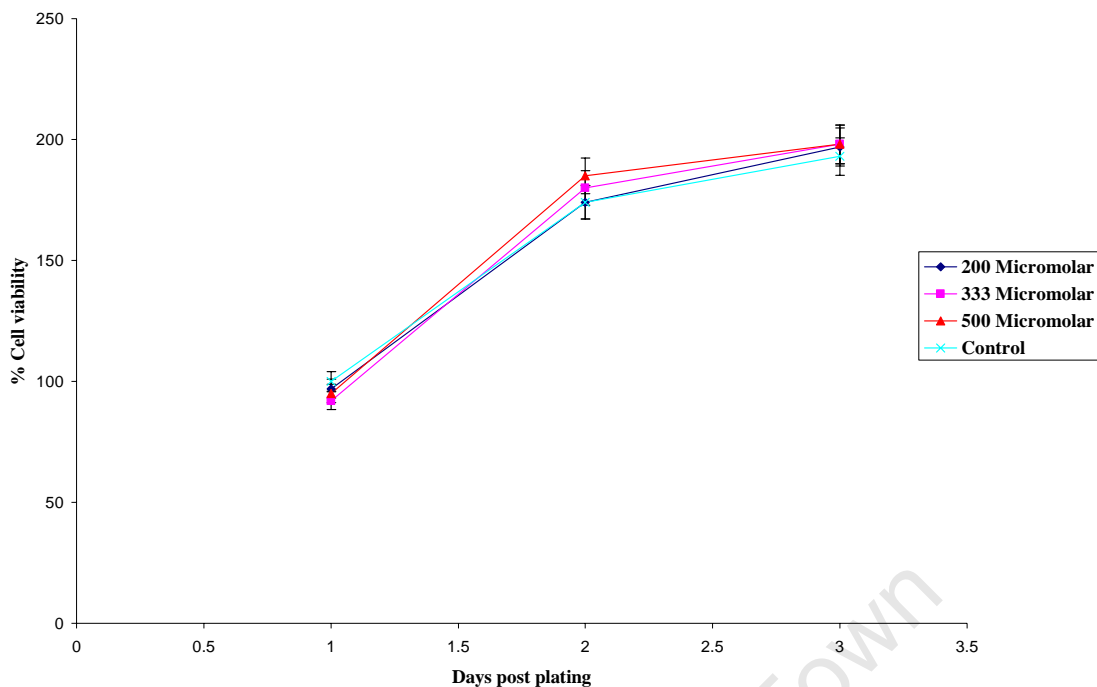
To further explore the effect of the putative antioxidants (compound **33** and products **30**, **35**), a dose and time response study was carried out to determine the long term effect of the putative antioxidants. HaCaT cells were grown in the presence of the concentration ranges of the putative antioxidants which had been proved to be non-toxic to HaCaTs during the 24hr incubation period, as described in the previous paragraph. All the concentrations that proved non-toxic over a 24hr period, also proved to be non-toxic over a 3 day period (Figure 4.14, 4.15, 4.16). Over the 3 day period the cells displayed a standard sigmoidal cell growth curve indicative of no cell loss (Figure 4.14, 4.15, 4.16) and these concentrations were used for all subsequent experiments.



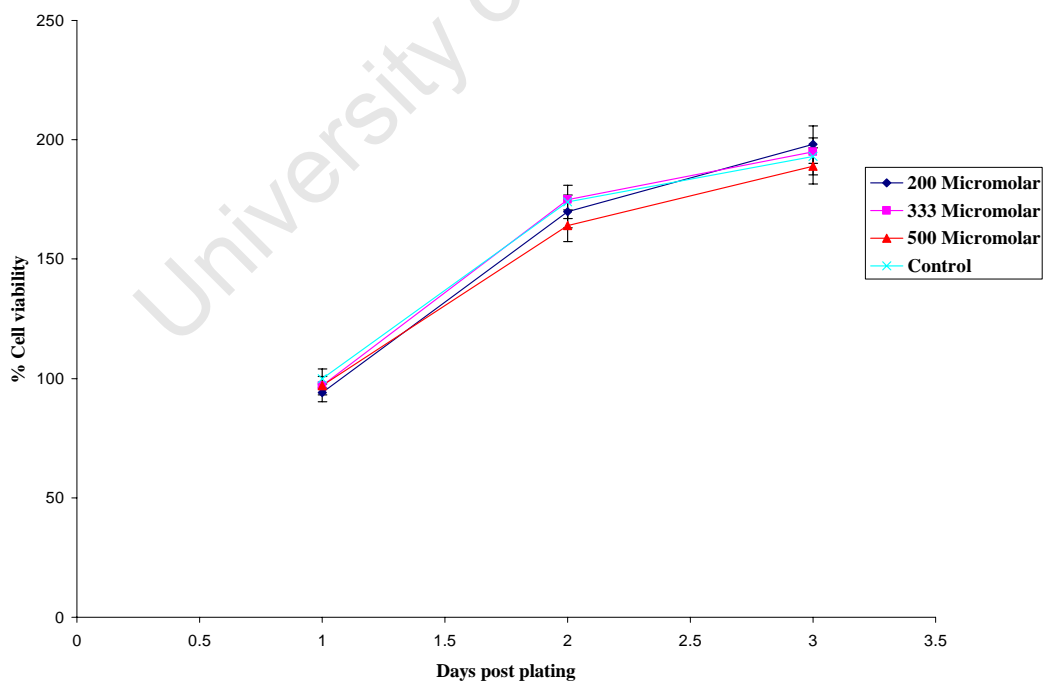
**Figure 4.13.** Effect of the various concentrations of putative antioxidants on HaCaT cell viability evaluated using the XTT assay (Roche, 2008). Results are expressed as a percentage of viability related to the control (n=3). H (hydroxytyrosol), HL (product **35** from hydroxytyrosol-laccase reaction), FL (product **30** from ferulic acid-laccase reaction) (\*P < 0.05)



**Figure 4.14.** Long term effect of increasing concentrations of compound **33** (hydroxytyrosol) on HaCaT cell viability evaluated using the XTT assay over a period of 3 days (n=3) (\*P < 0.05)



**Figure 4.15.** Long term effect of increasing concentrations of product **35** (from the hydroxytyrosol-laccase reaction) on HaCaTs cell viability, evaluated using the XTT assay over a period of 3 days (n=3) (\*P < 0.05)



**Figure 4.16.** Long term effect of increasing concentrations of product **30** (ferulic acid/laccase reaction) on HaCaTs cell viability evaluated using the XTT assay over a period of 3 days (n=3) (\*P < 0.05)

#### 4.8 ROS assay to elucidate the effect of putative antioxidants on the production of ROS due to irradiation

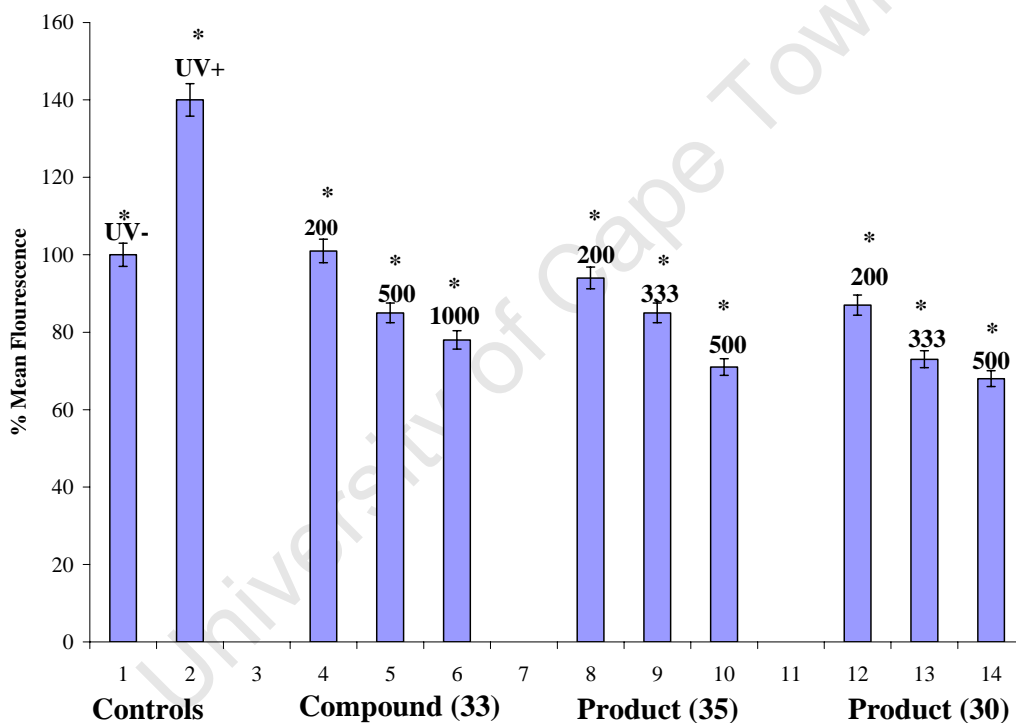
UVA irradiation of cultured human skin cells results in over-elevated levels of H<sub>2</sub>O<sub>2</sub> and other ROS, with H<sub>2</sub>O<sub>2</sub> being the predominant species (Vile and Tyrell, 1995; Petersen *et al.*, 2000). Dihydrorhodamine (DHR 123) can be used to detect H<sub>2</sub>O<sub>2</sub> produced following irradiating the cells with UVA; it has been shown to react with H<sub>2</sub>O<sub>2</sub> in the presence of peroxidase and is extensively used as a probe for the detection of intracellular H<sub>2</sub>O<sub>2</sub> (Katiyar *et al.*, 2001, Katiyar *et al.*, 2001 and Yang *et al.*, 2000). The mean fluorescence (MF), read on a FACS machine, thus indicates intracellular H<sub>2</sub>O<sub>2</sub> production. To elucidate the effect that our putative antioxidants (compound **33**, products **30**, **35**) would have on the excess production of ROS due to irradiation, H<sub>2</sub>O<sub>2</sub> production in keratinocytes was measured. The cells were first treated with the various concentrations of the putative antioxidants, irradiated with UVA and then exposed to the dye DHR 123 (Section 3.6).

The quantitative analysis of the data showed that the putative antioxidants (compound **33**, products **30**, **35**) inhibited UVA-induced H<sub>2</sub>O<sub>2</sub> production in a dose-dependent way (Figure 4.17). The results indicate that compound **33** and products **35** and **30**, at concentrations 200 µM, reduced ROS levels to normal physiological levels of 87-100 % mean fluorescent relative to the control of 100 % mean fluorescent. At a concentration of 500 µM, compound **33** resulted in the same relative ROS reduction effect as a concentration of 333 µM for product **35**, both with a % mean fluorescence of 85%. At a concentration of 333 µM, product **30** resulted in the lowest % mean fluorescence of 73%, when compared to compound **33** (500 µM) and **35** (333 µM) with % mean fluorescence of 85%. The greatest decrease in UVA-induced ROS was observed in the highest concentrations of compound **33** (hydroxytyrosol), **35** (hydroxytyrosol-laccase product), **30** (ferulic acid-laccase product) with a % mean fluorescence of 78, 71, 68% respectively. Thus, ROS reduction was observed in the following order product **30**> product **35**> compound **33** (Figure 4.17).

These results directly demonstrate that compound **33** and products **30** and **35** have potent radical scavenging activity which can prevent the over-production of intracellular H<sub>2</sub>O<sub>2</sub> through a free radical scavenging pathway. This study suggests that the protective effects of the putative antioxidants against UV-induced ROS may take place through an interference with the reactions initiated by ROS. This interference might either be by directly neutralizing these intermediates (ROS), preventing formation of superoxide and/or hydrogen peroxide, or by regenerating the antioxidant system of the cells, because UV irradiation is thought to deplete the antioxidants' involved in the defence of the cells (Podda *et al.*, 1998 and Poquet *et al.*, 2008). Polyphenolic

antioxidants are thought to have an additive or synergistic effect with endogenous antioxidants, resulting in a more protective effect against ROS damage (Rietjens *et al.*, 2006).

The results also indicate that there was an increase in the degree of ROS reduction correlating with an increase in the mass-to-charge of compounds **33** and products **30** and **35**. Figure 4.17 shows that ROS reduction greatest by product **30** (MW 589), followed by product **35** and compound **33** (respective MW 451 and 153). Thus it can be suggested that the effect of polymerization was an increase in antioxidant activity such that H<sub>2</sub>O<sub>2</sub> levels were brought to normal physiological levels. In summary, this study provides the first evidence that products **35** and **30** are potent protective agents for keratinocytes after UVA irradiation and further evidence of the protective effect of compound **33** (O'Dowd *et al.*, 2004).



**Figure 4.17.** Results of the ROS assay to determine the effect of the various concentrations of the putative antioxidants on UVA-induced ROS levels following UVA irradiation (MF: mean fluorescence) n=3 (\*P < 0.05)

## 4.9 Evaluating the effect of putative antioxidants on UVA-induced apoptosis in HaCaTs using western Blot analysis

Apoptosis refers to a process of programmed cell death, which is considered a vital part of various processes including normal cell turnover, proper development, functioning of the immune system, chemical induced cell death and UVR over-exposure. Necrosis is the alternative to apoptosis, and is considered a toxic process where the cell is a passive victim and follows an energy-independent mode of death (Elmore, 2007). This study focuses on apoptosis as a mode of cell death induced by UVA over-exposure.

Apoptosis can take place through the extrinsic pathway or the intrinsic pathway. The extrinsic pathway involves transmembrane receptor-mediated interactions which involve members of the tumor necrosis factor (TNF). The intrinsic pathway is a mitochondrial-initiated event and thus is of interest for this study. It involves pro-apoptotic proteins involved in a caspase-independent mechanism of apoptosis called AIF (apoptosis inducing factor), which are released during apoptosis when the cell is dying. This intrinsic pathway is regulated by members of the Bcl-2 family of proteins (Elmore, 2007). This family of proteins is responsible for regulating the permeability of the mitochondrial membrane (release of AIF). These proteins can either be pro-apoptotic or anti-apoptotic; Bcl-2 is anti-apoptotic while Bax is pro-apoptotic (Zhuang *et al.*, 2000). The cells' sensitivity to apoptotic stimuli depends on the balance of pro-apoptotic (Bax) and anti-apoptotic (Bcl-2) proteins. We hypothesise that a protective effect by putative antioxidants may be indicated by a possible up-regulation of Bcl-2 and down-regulation of Bax (Gautam *et al.*, 2004) ([www.sgul.ac.uk/dept/immunology/~dash](http://www.sgul.ac.uk/dept/immunology/~dash)). The ratio of these proteins seems to suggest the "apoptotic status" of the cell. An excess of Bax results in the cells being more susceptible to apoptosis, and an excess of Bcl-2 renders the cells more resistant to apoptosis. To determine the nature of the anti-apoptotic effect mediated by the putative antioxidants (compound **33** and products **35**, **30**) due to UVA irradiation, the Bax/ Bcl-2 ratio was studied using western blot analysis.

To evaluate the expression of Bcl-2, Bax and AIF, the cells were cultured (Section 3.5.3) and treated with varying concentrations between 200 and 1000  $\mu\text{M}$  of the putative antioxidants compound **33** and products **35**, **30** for 18hrs, and then irradiated at  $22.3 \text{ J/cm}^2$ . Protein extraction was conducted (Section 3.5.4) and proteins were quantified using the BCA method (Section 3.5.5). Sham controls were included throughout and none were different from non-UV irradiated controls. Sham controls are put in the UV chamber, but covered with foil to ensure that they are not irradiated. The purpose of this control is to ensure that it is only the UVA rays that result in

the observed effects, and not the heat in the chamber. Alpha tubulin was used as a loading control in each experiment to ensure that the results were quantified correctly. Since the western blot results are quantified by band size, it is important to ensure that each sample had the same amount of protein when loaded and the loading control helps to determine this. The band obtained when the proteins are probed with the loading control represent the amount of protein in that loaded sample.

The results of western blot analysis showed that the putative antioxidants (compound **33**, products **30**, **35**) considerably reduced the expression of Bax (Figure 4.18, 4.21, 4.24), AIF (Figure 4.20, 4.23, 4.26) and induce the over-expression of Bcl-2 (Figure 4.19, 4.22, 4.25) in a concentration dependent manner. Please note that UV+ and UV- controls do not always occur in the same order, they were sometimes loaded differently.

#### **4.9.1. Evaluating the effect of compound 33, product 30 and 35 on UVA-induced apoptosis in HaCaTs using western Blot analysis**

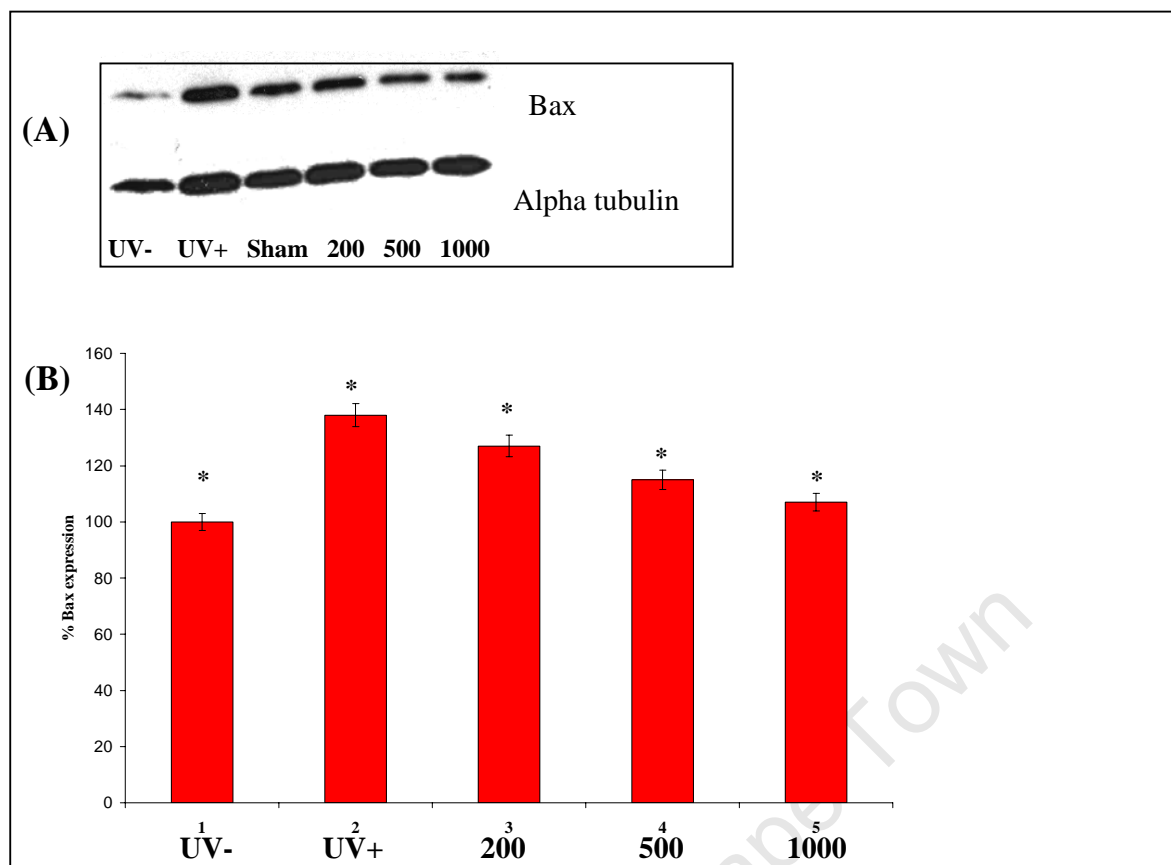
Compound **33**, at a concentration of 1000  $\mu\text{M}$ , resulted in the greatest decrease in Bax expression, by bringing Bax levels to the same expression levels as the control (Figure 4.18). Product **35** at a concentration of 200  $\mu\text{M}$  reduced Bax levels by 75%, while the other two concentrations tested (333 and 500  $\mu\text{M}$ ) reduced Bax levels by 87.5% relative the control (Figure 4.21). Product **30** reduced Bax levels, with the most notable decrease, at a concentration of 333  $\mu\text{M}$ , where Bax levels were brought to normal levels of expression in relation to the control, and concentration 500  $\mu\text{M}$  where Bax levels were reduced by 10% in relation to the control (Figure 4.24).

Compound **33**, at a concentration of 200  $\mu\text{M}$ , brought AIF levels of expression to normal levels in relation to the control. At the highest concentration (1000  $\mu\text{M}$ ), compound **33** reduced AIF levels by 15% relative to the control (Figure 4.20). Product **35** reduced AIF levels in a concentration dependent manner, to 28% for the 200  $\mu\text{M}$ , and 32% for concentrations 333 and 500  $\mu\text{M}$ , relative to the control (Figure 4.23). Product **30** at concentrations 200 and 333  $\mu\text{M}$  brought AIF levels to normal levels of expression relative to the control. At the highest concentration, 500  $\mu\text{M}$ , AIF levels were reduced by 37.5% relative to the control (Figure 4.26).

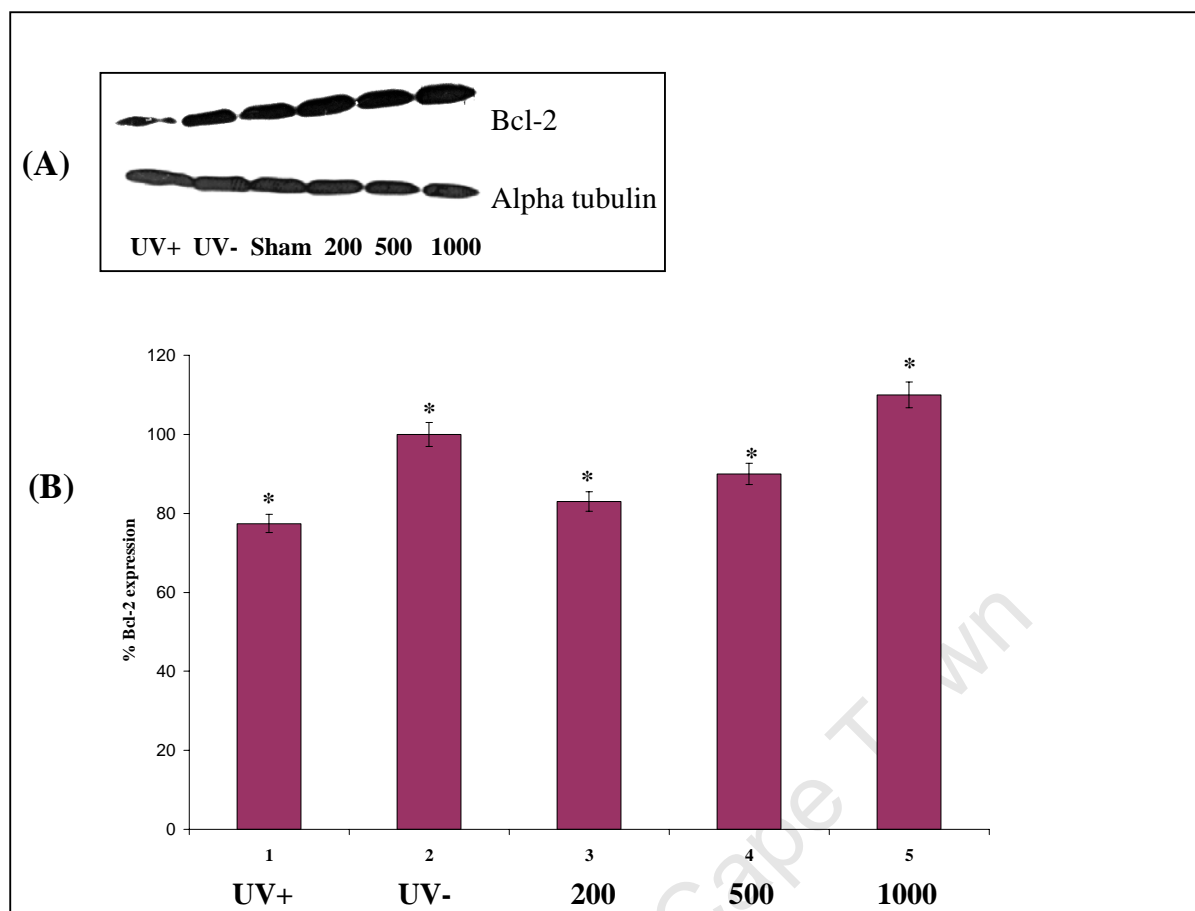
The presence of compound **33** caused an increase in Bcl-2 expression in a concentration-dependent manner. Concentrations 200 and 500  $\mu\text{M}$  brought Bcl-2 expression to normal levels of expression relative to the control. The highest concentration, 1000  $\mu\text{M}$ , lead to an increase in Bcl-2 levels of expression of 7% relative to the control (Figure 4.19). Product **35** increased Bcl-2

levels in a concentration-dependent manner; the highest concentration (500  $\mu\text{M}$ ) increased Bcl-2 levels by 16% relative to the control (Figure 4.22). Product **30** also lead to increased Bcl-2 expression in a concentration-dependent manner. At concentration 333 and 500  $\mu\text{M}$ , the Bcl-2 expression was increased by 12% and 22% respectively (Figure 4.25).

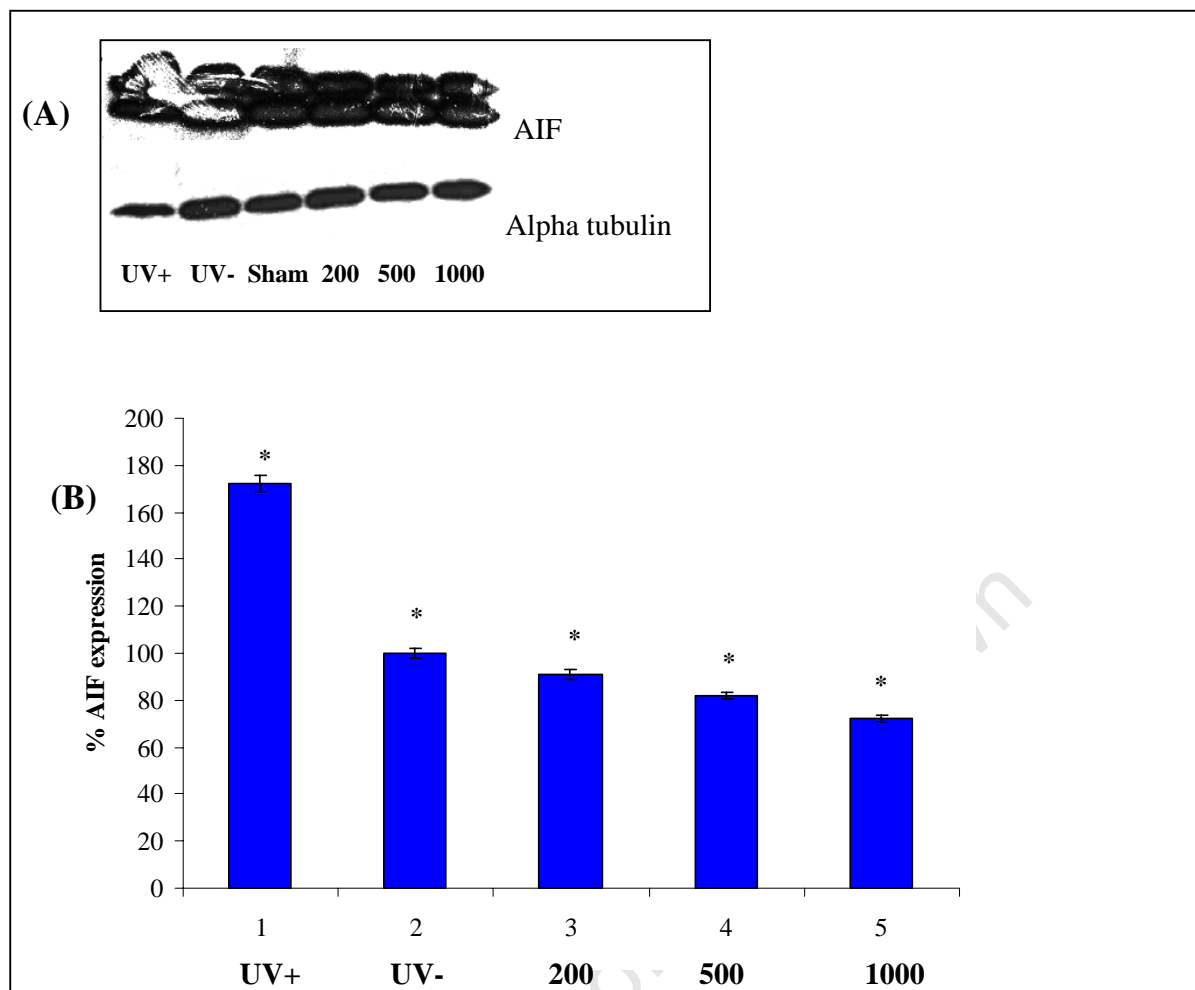
It is suggested that the putative antioxidants passively diffused into the cells, thereby provoking anti-apoptotic intracellular signals (Kondo *et al.*, 2004). The fact that the putative antioxidants affected the proteins of the Bcl-2 gene family in pre-treated, UVA irradiated cells suggests that these compounds inhibit apoptosis due to irradiation by inhibiting the intrinsic pathway of apoptosis (Hisayoshi *et al.*, 2004 and D'Errico *et al.*, 2006). The results suggest a possible mechanism in which Bcl-2 over-expression, due to the putative antioxidants, stabilize the mitochondrial functions and block the release of cytochrome c by increasing the antioxidant capacity of the cells (Assefa *et al.*, 2003). Recent evidence suggests that Bcl-2 can also guard other organelles, by fortifying the cellular antioxidant defence (Assefa *et al.*, 2003). This was clearly the case in the UVA-induced antioxidant treated cells, where Bcl-2 over-expression due to pre-treatment with putative antioxidants prevented mitochondrial membrane depolarization as the putative antioxidants were able to reduce Bax levels of expression (decreased mitochondrial membrane permeability) AIF and ROS formation.



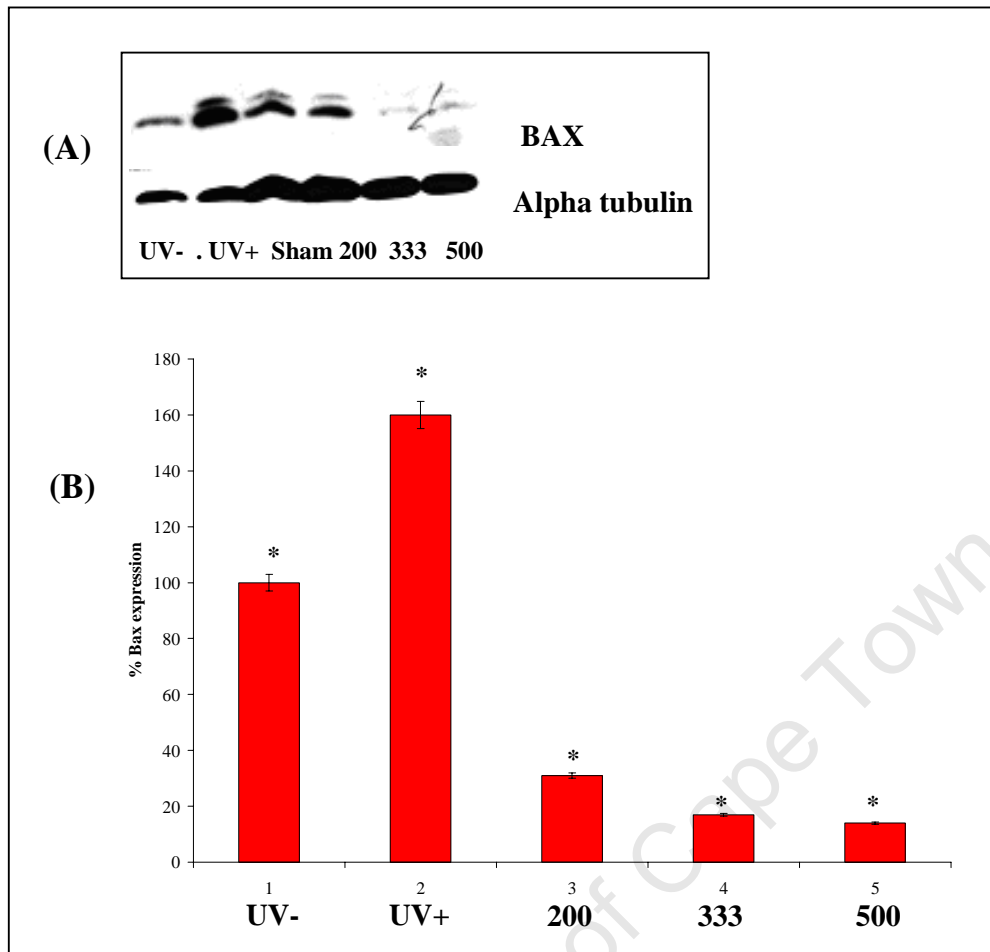
**Figure 4.18.** Results of the western blot to determine the effect of compound **33** on Bax expression using HaCaT cells following UVA exposure. (A) Shows the western Blot gel and (B) is the graph showing Bax expression in the presence of varying concentrations of the putative antioxidants. Cells were cultured and treated with varying concentrations (200-1000)  $\mu\text{M}$  of the putative antioxidant, compound **33** (hydroxytyrosol) for 18hrs and then irradiated at  $22,3\text{J}/\text{cm}^2$ . Protein extraction was conducted and proteins were quantified using the BCA method  $n=2$  (\* $P < 0.05$ )



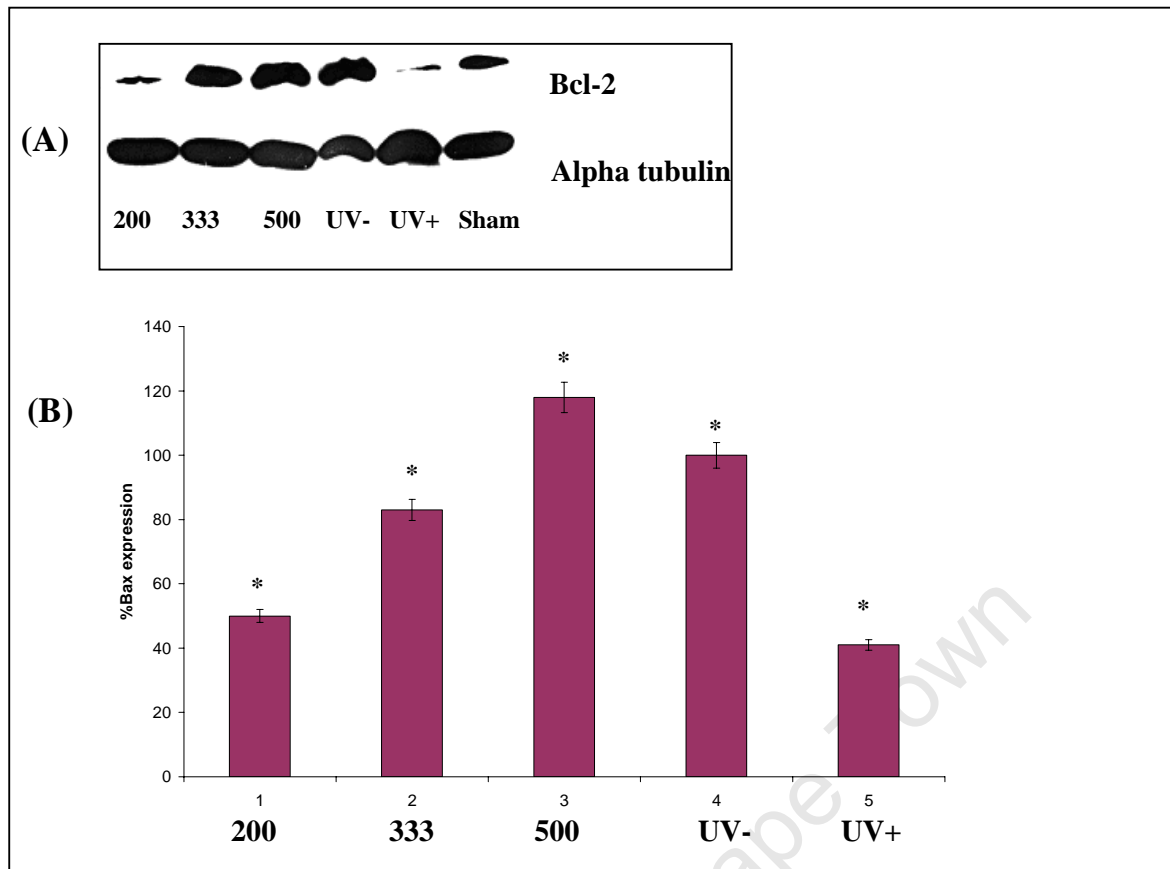
**Figure 4.19.** Results of the western blot to determine the effect of compound **33** on Bcl-2 expression using HaCaT cells following UVA exposure. (A) Shows the western Blot gel and (B) is the graph showing Bcl-2 expression in the presence of varying concentrations of the putative antioxidants. Cells were cultured and treated with varying concentrations (200-1000)  $\mu\text{M}$  of the putative antioxidant, compound **33** (hydroxytyrosol) for 18hrs and then irradiated at  $22,3\text{J}/\text{cm}^2$ . Protein extraction was conducted and proteins were quantified using the BCA method  $n=2$  (\* $P < 0.05$ )



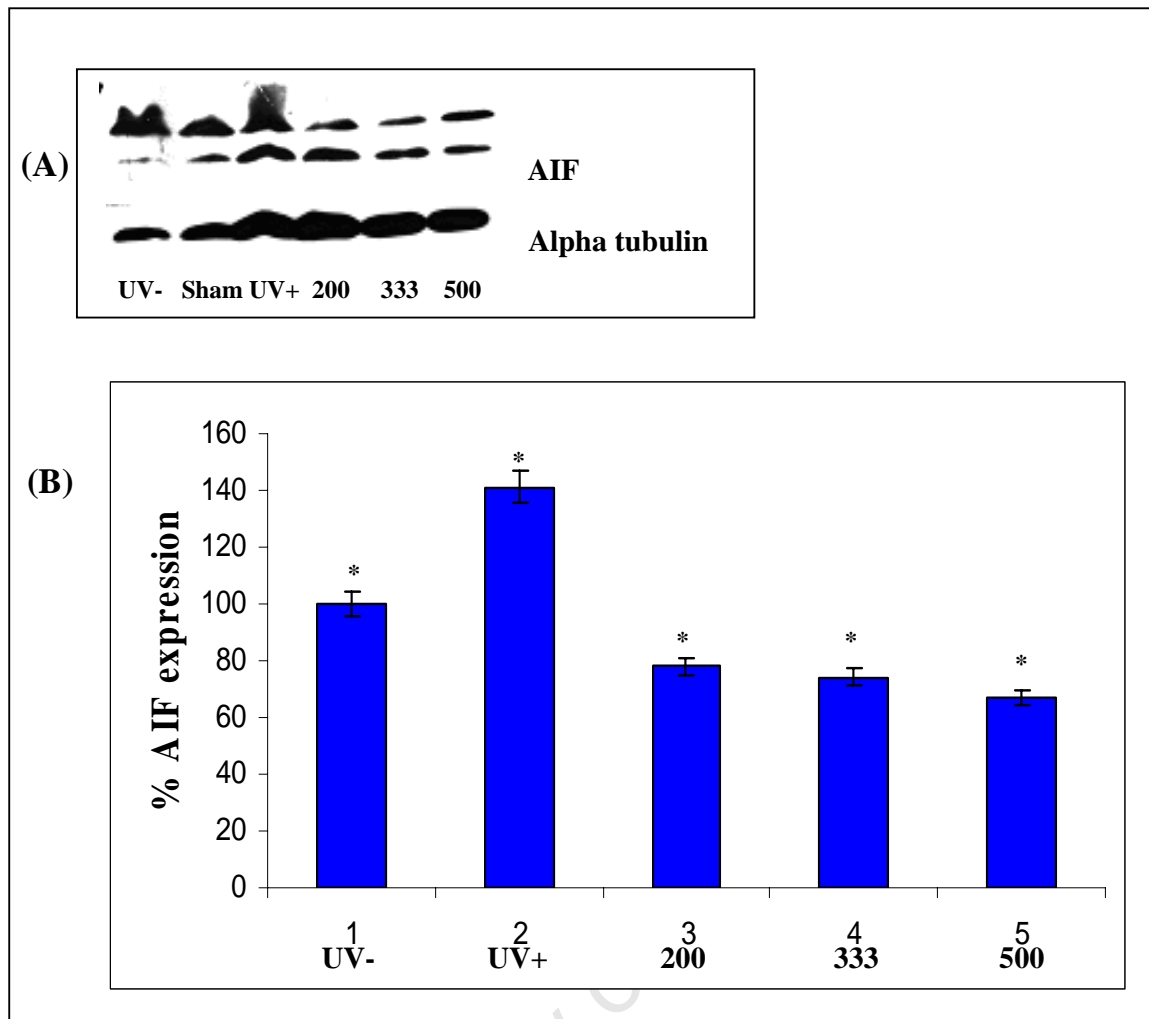
**Figure 4.20.** Results of the western blot to determine the effect of compound **33** on AIF expression using HaCaT cells following UVA exposure. (A) Shows the western Blot gel and (B) is the graph showing AIF expression in the presence of varying concentrations of the putative antioxidants. Cells were cultured and treated with varying concentrations (200-1000)  $\mu\text{M}$  of the putative antioxidant, compound **33** (hydroxytyrosol) for 18hrs and then irradiated at  $22,3\text{J}/\text{cm}^2$ . Protein extraction was conducted and proteins were quantified using the BCA method  $n=2$ . The bands increased at the same ratio, both bands were used for quantification (\* $P < 0.05$ )



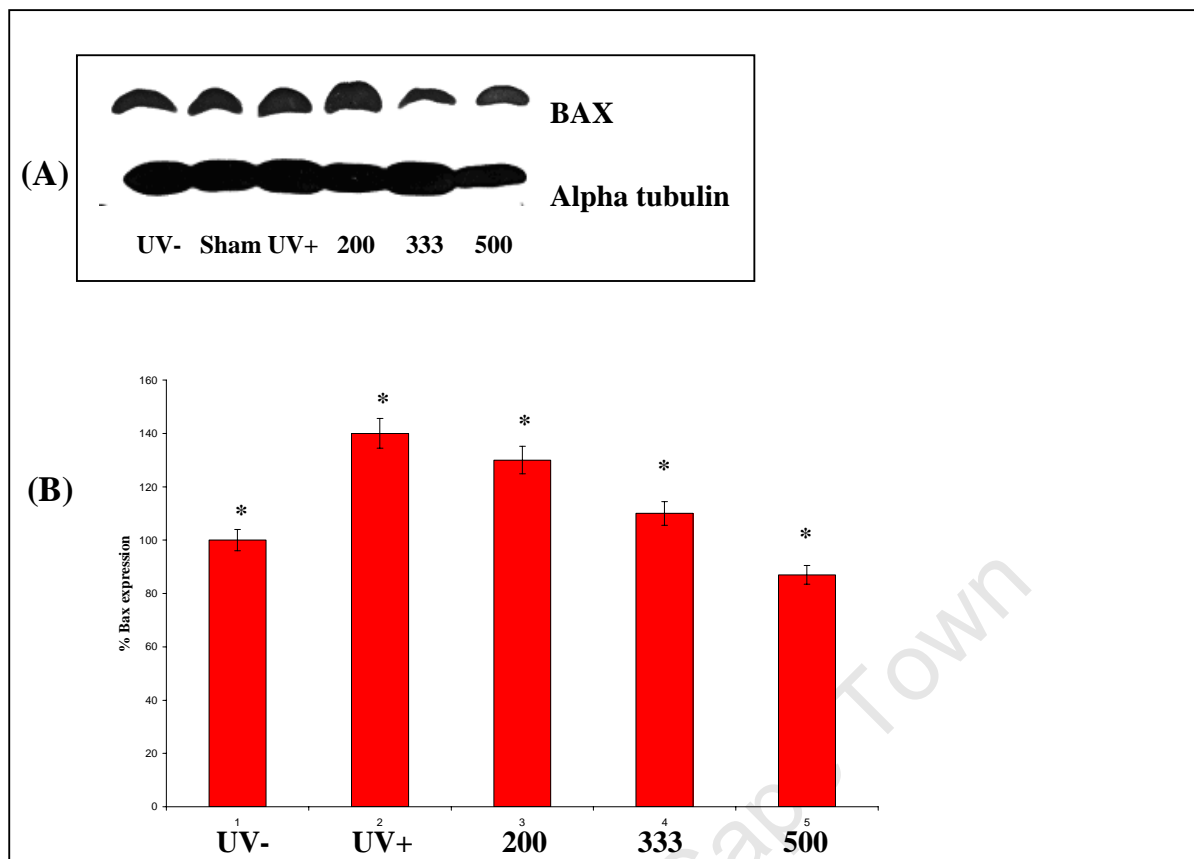
**Figure 4.21.** Results of the western blot to determine the effect of product **35** on Bax expression using HaCaT cells following UVA exposure. (A) Shows the western Blot gel and (B) is the graph showing Bax expression in the presence of varying concentrations of the putative antioxidants. Cells were cultured and treated with varying concentrations (200-500)  $\mu$ M of the putative antioxidant, product **35** (hydroxytyrosol laccase reaction) for 18hrs and then irradiated at  $22,3\text{J}/\text{cm}^2$ . Protein extraction was conducted and proteins were quantified using the BCA method  $n=2$  (\* $P < 0.05$ )



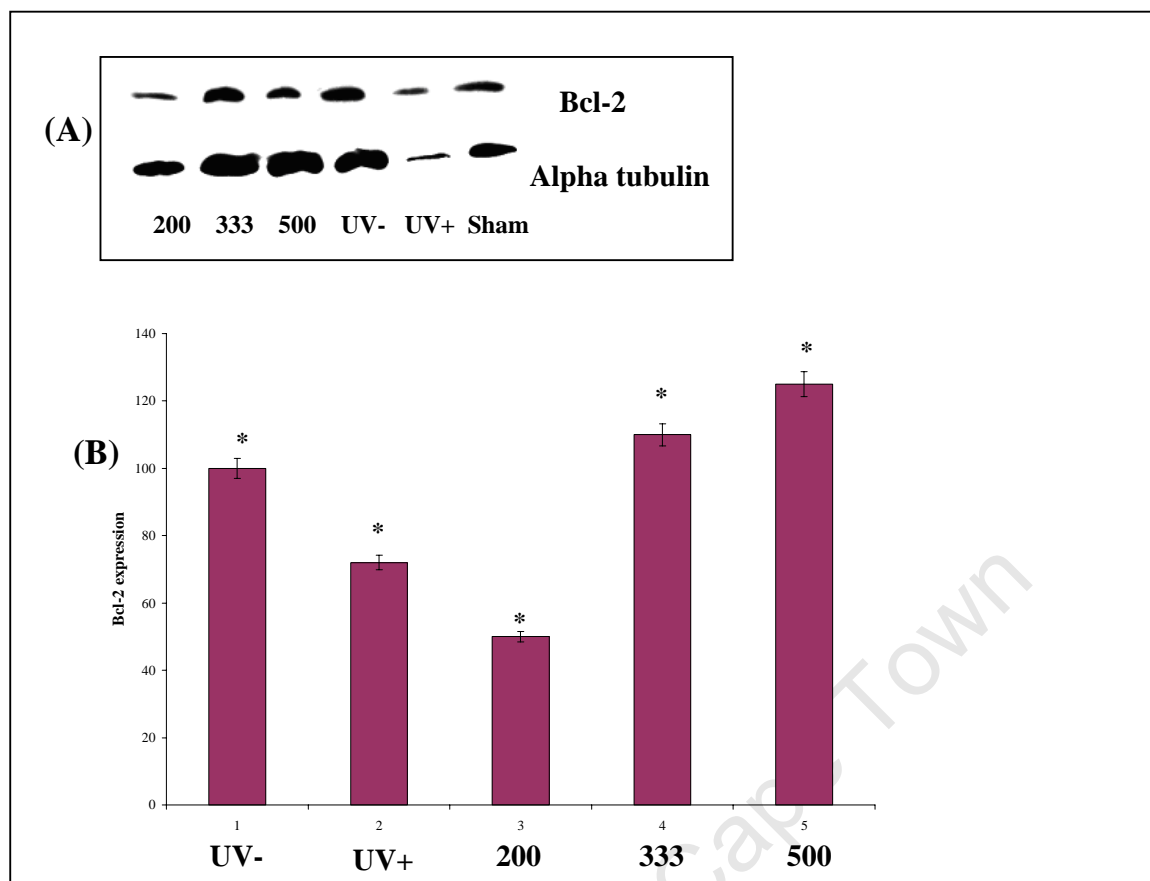
**Figure 4.22.** Results of the western blot to determine the effect of product **35** on Bcl-2 expression using HaCaT cells following UVA exposure. (A) Shows the western Blot gel and (B) is the graph showing Bcl-2 expression in the presence of varying concentrations of the putative antioxidants. Cells were cultured and treated with varying concentrations (200-500)  $\mu$ M of the putative antioxidant, product **35** (hydroxytyrosol laccase reaction) for 18hrs and then irradiated at  $22,3\text{J}/\text{cm}^2$ . Protein extraction was conducted and proteins were quantified using the BCA method  $n=2$  (\* $P < 0.05$ )



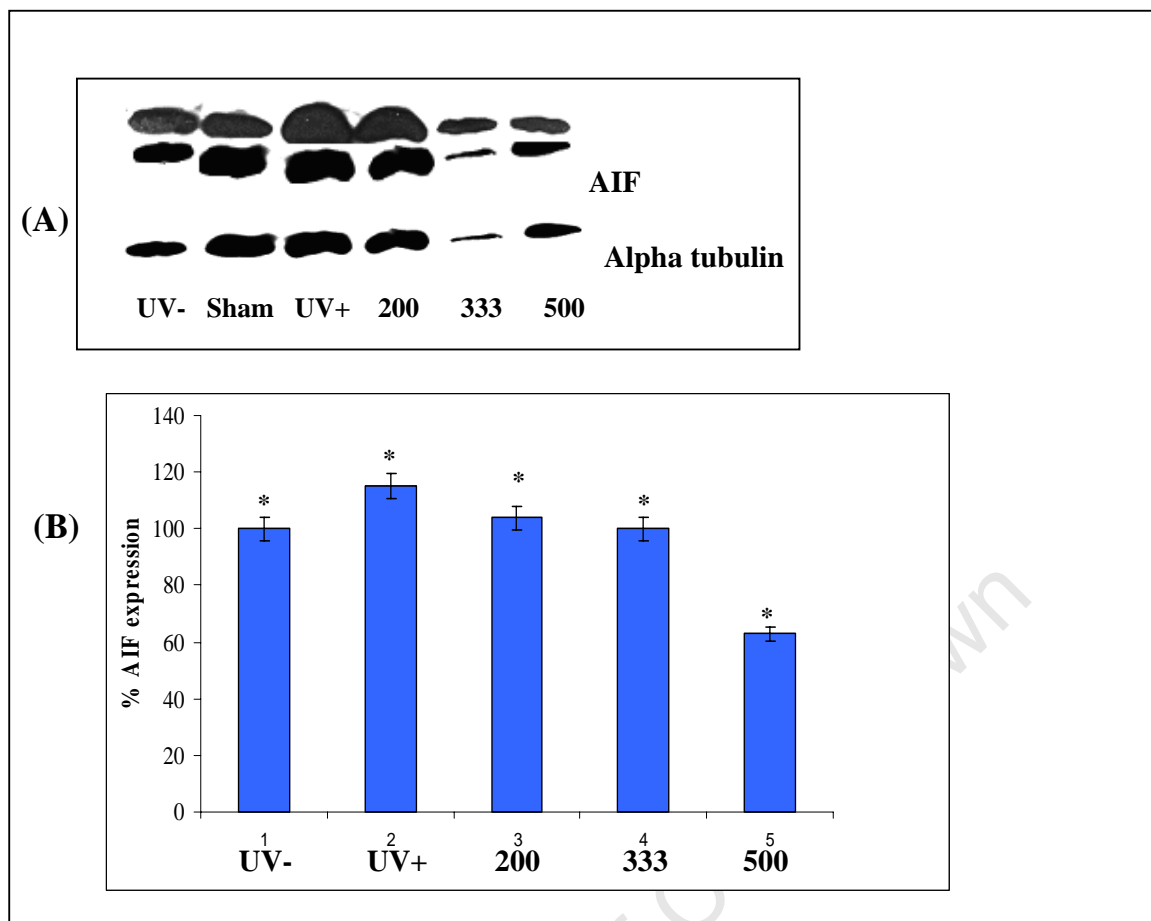
**Figure 4.23.** Results of the western blot to determine the effect of product **35** on AIF expression using HaCaT cells following UVA exposure. (A) Shows the western Blot gel and (B) is the graph showing AIF expression in the presence of varying concentrations of the putative antioxidants. Cells were cultured and treated with varying concentrations (200-500)  $\mu$ M of the putative antioxidant, product **35** (hydroxytyrosol laccase reaction) for 18hrs and then irradiated at  $22,3\text{J}/\text{cm}^2$ . Protein extraction was conducted and proteins were quantified using the BCA method  $n=2$ . The bands increased at the same ratio, both bands were used for quantification (\* $P < 0.05$ )



**Figure 4.24.** Results of the western blot to determine the effect of product **30** on Bax expression using HaCaT cells following UVA exposure. (A) Shows the western Blot gel and (B) is the graph showing Bax expression in the presence of varying concentrations of the putative antioxidants. Cells were cultured and treated with varying concentrations (200-500)  $\mu$ M of the putative antioxidant, product **30** (ferulic acid laccase reaction) for 18hrs and then irradiated at 22,3J/cm<sup>2</sup>. Protein extraction was conducted and proteins were quantified using the BCA method n=2 (\*P < 0.05)



**Figure 4.25.** Results of the western blot to determine the effect of product **30** on Bcl-2 expression using HaCaT cells following UVA exposure. (A) Shows the western Blot gel and (B) is the graph showing Bcl-2 expression in the presence of varying concentrations of the putative antioxidants. Cells were cultured and treated with varying concentrations (200-500)  $\mu\text{M}$  of the putative antioxidant, product **30** (ferulic acid laccase reaction) for 18hrs and then irradiated at  $22,3\text{J}/\text{cm}^2$ . Protein extraction was conducted and proteins were quantified using the BCA method  $n=2$  (\* $P < 0.05$ )



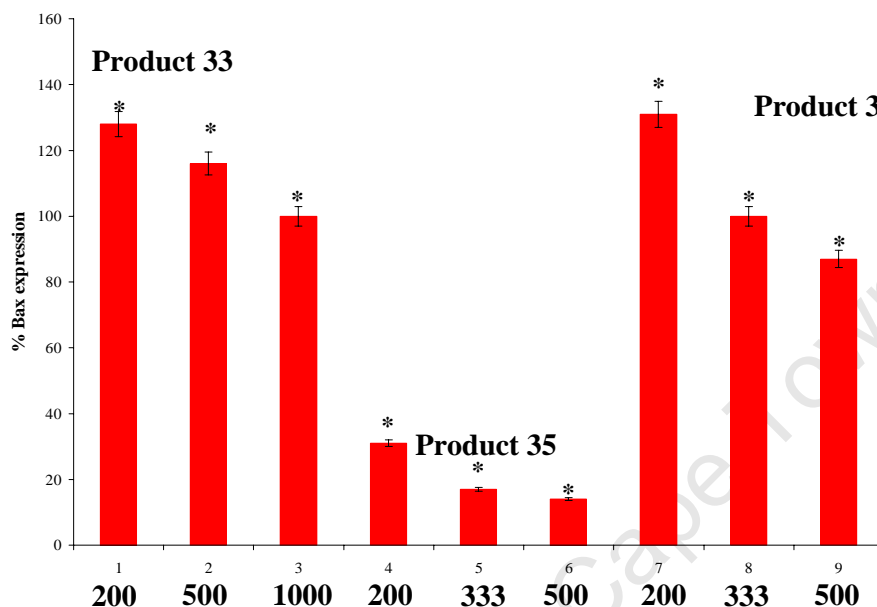
**Figure 4.26.** Results of the western blot to determine the effect of product **30** on AIF expression using HaCaT cells following UVA exposure. (A) Shows the western Blot gel and (B) is the graph showing AIF expression in the presence of varying concentrations of the putative antioxidants. Cells were cultured and treated with varying concentrations (200-500)  $\mu$ M of the putative antioxidant, product **30** (ferulic acid laccase reaction) for 18hrs and then irradiated at  $22,3\text{J}/\text{cm}^2$ . Protein extraction was conducted and proteins were quantified using the BCA method  $n=2$ . The bands increased at the same ratio, both bands were used for quantification (\* $P < 0.05$ )

#### 4.9.2. Comparison of different concentrations of compound **30**, product **33** and **35** on protein expression

The results shown in Figures 4.27, 4.28 and 4.29 indicate that the highest concentrations of each of the putative antioxidants (compound **33**, products **30**, **35**) was most effective in reducing Bax and AIF levels and inducing the over expression of Bcl-2.

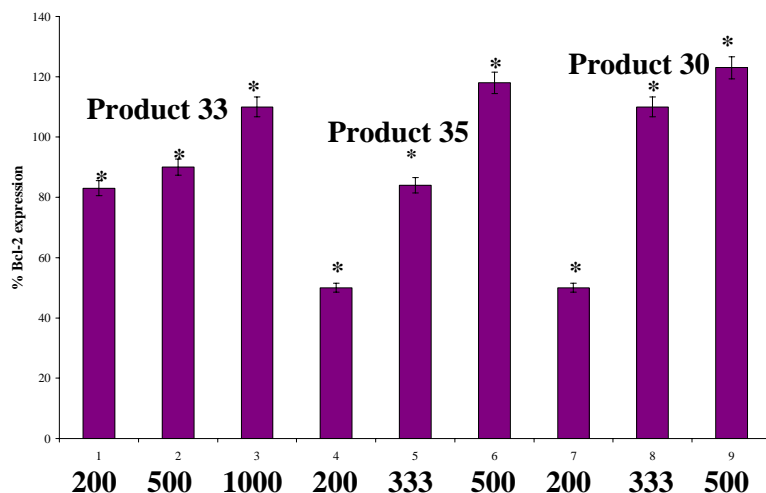
The highest concentrations of the putative antioxidant compound **33** and product **30** caused the greatest reduction in Bax levels of expression relative to the control (100%), by bringing

expression levels to 100% and 90% respectively, which are normal physiological levels of expression relative to the control in this experiment. Product **35** showed the greatest reduction in Bax levels, at its lowest concentration (200  $\mu\text{M}$ ), Bax levels were 70% lower than that of the control (100%). The lowest concentration of product **35** decreased Bax levels by 85% relative to the control (100%). Thus, it can be concluded that product **35** show greater putative antioxidant activity than compound **33**, or product **30**.



**Figure 4.27.** Results of the western blot to determine the effect of the various concentrations of the putative antioxidants (compound **33**, products **30**, **35**) on Bax expression using HaCaT cells following UVA exposure. Cells were cultured and treated with varying concentrations (200-1000)  $\mu\text{M}$  of the putative antioxidants compound **33** (hydroxytyrosol), product **35** (hydroxytyrosol laccase reaction), product **30** (ferulic acid laccase reaction) for 18hrs and then irradiated at  $22,3\text{J}/\text{cm}^2$ . Protein extraction was conducted and proteins were quantified using the BCA method (\* $P < 0.05$ )

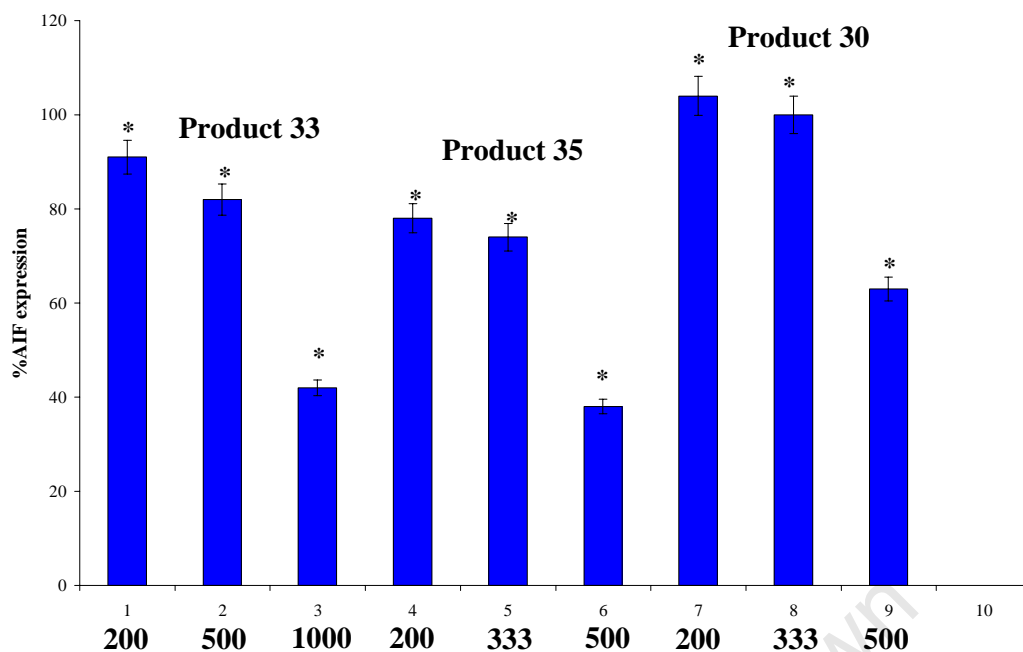
For compound **33**, the concentration that had the greatest effect on reducing Bax levels was 1000  $\mu\text{M}$ , whereas for product **30** and **35** it was 500  $\mu\text{M}$  (Figure 4.27, 4.29 and Table 4.6). Similarly; for compound **33**, the concentration that had the greatest effect on inducing Bcl-2 levels was 1000  $\mu\text{M}$ , for product **30** and **35** it was 500  $\mu\text{M}$  (Figure 4.28 and Table 4.6).



**Figure 4.28.** Results of the western blot to determine the effect of the various concentrations of the putative antioxidants (compound **33**, products **30**, **35**) on Bcl-2 expression using HaCaT cells following UVA exposure. Cells were cultured and treated with varying concentrations (200-1000)  $\mu\text{M}$  of the putative antioxidants compound **33** (hydroxytyrosol), product **35** (hydroxytyrosol laccase reaction), product **30** (ferulic acid laccase reaction) for 18hrs and then irradiated at  $22,3\text{J}/\text{cm}^2$ . Protein extraction was conducted and proteins were quantified using the BCA method (\* $P < 0.05$ )

**Table 4.6** Summary of effect of the various concentrations of the putative antioxidants (compound **33**, products **35**, **30**) on Bcl-2, Bax and AIF expression using HaCaT cells following UVA exposure

Effect	Concentrations ( $\mu\text{M}$ )								
	Product <b>30</b> (Ferulic acid laccase)			Compound <b>33</b> (Hydroxytyrosol)			Product <b>35</b> (Hydroxytyrosol laccase)		
	200	333	500	200	333	1000	200	333	1000
% Bax Expression	131	100	87	128	116	100	31	17	14
% Bcl-2 Expression	50	110	123	83	90	110	50	84	118
% AIF Expression	104	100	63	91	82	42	78	74	38



**Figure 4.29.** Results of the western blot to determine the effect of the various concentrations of the putative antioxidants (compound **33**, products **30**, **35**) on AIF expression using HaCaT cells following UVA exposure. Cells were cultured and treated with varying concentrations (200-1000)  $\mu\text{M}$  of the putative antioxidants compound **33** (hydroxytyrosol), product **35** (hydroxytyrosol laccase reaction), product **30** (ferulic acid laccase reaction) for 18hrs and then irradiated at  $22,3\text{J}/\text{cm}^2$ . Protein extraction was conducted and proteins were quantified using the BCA method (\* $P < 0.05$ )

#### 4.9.3. Overall assessment of antioxidant activities of compound **30**, product **33** and **35**

Product **35** showed stronger antioxidant activity than compound **33** and product **30**, as it was observed to induce greater levels of over-expression of Bcl-2, and decreased the over-expression of AIF and Bax. The antioxidant activity of product **35** was greater than that of its precursor hydroxytyrosol (compound **33**), suggesting that the polymerization of product **33** resulted in a product **35**, with increased antioxidant activity and a more cytoprotective effect. This is in keeping with a study that was conducted *in vitro* which indicated that the polymerisation of monophenols can result in polymer products that have increased antioxidant activity (Desantis-Mendoza *et al.*, 2006). However, product **30**, with a mass-to-charge of 589 m/z, and thus the largest of the putative antioxidants, did not show the highest antioxidant activity compared to product **35** (molecular weight of 451 m/z).

Antioxidants are extensively metabolized *in vivo*, resulting in metabolized forms of the parent antioxidant. The eventual antioxidant effect of these metabolites could result in increased antioxidant activity or decreased antioxidant activity as compared with the original antioxidant. The *in vitro* antioxidant assays, DPPH (Section 4.6.1), FRAP (Section 4.6.2), LDL (Section 4.6.3) all showed product **35** (hydroxytyrosol laccase reaction) and product **30** (hydroxytyrosol laccase reaction) to have the highest antioxidant activity, followed by their substrates compound **33** and compound **31** respectively. The same trend was observed in the UVA study with HaCaT cells where both polymeric products (products **35** and **30**) were found to have better antioxidant activity than the monomer compound **33** (hydroxytyrosol). This suggests that the metabolism did not have an effect on the antioxidant activity of the putative antioxidants by altering the chemistry of the parent compound, and further, it suggests that the metabolites formed worked synergistically with the parent compound to be able to reduce cytotoxicity of UV radiation (Poquet *et al.*, 2008).

# Chapter 5

## General Conclusion

The broad aim of this study was to generate new knowledge about the antioxidant capacity of selected synthesised compounds, which were synthesized using laccase and tyrosinase as biocatalysts. The target substrates for biocatalysis included tyrosol, hydroxytyrosol and ferulic acid. All of these compounds have known antioxidant activity. The synthesized products were expected to have added antioxidant activity to that of the starting materials. The aim was also to develop methodologies to measure their antioxidant effect in living cells, thus allowing an assessment of the protective role of the compounds as antioxidants, at a metabolic level.

These objectives as set out in Section 2.1.11.2 were accomplished, and the major findings of this work can be summarised as follows:

- i) The enzymatic process to synthesize product **30** using laccase obtained from *T. pubescens* was achieved. From this study we established that the resulting polymer which consisted of 7 repeats of 68 mass units which could not be fully identified. The polymeric product, with average mass-to-charge 589 m/z, was nevertheless tested for antioxidant activity. This polymeric product was found to have slow kinetic behaviour (76 minutes), but greater antioxidant activity than the parent compound ferulic acid.
- ii) Hydroxytyrosol was oxidised with laccase obtained from *T. pubescens*. Hydroxytyrosol is currently not available commercially, and thus it was necessary to produce this compound, through methods based on that of Espin *et al.* (2001). The hydroxytyrosol was oxidised by laccase in 50% methanol, as a study by Ncanana (2007) found that this system resulted in one major polymeric product. Using antioxidant assays to establish the antioxidant activity of this polymer showed that this polymer had significantly stronger antioxidant activity than its parent compound.
- iii) To test the antioxidant activity of these synthesized polymers further, *ex vivo* studies were carried out using the ROS assay. The initial step to this study involved working out a concentration range for the respective putative antioxidants which did not have adverse effects on the cells, and then exposing the cells to UVA in the presence and absence of these concentration ranges. In the study, it was found that each of the respective compounds showed good antioxidant activity. ROS production was decreased in a dose-dependent way for each of the putative antioxidants in the following order, product **30**> product **35**> compound **33**.

- iv) Protein expression studies were conducted using western blot analysis, to evaluate the effect that the putative antioxidant had on the expression of proteins, involved in the apoptotic process. Initially, the cells were exposed to UVA in the presence and absence of the varying concentrations of the putative antioxidants. In this study, the putative antioxidants were all able to decrease the over-expression of Bax and AIF, and increase the over-expression of Bcl-2 in a concentration-dependent manner. The *in vitro* antioxidant assays showed that the polymeric products had better antioxidant activity than the parent compound. The same trend was observed in *ex vivo* studies using HaCaTs. Since the putative antioxidants would have been metabolized *ex vivo*, which could affect antioxidant activity of the resulting compound, this suggests that the metabolism did not have an affect on the antioxidant activity of the putative antioxidant. This study supported the proposal that polymerisation of monophenols results in polyphenolic products with enhanced antioxidant activity.
- v) Biotransformation of hydroxytyrosol and ferulic acid by laccase lead to polymeric products with added antioxidant activity compared to the parent compound *in vitro* and *ex vivo*. The implication of this study can be considered for use in health, nutraceutical or cosmetic markets.

Compound **33**, products **30** and **35** significantly protected HaCaT cells against the cytotoxic effects induced by UVA. This suggests that the protection of compound **33**, products **30** and **35** towards the damaging effects of UVA, which can lead to apoptosis, may happen through an interference with the reaction initiated by ROS such as H<sub>2</sub>O<sub>2</sub>. This could happen either by directly neutralizing these intermediates or by regenerating the antioxidant system of the cells. Thus, it can be concluded that compound **33**, products **30** and **35** are potential candidates for the protection of cells against the damaging effects of UVA irradiation. The results suggest that it is possible that the putative antioxidants might display the same effects *in vivo*, provided they reach the skin. However, it must be noted that *in vivo*, the quantity of compound **33**, products **30** and **35** which will reach the skin after ingestion will be much lower than used *in vitro*. This study provides the first evidence that compound **33**, products **30** and **35** are potent protective agent for keratinocytes after exposure to UVA. This has implications that compound **33**, products **30** and **35** can be used commercially in skin care products, in health and nutraceuticals.

Future studies are required with regards to metabolism of compound **33**, products **30** and **35** and the products formed. This could shed some light the type of synergistic interactions between the parent and metabolized compound. Further investigation is also required in the polymerization mechanism of product **30**. Since apoptosis due to ROS damage starts with DNA damage, future studies could involve exploring whether these putative compounds are indeed able to prevent the

initial DNA damage. The skin is a complex organ comprising of melanocytes and fibroblasts, thus a study involving these cells and the putative antioxidants may be interesting in addressing whether these putative antioxidants have the same protective effect. It would also be interesting to explore the point of pre vs post exposure of cells to these putative compounds to elucidate which better protects the cells against UVR.

## Appendix

### Appendix A

#### Gel preparation

	<b>12% Running Gel</b>	<b>5% Stacking Gel</b>
dH <sub>2</sub> O	2.45 ml	2.44 ml
Acrylamide: bis 30%	3 ml	530 ml
1.5 M Tris pH 8.8	1.9 ml	
0.5 M Tris pH 6.8		1 ml
10% SDS	75 µl	40 µl
10% Ammonium Persulfate (AMPs)	75 µl	50 µl
Temed	7.5 µl	10µl

The 10% AMPs solution was made up fresh before every blot. The apparatus was assembled ready to make up the gel for running. Of the 12% without AMPs and Temed, 1 ml was taken and 10µl of the AMPs and Temed were added. This solution was added first to serve as a plug. This was allowed to polymerize, and the rest of the 12% was added three quarters full and allowed to polymerise (for about 30min). Following that, the stacking gel was added and the combs inserted. This was also allowed to polymerize. The assembled apparatus with the gels were placed in the tank and the running buffer added. The combs were then removed.

#### Sample preparation for loading the gel

The volume of the loading sample is obtained by using the concentration of the sample obtained from the BCA (bicinchoninic acid) method and the total volume to load in the gel (20µl/protein concentration = volume of stock to be used). The volume of the stock obtained was made up to 16µl with incomplete extraction buffer and that dilution was made to 20µl by adding 6X Protein dye. The protein dilutions were then heated for 2 minutes for 100<sup>0</sup>C. The protein samples were then loaded into the respective wells (20µl), and the protein marker used (peqGOLD, 5µl) was added to the first well.

## Running the gel

Following filling the tank with running buffer, the gel was run at 100V up till the last protein markers band could be observed and it had moved to the bottom of the gel. The apparatus was then disassembled and the gel removed and washed in TBS-T (Tris-Buffered Saline Tween-20) for 10 min to equilibrate the gel.

## Protein transfer

Nitrocellulose membrane (Hybond-C) was cut out to the respective size of the gel and put in TBS-T to equilibrate. The sponge and the filter paper used during transfer were also equilibrated in transfer buffer. The separated proteins were transferred (Towbin *et al.*, 1979) from gel to nitrocellulose paper (Hybond-C) at 100V with transfer buffer.

This order of stacking was followed:

Black cassette

Sponge

Filter paper

Gel

Membrane

Filter paper

Sponge

Red cassette

The cassette was placed in transfer apparatus with the black side to the negative. The tank was filled with transfer buffer, and ice packs were placed in the tank to keep the temperature of the buffer low. The transfer was run at 100V for 1 hour, while stirring. Following transfer, the membrane was washed twice in TBS-T for 10 minutes.

## Detection

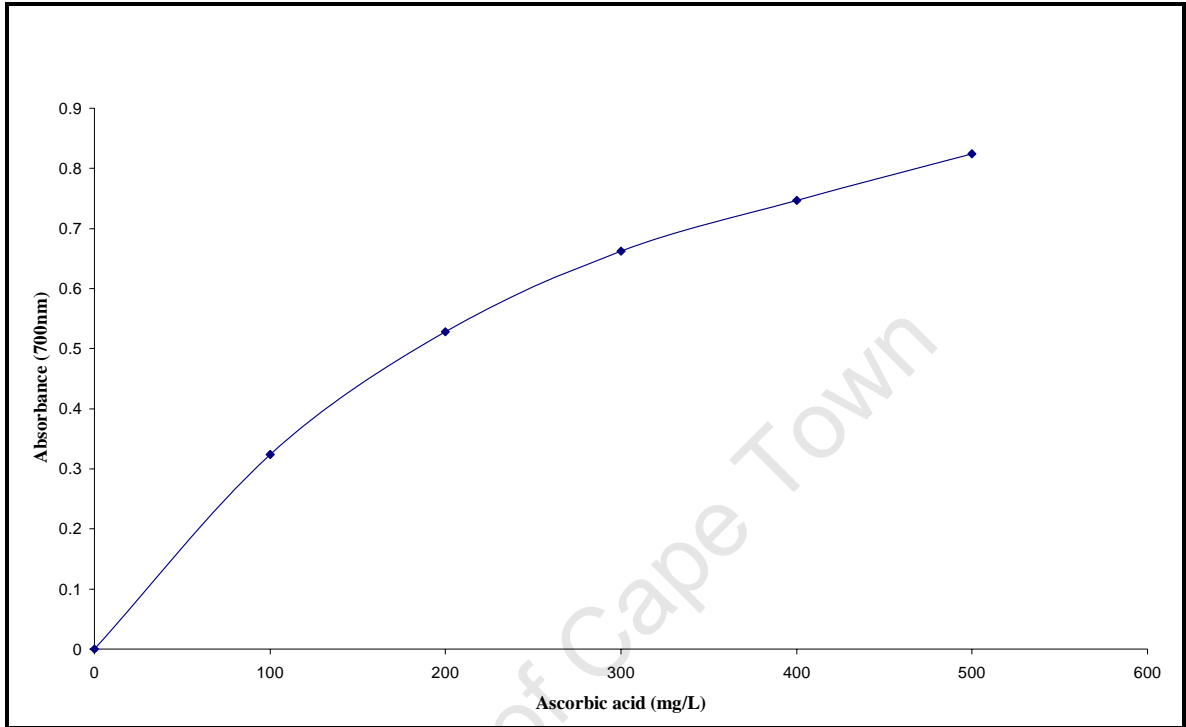
Chemiluminescent was achieved by using the reagent ECL (enhanced chemiluminescent) where equal volumes of the ECL reagents were added for the reaction mixture (volume needed =  $0.125\text{ml}/\text{cm}^2$ ). Two size fitting transparencies were cut and the blot was drained and placed on the one. The chemiluminescent (Super Signal) was added to the blot to react (1min) it with the horseradish peroxidase-linked secondary antibody resulting in a luminescence, in proportion to the amount of protein. The other transparency was put on top to form a sandwich and the excess

liquid drained. In the dark room, the sandwiched blot was in contact with Fuji X-ray film in an autoradiographic cassette. Exposure was done for 1, 5, 30 minutes depending on the rapid the bands could be observed. Each film following exposure was put in developer (2 min), washed (1 min), fixer (2 min), washed (2 min) and then air dried. All washing was done under constant running water. On the transparency, the outlines of the blot were drawn for identifying the bands size. The blot was then washed in TBS-T overnight to reprobe.

SDS (10 %):	SDS (10 g) Make up to 100 ml with distilled water.
Tank buffer (1L):	3g Tris 14.4g Glycine 10ml 10% SDS Make up to 1litre with dH <sub>2</sub> O
4X Transfer Buffer: (Make just before use)	250ml 4x transfer Buffer 200ml Methanol Make up to 1 litre with dH <sub>2</sub> O
PAGE-blue stain:	0.59g PAGE-blue 83 125ml methanol 100ml dH <sub>2</sub> O 17.5ml acetic acid Make up to 250 ml with dH <sub>2</sub> O
PAGE-blue destain:	400ml methanol 40ml glacial acetic acid Make up to 2L with dH <sub>2</sub> O
TBS-Tween (TBS-T):	50ml 2M Tris pH 7.4 8.7g NaCl Make up to 1L with dH <sub>2</sub> O Add 500µl Tween (roughly 20 drops from a Gilson)
Blocking solution:	10% milk in TBS-T Make as much as you require

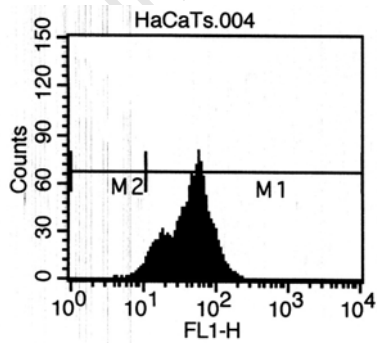
Transfer buffer: 250 ml 4X transfer buffer  
200ml methanol  
Make up to a litre

### Appendix B

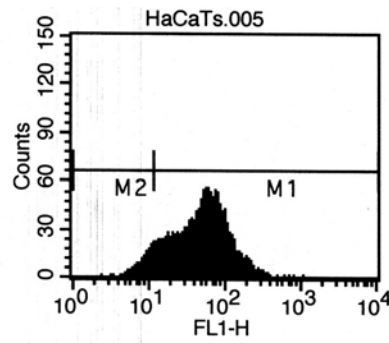


Standard curve of the ascorbic acid standard

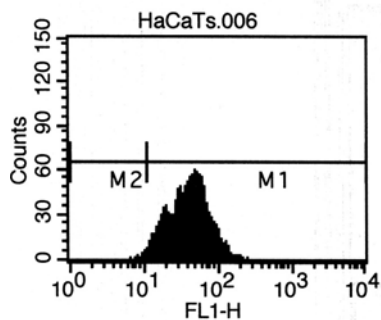
### Appendix C



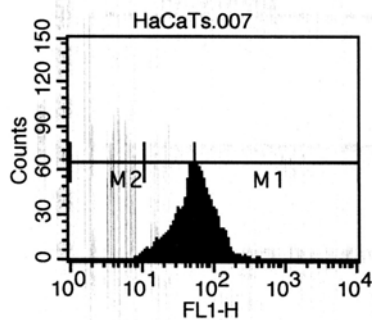
(A) UV-(MF: 47.55)



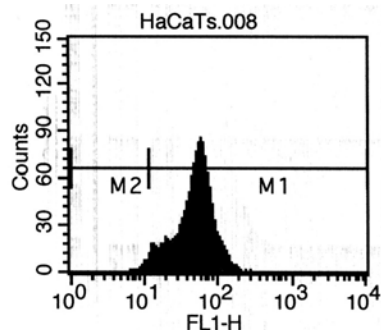
(B) UV+ (MF: 66.76)



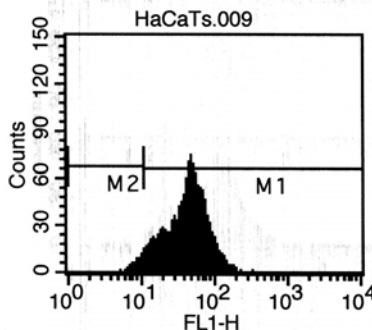
(C) Sham (MF: 44.16)



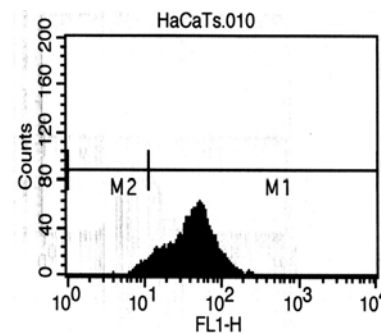
(D) Compound (33) 200  $\mu$ M (MF: 47.73)



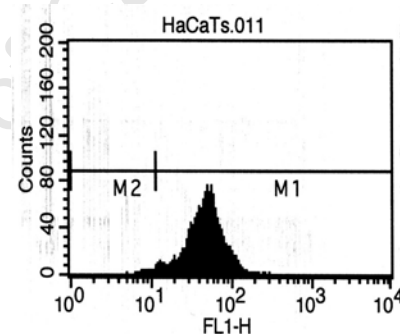
(E) Compound (33) 500  $\mu$ M (MF: 40.69)



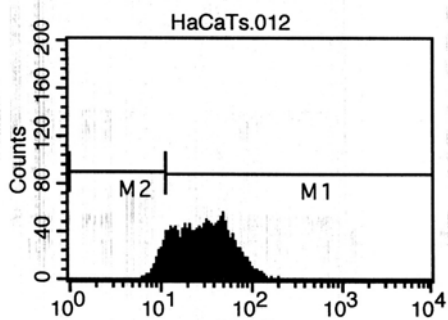
(F) Compound (33) 1000  $\mu$ M (MF: 35.08)



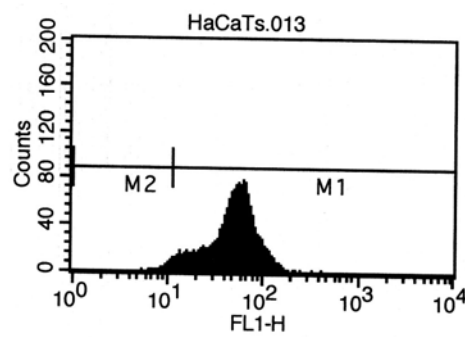
(G) Product (35) 200  $\mu$ M (MF: 44.88)



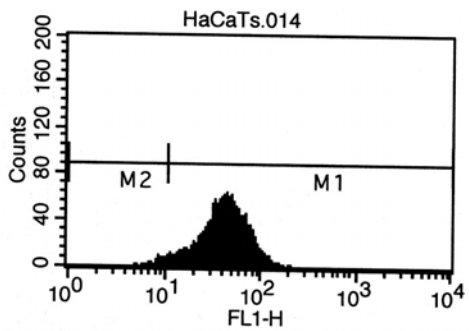
(H) Product (35) 333  $\mu$ M (MF: 40.27)



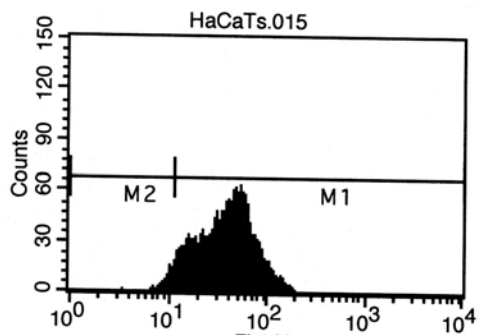
(I) Product (35) 500  $\mu$ M (MF: 33.79)



(J) Product (30) 200  $\mu$ M (MF: 41.74)



(K) Product (30) 333  $\mu$ M (MF: 35.03)



(L) Product (30) 500  $\mu$ M (MF: 32.50)

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