Protective Effects of Water Extracts from *Agrocybe aegerita* on H_2O_2-induced Oxidative Damage

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Abstract

Reactive oxygen species (ROS) generated as byproducts of normal cellular metabolism can attack many biological molecules to induce oxidation, leading to membrane damage, protein modification and DNA damage. Hydrogen peroxide (H_2O_2) is one of the most common ROS generated in human body, and is increasingly recognized as a toxic intermediate in a wide variety of physiopathologies such as cancer, aging, and ischemia-reperfusion injury. It is believed that ROS-induced injury may be prevented through supplementation of antioxidants. *Agrocybe aegerita*, an edible mushroom, has been shown to be a potential source of natural antioxidants and its protective effects against H_2O_2-induced oxidative damage in normal human skin cells were studied in this project.

In this study, three chemical assays ($ABTS^{\cdot+}$, hydroxyl radical and H_2O_2 scavenging activity assays) were first used for evaluating and comparing the antioxidant activities of hot (70℃) and cold (25℃) water extracts from both dried and fresh *A. aegerita*. The results were similar for the four water extracts from *A. aegerita* in $ABTS^{\cdot+}$ radical cation and hydroxyl radicals scavenging activities, but only cold water extract from fresh *A. aegerita* (CfAa) showed very strong H_2O_2 scavenging ability (nearly 100 % scavenging activity at concentration over 0.5 mg/ml for 0.1 mM H_2O_2).

CfAa could strongly enhance cell viability in 1 mM H_2O_2-treated adult human skin fibroblast (HDFa) cells. At a concentration of 100 μg/ml, the protection of CfAa against H_2O_2-induced cell death was over 80 % and 71 %, respectively by MTT and

LDH assay. When HDFa cells were co-incubated with CfAa (200-500 μg/ml) and H_2O_2, about 50 % protection on H_2O_2-induced DNA damage in comet assay was observed. It was also found that CfAa could restore the H_2O_2-induced depression in intracellular total glutathione level and enzyme activities (SOD, CAT and GPX) back to their normal levels. All these results indicate that CfAa may have potential to reduce the oxidative stress in human body.

Remarkable protective effect was only observed in cold water extract of fresh Aa, its genoprotective activity is believed to be associated with heat-labile component (s), which could be destroyed during the drying and heating process as shown in dried Aa extract and hot water extract of fresh Aa. The protective effect of CfAa could be either due to its strong H_2O_2 scavenging activity so that H_2O_2 was directly destroyed before acting on the human cells, or due to its ability to maintain the high level of intracellular antioxidant defense system (redox status and enzyme activities). Further studies will be focused on confirming the protective mechanism of CfAa and its application to reduce oxidative stress, as well as on the isolation of the genoprotective component (s).

摘要

活性氧 (ROS)，作爲生物生化過程中正常細胞新陳代謝的副產品，能攻擊許多生物分子因而引起氧化作用， 導致細胞膜受損，蛋白質變異以及脫氧核糖核酸 (DNA) 的損害。 過氧化氫 (H_2O_2) 是人體內最常產生的ROS之一，並且逐漸被認作多爲一種有害的中間物於多種生理病學中如癌症，衰老以及缺血再灌注損傷。 透過抗氧化物的增補相信能預防由ROS引起的傷害。 茶薪菇 (*Agrocybe aegerita*) ， 一種食用菌， 被視爲一種潛在的天然抗氧化物的來源，而本研究的重心旨在了解茶樹菇對由過氧化氫 (H_2O_2) 所誘導而成的氧化破壞在人類正常皮膚細胞中所起的保護作用。

在本研究中，首先用了三種化學測試 (清除ABTS 陽離子自由基法, 清除氫氧自由基法及清除H_2O_2法)來評估和比較乾茶薪菇以及新鮮茶薪菇的熱水 (70℃) 和冷水 (25℃) 提取物的抗氧化活性。 結果顯示，四種茶薪菇的水相提取物的 ABTS 陽離子自由基和氫氧自由基的清除能力大致相約，但只有新鮮茶薪菇的冷水提取物 (CfAa) 才顯示出強勁的H_2O_2清除能力，當濃度爲 0.5 毫克/毫升或以上時，清除 H_2O_2 (1 毫摩爾/升) 的能力接近百分之百。

研究亦發現 CfAa 能強烈提升經過 1 毫摩爾/升 的 H_2O_2 處理的成年人皮膚纖維組織細胞 (HDFa) 的生存能力。 在 100 微克/毫升的濃度下， CfAa 於 MTT 和 LDH 測試中顯示出對 H_2O_2 引起的細胞死亡的保護作用分別爲超過百分之八十以及百分之七十一。在彗星分析法中，當 HDFa 細胞與 CfAa (200-500 毫克/毫升) 及 H_2O_2 共同培養時，CfAa 對 H_2O_2 誘導的 DNA 損害起了大約百分之五十的保護作用。 此外，CfAa 還能使細胞內受 H_2O_2 壓抑的總麩胱甘肽水平及多種酶的活性 (超氧歧化酶 SOD，觸酶 CAT 和麩胱甘肽過氧化酶 GPx) 恢復至原來的正常水平。上述結果均表明 CfAa 有潛能幫助降低人體內的氧化壓力。

基於只有新鮮茶薪菇的冷水提取物才顯示出非凡的保護效能， 相信其基因保護作用亦與熱變的成分有關，而這些熱變的成分可能在乾式法或加熱期間受到破壞， 以致令乾茶薪菇的提取物及新鮮茶薪菇的熱水提取物失去保護作用。 CfAa 的保護作用可能是基於其清除 H_2O_2 的強勁能力，致使 H_2O_2 在對人類細胞起作用之前已直接被消除。不過，保護作用亦可能是由於 CfAa 能保持細胞內的高水平抗氧化防禦系統 (氧化還原狀態和酶的活性)。 未來的研究方向將會著重於確認 CfAa 的保護機制，其降低氧化壓力的應用以及隔離具有基因保護作用的成分。

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Abbreviations

Aa	*Agrocybe aegerita*
Abs	Absorbance
ABTS	2,2'-azinobis-(ethyl-benzothiazoline-6-sulfonic acid
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
CdAa	Cold water extract of dried *Agrocybe aegerita*
CE	Catechin equivalent
CfAa	Cold water extract of fresh *Agrocybe aegerita*
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DPPH	1,1-diphenyl-2-picrylhydrazyl
EC_{50}	50% effective concentration
EDTA	Ethylenediaminetetra-acetic acid
FBS	Fetal bovine serum
Fe^{2+}	Ferrous ion
Fe^{3+}	Ferric ion
GPx	Glutathione peroxidase
GSH	Reduced glutathione
GR	Glutathione reductase
GSSG	Oxidized glutathione
H_2O_2	Hydrogen peroxide
HdAa	Hot water extract of dried *Agrocybe aegerita*

HDFa	Adult human skin dermal fibroblast cell line
HfAa	Hot water extract of fresh *Agrocybe aegerita*
HO•	Hydroxyl radical
HOO•	Peroxyl radical
HRP	Horseradish peroxidase
L•	Lipid radical/allyl radical
LDH	Lactate dehydrogenase
LH	Unsaturated lipids
LO•	Alkoxyl radical
MDA	Malondialdehyde
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NADPH	Nicotinamide phosphate
NO•	Nitric oxide radical
O_2^{-}•	Superoxide anion radical
OH	Hydroxyl group
PBS	Phosphate buffer saline
PUFA	Polyunsaturated fatty acids
R^2	Pearson's correlation coefficient
RO•	Alkoxyl radical
ROOH	Alkylhydroperoxide
ROS	Reactive oxygen species
SD	Standard deviation
SOD	Superoxide dismutase
SPSS	Statistical Package for Social Sciences version

TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TEAC	Trolox equivalent antioxidant capacity
Trolox	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
UV	Ultraviolet

Content

Chapter 1: Introduction

1.1 Reactive oxygen species (ROS)

1.1.1 Definition and examples

Reactive oxygen species (ROS) is the collective term used to describe forms of oxygen that are energetically more reactive than molecular oxygen (O_2). ROS are typically molecular species that have undergone electron addition(s) and are thus reduced forms of oxygen (Smirnoff, 2005). ROS include oxygen ions, free radicals and some nonradical derivatives of oxygen that are either oxidizing agent and/or are easily converted into radicals. Examples of ROS are shown in Table 1. Free radicals are defined as any species capable of independent existence (hence the term "free") that contain one or more unpaired electrons (Yashikawa *et al.*, 1997). With the possession of the unpaired electrons, free radicals are usually unstable and highly reactive as they require another electron to fill the orbital and become stable.

Table 1 Examples of ROS

Radicals	Nonradicals
Superoxide anion, $O_2^{\cdot -}$	Hydrogen peroxide, H_2O_2
Hydroxyl radical, $^{\cdot}OH$	Organic hydroperoxide/Alkylhydroperoxide,
Alkoxy radical, $L(R)O^{\cdot}$	L(R)OOH
Peroxy radical, $L(R)OO^{\cdot}$	Singlet oxygen, $^1\Delta O_2$
Hydroperoxyl radical, $HOO^{\cdot}$	Ozone, O_3

'Reactive' is a relative term, as H_2O_2 and $O_2^{\cdot -}$ react quickly with only a few molecules, whereas $^{\cdot}OH$ reacts quickly with almost anything. $RO_2^{\cdot}$, $RO^{\cdot}$ and O_3 have intermediate reactivities.

1.1.2 Generation of ROS in biological systems

Many biochemical reactions (such as oxidative phosphorylation) vital to normal aerobic metabolism of human require the transfer of four electrons to a molecule of oxygen to form H_2O. Under most circumstances, this transfer occurs simultaneously without the formation of other intermediates. However, occasionally, molecular oxygen undergoes sequential univalent reduction to form other oxygen intermediates with different toxicities prior to the generation of H_2O (Figure 1.1).

Addition of single election to ground state O_2 molecule produces superoxide radical ($O_2^{\cdot -}$). At physiologic pH, $O_2^{\cdot -}$ rapidly reduces itself to form more cytotoxic divalent oxygen reduction product, hydrogen peroxide (H_2O_2), by addition of one more electron. Besides, approximately 1% of $O_2^{\cdot -}$ will be protonated to the more reactive peroxyl radical ($HO_2^{\cdot}$) (Borg, 1993). Through Haber-Weiss/Fenton reaction (equation 1.1 and 1.2), hydroxyl radicals ($^{\cdot}OH$) are produced from H_2O_2 in the presence of transition metal catalyst (M).

$$O_2^{\cdot -} + H_2O_2 \rightarrow O_2 + {}^{\cdot}OH + OH^- \qquad (1.1)$$

$$M^{n+} + H_2O_2 \rightarrow M^{n+1} + {}^{\cdot}OH + OH^- \qquad (1.2)$$

Besides, $O_2^{\cdot -}$ and H_2O_2 are produced by the chemical reaction of O_2 with some molecules such as adrenaline, dopamine and tetrahydrofolates (Halliwell, 1996). Low-wavelength electromagnetic radiation (e.g. gamma rays) splits water to generate $^{\cdot}OH$, while UV light can cause the homolytic cleavage of O-O bond in H_2O_2 to give 2 $^{\cdot}OH$ (von Sonntag, 1987). Sometimes, ROS are generated deliberately for useful purposes. For example, activated phagocytes generate $O_2^{\cdot -}$ and H_2O_2 as one of their mechanisms for killing foreign organisms (Babior & Woodman, 1990).

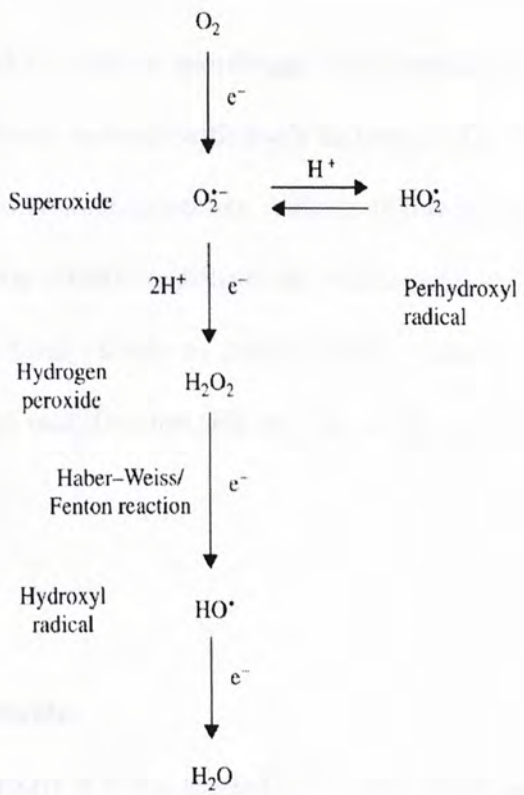

Figure 1.1 Generation of ROS through sequential reduction of molecular oxygen (Smirnoff, 2005)

1.1.3 Features of specific ROS

1.1.3.1 Superoxide anion

Superoxide anion ($O_2^{\bullet -}$) is a moderately reactive compound capable of acting as an oxidant or reductant in biologic systems. Such relative inactivity allows it to diffuse for a considerable distance before it exerts its toxic effects. Extracellularly generated $O_2^{\bullet -}$ can gain access to intracellular targets via cellular anion channels (Roos, *et al.*, 1984). $O_2^{\bullet -}$ can decrease the activity of some enzymes such as NADH dehydrogenase (involved in energy metabolism), ribonucleotide reductase (producing precursors for DNA synthesis), and some antioxidant defense enzymes such as catalase, and glutathione peroxidase (Willcox *et al.*, 2004).

1.1.3.2 Peroxyl radical

Peroxyl radical ($HOO^{\cdot}$) is more membrane permeable than $O_2^{\cdot-}$ because of its neutrality. It is more likely to react with itself to form H_2O_2. The oxidative damages in biological systems attributed to peroxy radicals is due to their comparatively long half-life and thus greater ability to diffuse into biological fluids in cells and tissues. Peroxyl radicals have been shown to induce DNA breakage, which involves both strand scission and base modification (Hu & Kitts, 2001).

1.1.3.3 Hydrogen peroxide

Hydrogen peroxide (H_2O_2) is not a radical. It is very diffusible within and between cells and it can cross cell membranes rapidly. It can attack certain enzymes such as glyceraldehyde-3-phosphate dehydrogenase, an enzyme in the glycolytic pathway, affecting the cellular energy-producing system. It can also oxidize certain keto-acids such as pyruvate. Besides, it leads to the depletion of ATP, reduced glutathione, and NADPH. Furthermore, it induces an increase in free cytosolic Ca^{2+} and activates ADP ribose polymerase that leads to cell death. The toxicity of H_2O_2 is also attributed to its ability to react with free iron and copper ions to form much more damaging species such as hydroxyl radical ($^{\cdot}OH$) and peroxynitrite (Willcox *et al.*, 2004).

1.1.3.4 Hydroxyl radical

Hydroxyl radical (·OH) is the most reactive radial species, capable of interacting with almost every type of molecule found in living cells. If ·OH contacts the cellular compound, reaction occurs immediately (with second-order rate constants of 10^9 to 10^{10} M^{-1} sec^{-1}) (Halliwell & Cross, 1994). Owing to its high reactivity, it is short-lived and is diffusion-limited such that once formed in a biological system, it usually travels only a very short distances before it encounters an oxidizable substrate. Therefore, in order to mediate injury directly, ·OH has to be generated in close proximity to a critical cellular target molecules.

Since ·OH generated from reaction of H_2O_2 with transition metal ions already bound to a biological molecule tends to react with that molecule rather than with any added scavenger, ·OH will be very difficult to be removed by scavenger. When ·OH is generated adjacent to DNA, it attacks both the deoxyribose sugar and the purine and pyrimidine bases resulting intermediates radicals, which are the immediate precursors for DNA base damage (Jaruga & Dizdaroglu, 1996).

1.1.4 Damaging effects of ROS on biomolecules

ROS are toxic and they can attack cellular molecules such as lipids in cell membranes, proteins in tissues and enzymes, and carbohydrate and DNA, which leads to membrane damage, protein modification and DNA damage, respectively. Their destructiveness depends on the reactivity and concentration. These oxidative damages are considered to result in aging and several degenerative diseases which will be discussed in later session (1.3.2).

1.1.4.1 Lipid peroxidation

Oxidative degradation of lipids is refered as lipid peroxidation. Due to the presence of multiple double bonds, polyunsaturated fatty acids (PUFA) in phospholipid bilayer of membrane are extremely sensitive to oxidation. Lipid peroxidation in membrane would lead to changes in membrane density, fluidity, and permeability, thus affecting cellular functions (Willcox *et al.*, 2004).

Lipid peroxidation can proceed by a free radical-mediated chain reaction mechanism, which consist initiation, propagation and termination steps.

Initiation: $LH + R^{\cdot} \rightarrow L^{\cdot} + RH$ (1.3)

where LH and $R^{\cdot}$ represent the substrate molecule such as unsaturated lipid, and the initiating oxidizing radical respectively. $R^{\cdot}$ abstract a hydrogen atom from LH to produce highly reactive allyl radical/lipid radical ($L^{\cdot}$).

Propagation: $L^{\cdot} + O_2 \rightarrow LOO^{\cdot}$ (1.4)

$LOO^{\cdot} + LH \rightarrow L^{\cdot} + LOOH$ (1.5)

$L^{\cdot}$ rapidly reacts with oxygen to form peroxyl radical ($LOO^{\cdot}$). Peroxyl radicals are the chain carriers of the reaction that can further oxidize another lipid molecule, producing lipid hydroperoxides (LOOH). In this way, a single initiating radical can result in the damage of hundreds of lipid molecules.

Lipid hydroperoxides (LOOH) could easily be broken down to a wide range of compounds, including alcohols, aldehydes, alkyl formats, ketones,and hydrocarbons (Antolovich *et al.*, 2001). Some of the aldehyde end products like malondialdehyde (MDA) and 4-hydroxynonenal can cause damage to both proteins and DNA (El Ghissassi *et al.* 1995).

The breakdown of LOOH would also result in the production of lipid peroxyl, alkoxyl radicals and hydroxyl radical.

$$LOOH \rightarrow LO^{\cdot} + HO^{\cdot} \qquad (1.6)$$

$$2LOOH \rightarrow LOO^{\cdot} + LO^{\cdot} + H_2O \qquad (1.7)$$

$$LOOH + M^{n+} + H^{+} \rightarrow LO^{\cdot} + M^{(n+1)+} + H_2O \qquad (1.8)$$

$$LOOH + M^{(n+1)+} + OH^{-} \rightarrow LOO^{\cdot} + M^{n+} + H_2O \qquad (1.9)$$

Termination reactions involve the combination of radicals to form non-radical products.

Termination:

$$\left.\begin{array}{ll} LO^{\cdot} + LO^{\cdot} & \rightarrow \\ LOO^{\cdot} + LOO^{\cdot} & \rightarrow \\ LO^{\cdot} + LOO^{\cdot} & \rightarrow \end{array}\right\} \text{non-radical products} \qquad (1.10)$$

1.1.4.2 DNA damage

ROS produce a number of lesions in DNA such as base lesions, sugar (deoxyribose) lesions, single-strand breaks, double-strand breaks, abasic site (a site where a DNA base is lost), DNA-protein cross-links and aldehyde adducts to nucleobases by a variety of mechanisms (Dizdaroglu & Karakaya, 1999).

Conversion of guanine to 8-hydroxyguanine (8-OHG) is a frequent result of ROS attack, and it has been found to alter the enzyme-catalysed methylation of adjacent cytosines, which is important for the regulation of gene expression. 8-OHG is regarded as the most commonly produced base lesion, and it is often measured as an index of oxidative DNA damage. It is sometimes measured as the nucleoside, 8-hydroxydeoxyguanosine (8-OHdG) (Wiseman & Halliwell, 1996).

DNA-protein cross-links may be formed by combination of a DNA radical and a protein radical, or by addition of a DNA radical to an aromatic amino acid in proteins (or addition of a protein radical to a DNA base) (Wiseman & Halliwell, 1996).

Structural alterations in DNA such as base pair mutations, rearrangements, deletions, insertions and sequence amplification could be caused by ROS attack. ROS can also produce gross chromosomal alterations in addition to point mutation. Oxidation reaction of DNA could result in development of cancer if DNA repair capacity in cell is insufficient (Wiseman & Halliwell, 1996).

1.1.4.3 Protein oxidation

There are many different modes of inducing damage on protein by ROS, including metal catalyzed oxidation, oxidation induced cleavage of peptide bonds, amino acid modification, and the conjugation of lipid peroxidation products (Cecarini *et al.*, 2007).

Metal-catalyzed oxidation is found to be one the most common mechanisms for inducing protein oxidation. Usually, this involves ROS attack on amino acid residues near the specific metal binding site within the protein (Cecarini *et al.*, 2007).

Amino acid modification is induced through side-chain reaction with ROS. The most sensitive amino acids are those containing aromatic side chain groups or sulfhydryl (–SH) groups. Examples of aromatic side-chain amino acid modification include the conversion of phenylalanine residues to hydroxy derivatives, tryptophan residues to N-formylkynurenine, histidine residues to 2-oxohistidine and tyrosine residues to a dihydroxy-derivative (dopa). Methionine and cysteine are oxidized by ROS via reactions at the site of sulfhydryl residues, resulted in the production of sulfoxide, sulfenic acids, and disulfide bridges. Besides, cysteine is vulnerable to oxidant-induced cross-linking (Stadtman & Levine, 2000; Cecarini *et al.*, 2007).

ROS-mediated protein oxidation gives rise to the formation of carbonyl groups. Carbonyl derivatives can also be formed as a consequence of secondary reactions of some amino acid side-chains with lipid peroxidation products such as 4-hydroxynonenal. It is notable that these carbonyl groups can further react with the α-amino groups of lysine residues in the same or another protein molecules leading to the formation of intra- or inter-molecular cross-links which can promote the

formation of protein aggregates. These aggregates are unable to be degraded by normal protein degradation mechanisms, and they can also inhibit proteolytic degradation of other oxidized protein. The consequence is the accumulation of oxidized proteins and enhancement of cellular dysfunction (Stadtman & Levine, 2000; Cecarini *et al.*, 2007).

Protein oxidation leading to changes in the three-dimensional structure and activity would hence affect the function of many receptors, enzymes and transport proteins. As a result, cell structure, cell signaling, and the various metabolisms would be affected (Cecarini *et al.*, 2007).

1.2 Antioxidants

1.2.1 Introduction

Antioxidant can be defined as "any substance that when present at low concentrations, compared to those of an oxidizable substrate, can significantly delays/inhibits oxidation of that substrate" (Halliwell & Gutteridge, 1995).

To protect organelles and cellular components against the deleterious effects of ROS, physiological antioxidant defense system has been developed, which involved a variety of antioxidants of endogenous and exogenous origin. Endogenous antioxidants refer to those that are synthesized in the body. Other antioxidants that are not synthesized in body are regarded as exogenous antioxidants. Relationship between some ROS and antioxidants is summarized in Figure 1.2.

1.2.2 Mode of action

Basically, the defense system includes preventive antioxidants as first line of defense, radical scavenging antioxidants as second line of defense and repair/de novo enzymes as third line of defense. Preventive antioxidants suppress the formation of radicals (e.g. by chelating catalytic metal ions or by removing oxygen or decreasing local oxygen concentrations). Radical scavenging antioxidants inhibit the chain initiation and prevent chain propagation. Enzymes such as lipases, proteases, DNA repair enzymes, and transferases act in the defense system by repairing oxidative damage, reconstituting membrances, and eliminating the damaged molecules. Besides, by promoting the death of cells (apoptosis) with excessively DNA damaged helps prevent transformed cells from arising. In fact, many antioxidants have more than one mechanism of action (Willcox *et al.*, 2004).

1.2.3 Endogenous Antioxidants

Endogenous antioxidants (biological antioxidants) can be divided into two main categories: antioxidant enzymes and antioxidant compounds. Antioxidant enzymes are enzymes that can remove ROS in a catalytic fashion while antioxidant compounds include certain proteins, and low-molecular-mass species of non-protein nature that can remove ROS nonenzymatically and have to be regenerated enzymatically or nonenzymatically in order to function in a catalytic fashion (Yagi, 1993). The levels and locations of these biological antioxidants must be tightly regulated for cell survival.

1.2.3.1 Antioxidant enzymes

Superoxide dismutase (SOD)

Superoxide dismutase (EC 1.15.1.1) is responsible for the conversion of superoxide radical to H_2O_2 and O_2 (1.11). In humans, there are three forms of SOD: cytosolic SOD (SOD-1), mitochondrial SOD (SOD-2) and extracellular SOD (SOD-3). SOD-1 is a dimer (consists of two identical subunits) while the other two are tetramers (four subunits). SOD-1 and SOD-3 contain copper and zinc, respectively, while SOD-2 contains manganese (Zelko *et al.*, 2002).

$$2\ O_2^{\cdot -} + 2H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2 \qquad (1.11)$$

Catalase (CAT)

Catalase (EC1.11.1.6) is an ubiquitous antioxidant enzyme that is present in most aerobic cells. In humans, the catalase particularly active in liver, kidney, lung and erythrocytes, where it is believed to account for the majority of hydrogen peroxide decomposition (Calabrese & Canada, 1989). Catalase is involved in the detoxification of hydrogen peroxide (H_2O_2) by two types of reactions, which proceed through a common intermediate (complex I). Complex I is formed by binding of H_2O_2 to the enzyme (1.12). Reaction of complex I with another H_2O_2 leads to the formation of one molecule of molecular oxygen and two molecules of water (1.13). If a hydrogen donor (AH_2) such as methanol or ethanol reacts with complex I, the products are water and formaldehyde or acetaldehyde (1.14).

$$CAT + H_2O_2 \rightarrow CAT - H_2O_2 \text{ (complex I)} \quad (1.12)$$

$$CAT - H_2O_2 + H_2O_2 \rightarrow CAT + 2H_2O + O_2 \text{ (catalytic activity)} \quad (1.13)$$

$$CAT - H_2O_2 + AH_2 \rightarrow CAT + 2H_2O + A \text{ (peroxidatic activity)} \quad (1.14)$$

Overall equations:

$$2H_2O_2 \xrightarrow{CAT} O_2 + 2H_2O \text{ (catalytic activity)} \quad (1.15)$$

$$H_2O_2 + AH_2 \xrightarrow{CAT} 2H_2O + A \text{ (peroxidatic activity)} \quad (1.16)$$

Glutathione peroxidases (GPx)

Glutathione peroxidase (EC1.11.1.9) is the general name of an enzyme family which catalyzes the reduction of hydroperoxides with concomitant conversion of reduced glutathione (GSH) to oxidized glutathione (GSSG). GPx reduces lipid

hydroperoxides to their corresponding alcohols (1.17) and reduces free hydrogen peroxide to water (1.18). There are four types of GPx including cytosolic (cGPx), plasma (pGPx), gastrointestinal (GI-GPx) and phospholipids-hydroperoxide (PH-GPx) glutathione peroxidase. All of the GPx enzymes except PH-GPx are tetramers of four identical subunits. Each subunit contains a selenocysteine in the active site which participates directly in the two-electron reduction of the peroxide substrate. Since PH-GPx is a monomer, this small size helps in interaction with hydroperoxides integrated in membranes. These enzymes use glutathione as the ultimate electron donor to regenerate the reduced form of the selenocysteine (Brigelius-Flohé, 1999).

$$ROOH + 2GSH \xrightarrow{GPx} ROH + GSSG + H_2O \qquad (1.17)$$

$$H_2O_2 + 2GSH \xrightarrow{GPX} 2H_2O + GSSG \qquad (1.18)$$

Glutathione reductase (GR)

Glutathione reductase (EC 1.6.4.2) is a flavoprotein enzyme that regenerates GSH from GSSG, with NADPH as a source of reducing power (1.19). This enzyme is essential for the GSH redox cycle in maintaining adequate levels of reduced cellular GSH of which importance will be discussed in the following (section 1.2.3.2).

$$GSSG + NADPH + H^+ \xrightarrow{GR} 2\,GSH + NADP^+ \qquad (1.19)$$

1.2.3.2 Antioxidant compounds

Glutathione

Glutathione, a low-molecular-weight tripeptide (γ-glutamylcysteinylglycine) present in all mammalian cells at millimolar concentrations, exists in both the reduced (GSH) and the oxidized (GSSG) forms which can be inter-converted by GPx and GR as discussed above (section 1.2.3.1). GSH is the predominant form and it is the most abundant non-protein intracellular sulfhydryl/thiol-containing compound (Mello & Kubota, 2007). It acts as a nucleophilic co-substrate to glutathione transferase in detoxification of xenobiotics and is an essential electron donor to glutathione peroxidases in reduction of hydroperoxides (Baillie & Latter, 1991). It also serves several cellular functions including amino acid transport and maintenance of protein sulfhydryl reduction status. Its central nucleophilic cysteine residue is responsible for its high reductive potential (Mello & Kubota, 2007) and its antioxidants functions are performed through hydrogen donation from the thiol moiety or by conjugation reactions (Baker *et al.*, 1990).

GSH can reduce tocopherol radicals (Escobedo *et al.*, 2004) and serve as a scavenger for $O_2^{\cdot-}$ and $OH^{\cdot}$ through donation of its hydrogen atom resulting in the formation of glutathione disulfide (GSSG) (Droge & Breitkreutz, 2000). In mammalian cells, the cycling between GSH and GSSG serves to remove ROS such as H_2O_2 produced due to either cellular respiration or metabolism of toxic substances (Lin & Yang, 2007).

A high GSH/GSSG ratio is essential for protection against oxidative stress. A change in this ratio has been considered an indicator of the oxidative state of the cell. Depletion of GSH in addition to a challenge by other oxidative reactions can lead to irreversible cell damage. Therefore, the measurement of GSH and its disulfide form (GSSG) provides information about cellular defense and cellular response to ROS challenges (Baker *et al.*, 1990).

1.2.4 Exogenous antioxidants

Exogenous antioxidants are often referred to dietary antioxidants. Diet plays an important role in the production of antioxidant defense system by providing essential nutrient antioxidants such as vitamin E, C, and β-carotene, antioxidant phenols including flavanoids, and essential minerals that form important antioxidant enzymes (e.g. zince for SOD and selenium for GPx).

α-Tocopherol (vitamin E) is the most important free radical scavenger within membranes. It inhibits lipid peroxidation by scavenging peroxyl radicals, which are intermediates in the chain reaction (Hallwell & Cross, 1994).

Diet also plays an important role in the oxidation process by affecting the substrates that are subjected to oxidation. For example, lipoprotein and fatty acid composition in the cell membrane is determined primarily by diet, so consumption of less polyunsaturated fatty acids provides less substrates for lipid peroxidation.

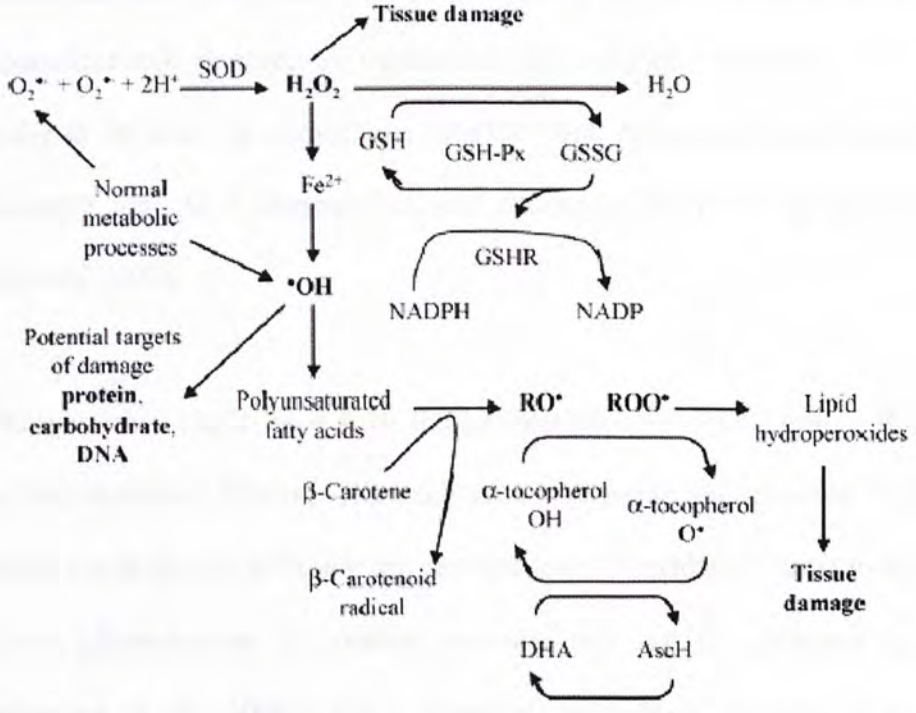

Figure 1.2 Relevant relationship between ROS and antioxidants (Adapted from Aruoma, 1994)

1.3 Oxidative stress

1.3.1 Balance between ROS and antioxidants

Oxygen is essential to many living organisms for the production of energy to fuel biological processes. However, ROS are usually generated as byproducts of normal cellular metabolism. They are constantly generated in cells and rapidly removed by the endogenous antioxidant system (non-enzymatic and enzymatic antioxidants) so as to prevent their harmful effects and maintain a pro-oxidant/antioxidant balance.

Oxidative stress is resulted in an imbalance between the production of ROS and a biological system's ability to readily detoxify the reactive intermediates or repair the resulting damage. Most aerobes can tolerate mild oxidative stress by synthesis of extra antioxidant defenses to restore the balance. However, severe oxidative stress produces major interdependent derangements of cell metabolism, which include DNA strand breakage, base/sugar modification, lipid peroxidation (accumulation of oxidized-damage molecules would lead to increase in level of dysfunctional marcomolecules), damage to membrane ion transporters (such as K^+ channels), increase in the level of intracellular free Ca^{2+} and decompartmentization of catalytic iron/copper ion. As a consequence, cell death may result by apoptosis or necrosis (Halliwell, 1996).

Oxidative stress could be due to the production of excess ROS or the decline in cellular antioxidant defense. High metabolic demands and external factors such as sunlight, smoking and pollution increase the level of oxidative stress to such an extent that our physiological antioxidant systems may not be adequate to cope with (Svobodova *et al.*, 2006). Toxic chemicals and drugs increase ROS production through cytochrome P-450-dependent oxidation resulting in the formation of

superoxide radicals as byproducts. ROS are also produced at sites of inflammation as a result of the phagocyte oxidative burst (Wiseman, 1996). Depletion of antioxidant levels could be caused by malnutrition which lowers antioxidant vitamin and glutathione levels.

1.3.2 Diseases associated with oxidative stress

Oxidative stress that resulted in deterioration of physiological functions would lead to the development of diseases and an acceleration of aging. Cancer, emphysema, cirrhosis, atherosclerosis, and arthritis have all been correlated with oxidative damage. The Free Radical Hypothesis of Aging (also called the Oxidative Stress Theory of Aging) suggests that age-related changes are manifestations of the body's inability to cope with oxidative stress that occurs throughout the lifespan. How the oxidative stress causes the aging is not clearly known, but it is believed to involve lipid and protein oxidation, increases in DNA oxidation products, and deficiency in calcium regulatory mechanisms that eventually lead to cell death (Willcox *et al.*, 2007). Mutagenic alteration of DNA by ROS can initiate carcinogenesis as mutations affect gene expression and/or function, particularly those of tumor suppressor genes and proto-oncogenes, which lead to unregulated growth typical of cancer cells. Inflammatory state, mediated in part by ROS, would result in damage to vascular endothelial and smooth muscle cells. Endothelial dysfunction would promote recruitment of monocytes, macrophages, growth factors and cellular hypertrophy, all of which contribute to the formation of atherosclerotic plaques (Seifried *et al.*, 2007).

1.4 Previous studies on edible mushroom antioxidants

Since antioxidants are essentially needed in body system to balance the oxidative stress and dietary antioxidant supplements may help offsetting the detrimental effects of ROS, many studies have been carried out to search and develop antioxidants of natural origin due to the safety concern of synthetic antioxidants. Among various naturally occurring substances, edible mushrooms have been intensively investigated as the potential source of antioxidants. Mushrooms are considered as macrofungi with a distinctive fruiting body that is large enough to be seen with naked eye (Chang & Miles, 1992). They have long been acknowledged for their medicinal properties in addition to their desirable flavors and nutritional value. A number of bioactive mushroom components were found to exhibit therapeutic effects such as anti-tumor, anti-inflammatory, anti-diabetic, anti-hypercholesterolemia and immunomodulatory (Wasser & Weis, 1999).

As many diseases are associated with oxidative stress (refer to section 1.3.2), the therapeutic effects of mushrooms might be closely related their antioxidative properties. Many mushrooms have been demonstrated to have the ability to scavenge ROS. It was reported that antitumor polysaccharoproteins from several mushrooms including *Coriolus versicolor, Ganoderma lucidum*, *Grifola umbellata*, *Volvariella volvacea, Tricholoma lobayense* and *Tremella fuciformis* exhibited significant superoxide and hydroxyl radical scavenging activities (Liu *et al.*, 1997). In the study by Mau *et al.* (2001), methanol extract of some ear mushrooms (black, red, jin and snow ears) showed excellent 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging effect, reducing power, ferrious ions chelating effect and inhibition effect on lipid peroxidation.

1.4.1 Previous studies on *Agrocybe aegerita*

Agrocybe aegerita (a synonym of *Agrocybe cylindracea*), also called black poplar mushroom, is an agaric fungus that colonizes deciduous wood and bark mulch, preferably stumps of poplar trees. This fungus can be found in North America, Europe and Asia, and it seems to prefer warm or mild climates (Ullrich *et al.*, 2004). *A. aegerita* could also be cultivated and commercial products of *A. aegerita* generally have long stipes and closed caps. *A. aegerita* is highly valued as functional food for its physiological benefits. It was found that fruiting bodies of this mushroom are active in several therapeutic effects including antitumor, antifungal, nerve tonic, hypercholesterolemia and hyperlipidemia (Wasser & Weis, 1999). Shon & Nam, (2001a) reported that *A. aegerita* possessed antimutagenic activities and might play a role in the prevention of cancer. Soybean fermented with *A. aegerita* also formed polysaccharides with cancer chemopreventive activity (Shon & Nam, 2001b). Furthermore, antigenotoxic activity of *A. aegerita* has been detected using Drosophila assays (Taira *et al.*, 2005). Recently, *in vitro* antioxidant properties of different extracts (such as methanol and hot water extracts) from *A. aegerita* have also been studied (Lo & Cheung, 2005; Tsai *et al.*, 2006). All these findings imply that *A. aegerita* is a promising source of natural antioxidants with physiological benefits. However, antioxidants studies of *A. aegerita* were limited in chemical assays and very few data are available on the correlation between antioxidant activity and genoprotective effect of antioxidants in *A. aegerita*.

1.5 Cell culture models for antioxidant research

Antioxidant research has been expanded dramatically since the mid-1990s with the development of several *in vitro* assays measuring the total antioxidant activity of pure compounds, foods and dietary supplements (Cao *et al.*, 1993; Ou *et al.*, 2001; Prior *et al.*, 2003). Other methods for testing antioxidant activity usually consider their ability to inhibit lipid peroxidation and free radicals (such as ABTS, DPPH, hydroxyl and superoxide radicals) scavenging activities (Antolovich *et al.*, 2002). Antioxidant properties were also assayed in terms of reducing power and chelating abilities on ferrous and cupric ions (Tsai *et al.*, 2006). However, these data are difficult to apply to biological systems, as these antioxidant activity assays in test tubes do not necessarily reflect the cellular physiological conditions and do not consider the bioavailability and metabolism issues (Liu & Finley, 2005). The mechanisms of action of antioxidants go beyond the free radicals scavenging activity in disease prevention and health promotion (Liu, 2004). Animal models and human studies are expensive and not suitable for the initial antioxidant screening of foods and dietary supplements. Therefore, there is a need for cell culture models to support antioxidant research and to access the bioactivity of antioxidants prior to animal studies and human clinical trials.

Cells in humans and other organisms are constantly exposed to a variety of oxidizing agents, which may present in the surrounding environment or be produced by metabolic activity within cells. Cells correspond to different tissues or located in different parts of the body may exhibit different response to antioxidants, so different cell lines could be used for different purposes in the investigation of oxidative stress and related diseases. Caco-2 and HepG2 cancer cells were suggested to be the potential models for studying bioavailability and metabolism of antioxidants (Liu &

Finley, 2005), so it was common to use cancer cell lines in many studies (even the focus was not in cancer research) due to their easy and fast culturing properties.

However, due to the concern that metabolism in cancer cells may be different from that of normal cells, results obtained from normal cell lines would be more reliable when studying the cellular damaging effects of ROS and protective effect of antioxidants.

Normal skin cells could be one of the potential models for antioxidant study. Due to its external position, the skin acts as the outermost barrier of the body. More than other tissues, the skin is in continual contact with external environment and is exposed to oxygen and numerous toxic agents including ultraviolet (UV) radiation and air pollution. Therefore, the skin is our first line of defence from environmental insults, which accentuates the need for cell defensive mechanisms. The skin is equipped with an elaborate system of antioxidant substances and enzymes including a network of redox active antioxidants. (De Pascale *et al.*, 2006). It was found that ROS including H_2O_2 are produced in the skin by UV radiation, causing oxidative damage to cellular components such as mitochondria and nuclear DNA, which in turn accelerates ageing and contributes to skin cancers (Miyachi, 1995; Peus *et al.*, 1999). It was also found that UVA irradiation could cause accumulation of extracellular H_2O_2 in the culture medium (Yu *et al.*, 2006). The pro-oxidative environment and constant exposure to ROS make skin cell a suitable model for studying oxidative stress and the effect of dietary antioxidants.

1.6 Objectives

Generation of ROS is a normal attribute of cellular metabolism and the increased production of ROS due to various factors may lead to oxidative stress when the cellular antioxidant defense system is overwhelmed. Oxidative stress, associated with the formation of ROS, plays an important role in the pathogenesis of various diseases. Among a great variety of ROS, hydrogen peroxide (H_2O_2) is worth investigating as it is generated from nearly all sources of oxidative stress and it can diffuse freely in and out of cells and tissues. Also, H_2O_2 is increasingly recognized as a toxic intermediate in a wide variety of physiopathologies such as cancer, aging, and ischemia-reperfusion injury (Aruoma, 1996). In spite of the fact that the mushroom *Agrocybe aegerita* has been studied for some time, few data are available on the protective effect of its antioxidant extract against H_2O_2-induced oxidative damage in human.

The objectives of this project are:

1. To evaluate and compare the antioxidant properties of hot and cold water extracts from *Agrocybe aegerita.*
2. To evaluate the pattern of H_2O_2-induced oxidative stress in normal human cell line by measuring cell viability, membrane integrity, protein content, lipid peroxidation and DNA damage.
3. To evaluate and compare the protective effects of hot and cold water extracts from *Agrocybe aegerita* on H_2O_2-induced oxidative damage in human cell line.
4. To evaluate the changes in the cellular antioxidant defense mechanisms by H_2O_2 and extracts from *A. aegerita* by examining the systems involved in the glutathione redox cycle and the concerted action of antioxidant enzymes.

Chapter 2 Materials and Methods

2.1 Materials

2.1.1 Mushroom fruiting bodies

Dried fruiting bodies of *Agrocybe aegerita* (*Aa*) (Figure 2.1) was purchased from Oriental Products Supplies Ltd while fresh fruiting bodies of *Aa* (Figure 2.2) was purchased from local market in Hong Kong.

Figure 2.1- Dried fruiting bodies of *Aa*

Figure 2.2 - Fresh fruiting bodies of *Aa*

2.1.2 Cell lines and their subcultures

Adult human skin dermal fibroblast cell line (HDFa) as shown in figure 2.3 was purchased from Cascade Biologics Inc., Portland (Cat. # C-102-5C).

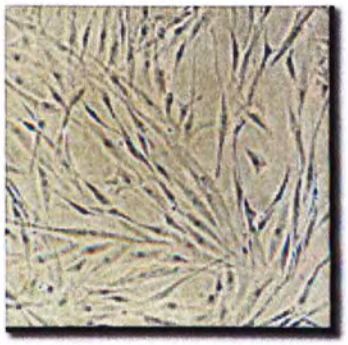

Figure 2.3 - HDFa

Cells were grown in Dulbecco's modified Eagle's medium (DMEM) (GIBCO, Cat. # 12100-046) in which 1.5 g/L sodium bicarbonate was added and pH was adjusted to 7.4. To complete the medium, it was supplemented with 10 % (v/v) heat-inactivated fetal bovine serum (FBS) (GIBCO, Cat. # 26140-079) and 1 % penicillin-streptomycin (GIBCO, Cat. # P0781). Cells were cultured in tissue-culture flask at a density of 2.5 x 10^3 cells/cm^2 and incubated in a humidified atmosphere with 5 % CO_2 at 37℃.

Cells were subcultured every three to four days. To subculture, medium in tissue-culture flask was first removed. After washing with phosphate buffer saline (PBS) (pH 7.4) to remove the remaining medium that contained trypsin inhibitor, the cells were treated with Trypsin/EDTA solution (Hyclone, Cat # SH30042.01) for 1-3 min until the cells became partially detached and rounded. The flask was then tapped gently to dislodge the cells from the surface of the flask. The detached cells together with several milliliter of medium were subjected to centrifugation at 180 x *g* for 5 min. Cell pellets was resuspended in fresh medium and its concentration was determined. Cells suspension was then transferred to new culture flasks.

2.2 Principle of Methods and Procedures

2.2.1 Sample preparation and extraction

Dried *Aa* sample was frozen in an ultra-low freezer (U85-18m, So-low, USA) at -80 ℃ and then freeze-dried by a freeze dryer (Labconco, England) to remove moisture content in sample. After that, the dried *Aa* sample was milled into powder through 0.5 mm sieve using a hammer mill (MF 10, IKA-WERKE, Germany). Fresh *Aa* sample was cleaned with a brush to remove dirt on the surface of mushroom and was then cut into small pieces.

Dried *Aa* powder (30 g) was extracted by continuous stirring in 600 ml distilled water at 70℃ and at ambient temperature (~25℃), respectively for 3 h. Fresh *Aa* (30 g mushroom pieces) was extracted by first homogenizing in a Waring blender and then suspended in 600 ml distilled water with continuous stirring at 70℃ and at ambient temperature (~25℃), respectively for 3 h. The extraction ratio was 1 g sample: 20 ml solvent. After the extraction, the water-soluble fraction was separated from insoluble residue by centrifugation (4800 rpm) and filtration (through a filter paper).

The mushroom crude extracts were obtained by lyophilizing the filtrates. The lyophilized powder (extract) was weighed and the extraction yield was calculated which was expressed as number of grams of extract obtained per gram of dried matter of mushroom sample. Moisture content of fresh *Aa*, which was measured by the difference in weight before and after freeze drying, was subtracted from the gross weight of fresh *Aa* in order to calculate the extraction yield. Extracts obtained at 70 ℃ were regarded as hot water extracts (coded as H) while that obtained at ambient temperature were regarded as cold water extracts (coded as C). There were altogether

four mushroom crude extracts, they were cold water extract of fresh *Aa* (CfAa), hot water extract of fresh *Aa* (HfAa) , cold water extract of dried *Aa* (CdAa) and hot water extract of dried *Aa* (HdAa). All extracts were then stored in plastic bottle inside a desiccator prior to analysis.

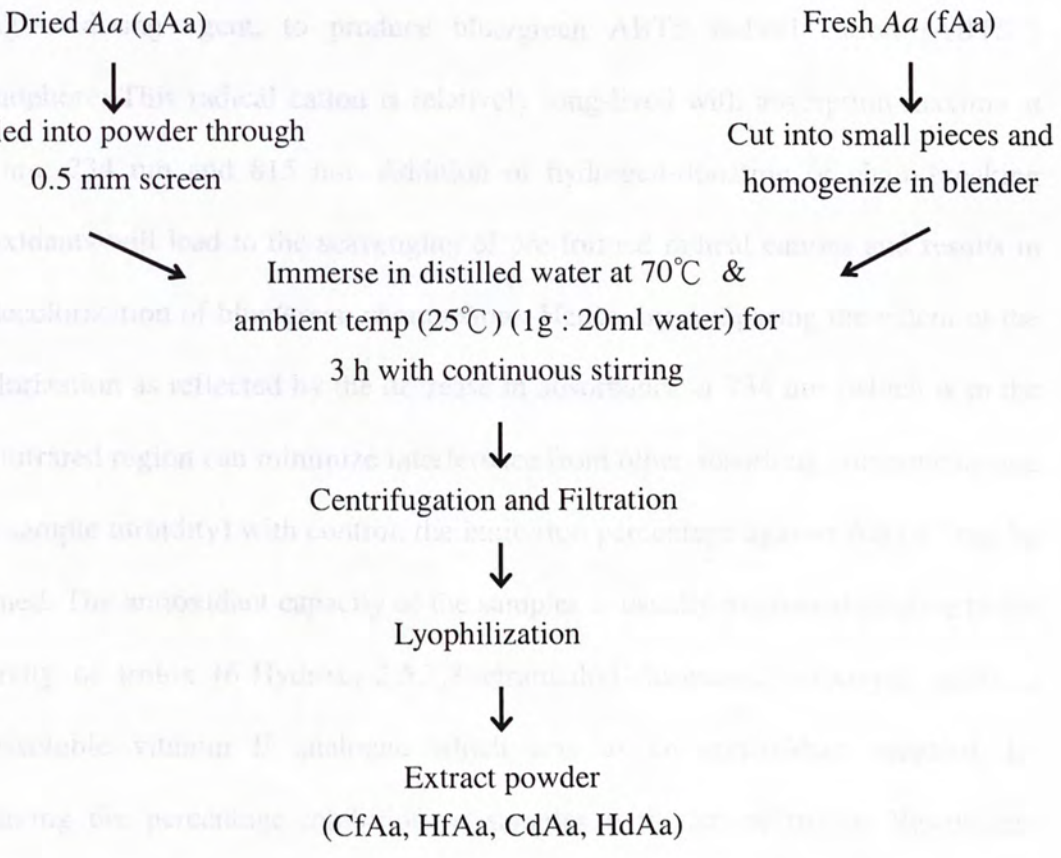

Figure 2.4 Extraction method of dried and fresh Aa

2.2.2 Chemical assays for *in vitro* antioxidative properties of mushroom extracts

2.2.2.1 $ABTS^{\cdot+}$ scavenging activity

Principle

This method was first reported by Miller *et al.* (1993) and was later improved by Re *et al.* (1999). This is a decolorization assay application to both lipophilic and hydrophilic antioxidants. In this assay, 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) is oxidized by potassium persulphate ($K_2S_2O_8$) which is a strong oxidizing agent, to produce blue/green ABTS radical cation ($ABTS^{\cdot+}$) chomophore. This radical cation is relatively long-lived with absorption maxima at 645 nm, 734 nm and 815 nm. Addition of hydrogen-donating or chain-breaking antioxidants will lead to the scavenging of pre-formed radical cations and results in the decolorization of blue/green chomophore. Hence, by comparing the extent of the decolorization as reflected by the decrease in absorbance at 734 nm (which is in the near infrared region can minimize interference from other absorbing components and from sample turbidity) with control, the inhibition percentage against $ABTS^{\cdot+}$ can be obtained. The antioxidant capacity of the samples is usually expressed relative to the reactivity of trolox (6-Hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid), a water-soluble vitamin E analogue which acts as an antioxidant standard, by comparing the percentage inhibition of samples with that of trolox. Results are expressed as Trolox Equivalent Antioxidant Capacity (TEAC) which reflects the concentration/amount of trolox having the same antioxidant capacity to 1 mg of sample.

Procedures

The scavenging activity of $ABTS^{\cdot+}$ of extracts was measured according to the method described by Re *et al.* (1999) with some modifications. ABTS (7 mM) and $K_2S_2O_8$ (140 mM) (Alrich) were prepared by dissolving 0.03841 g ABTS and 0.37846 g $K_2S_2O_8$ in 10 ml distilled water, respectively. The ABTS radical cation ($ABTS^{\cdot+}$) was produced by reacting 10 ml of 7 mM ABTS with 176 µl of 140 mM $K_2S_2O_8$. After the mixture was kept in the dark at room temperature for 12-16 hours to allow the completion of radical generation, it was diluted with 95 % ethanol so that its absorbance was adjusted to 0.70 ± 0.05 at 734nm. To determine the scavenging activity, 1 ml diluted $ABTS^{\cdot+}$ reagent was mixed with 10 µl of sample or negative control (water). Then the absorbance was measured at 734 nm 6 min after the initial mixing, using ethanol as blank. The percentage inhibition of the samples was calculated by the following equation:

Inhibition percentage (%) = [1-(Ab_{734} of sample/Ab_{734} of negative control)] x 100%

where Ab_{734} was the absorbance at 734 nm. (2.1)

Various concentrations (0.25 mM-2.5 mM) of Trolox was prepared in 95 % ethanol as standard. It should be noted that fresh working standard should be prepared daily. A dose-response curve of percentage inhibition against different concentrations of Trolox standard was prepared. The antioxidant activity of samples was expressed as Trolox Equivalent Antioxidant Capacity (TEAC) which represented the concentration (mM) of Trolox, having the same activity as 1 mg of sample.

2.2.2.2 Hydroxyl radical scavenging activity

Principle

The assay is known as deoxyribose method and was first described by Halliwell *et al.*, 1987. In the assay, hydroxyl radicals (·OH) are generated by reaction between iron-EDTA complex and hydrogen peroxide (H_2O_2) in the presence of ascorbic acid (2.2-2.5). The ·OH radicals generated will then attack the deoxyribose sugar (2-deoxy-D-ribose) and trigger a series of reactions resulted in the formation of malondialdehyde (MDA) upon heating under acidic condition (2.6). MDA is detected by its ability to react with thiobarbituric acid (TBA) to form a pink chromogen which can be measured at 532 nm (2.7). Antioxidants present compete with deoxyribose for the hydroxyl radical produced and hence diminishes MDA as well as chromogen formation, so the extent of depletion of the intensity of pink color represents the hydroxyl radical scavenging ability of the sample. Addition of ascorbic acid and extra H_2O_2 can accelerate the rate of deoxyribose degradation by regenerating Fe^{2+}-EDTA and reacting with Fe^{2+}-EDTA, respectively. Since the ability of sample in chelating the iron ions would affect the accuracy of the assay, the addition of EDTA not only can facilitate the formation of ·OH radicals but also prevent the sample from forming complexes with iron ions.

Fe^{3+}-EDTA+ asorbate → Fe^{2+}-EDTA + oxidized ascorbate (2.2)

Fe^{2+}-EDTA + O_2 → Fe^{3+}-EDTA + O_2^- (2.3)

$2O_2^- + 2H^+$ → $H_2O_2 + O_2$ (2.4)

Fe^{2+}-EDTA + H_2O_2 → OH^- + ·OH + Fe^{3+}-EDTA (2.5)

·OH + deoxyribose → fragments $\xrightarrow{\text{heat with TBA plus acid}}$ MDA (2.6)

2TBA+ MDA → chromogen (2.7)

Procedures

The scavenging activity of hydroxyl radical of extracts was measured according to the method described by Halliwell *et al.*, (1987) with some modifications.

One hundred microliters of different concentrations (2.5-10 mg/ml) of samples (or negative control or standard) was mixed with 690 µl of 2.5 mM 2-deoxy-D-ribose (Sigma) in 0.2 M phosphate buffer saline at pH 7.4. Then 100 µl of iron(III) chloride-EDTA mixture prepared by mixing equal volume of 2.0mM iron(III) chloride (Unilab) in deaerated water and 2.08 mM EDTA (Fluka) in 0.2 M PBS was added to the sample mixture. After that, 100 µl of 1.0 mM ascorbic acid (Sigma) and 10 µl of 0.1 M H_2O_2 (Wako) in deaerated water were added to the mixture. After incubation at 37℃ for 10 minutes, 1.0 ml cold 2.8 % (w/v) trichloroacetic acid (Sigma) was added to the mixture followed by 0.5 ml 1 % thiobarbituric acid (Sigma). The mixture was boiled at 100℃ for 8 min. After cooling to room temperature, the absorbance of mixture was measured at 532 nm. Distilled water was used as negative control while dimethyl sulphoxide (DMSO) was used as standard. All the samples were done in triplicate. Hydroxyl radical scavenging activity (%) was plotted against different concentrations of extracts.

Hydroxyl radical scavenging activity (%)

$= [1-(Ab_{532} \text{ of sample}/Ab_{532} \text{ of negative control})] \times 100\%$ (2.8)

2.2.2.3 Hydrogen peroxide scavenging activity

Principle

Measurement of hydrogen peroxide is based on the method described by Gupta (1973) and modified by Jiang *et al.* (1990). It relies upon the rapid peroxide-mediated oxidation of ferrous (Fe^{2+}) to ferric ion (Fe^{3+}) (catalysed by sorbitol) under acidic conditions followed by reaction of the Fe^{3+} with the dye, xylenol orange. In the assay, peroxide first reacts with sorbitol, converting it to a peroxyl radical, which in turn initiates the oxidation of Fe^{2+} to Fe^{3+}. In a sulfuric acid solution (H_2SO_4), Fe^{3+} complexes with the xylenol orange to form a purple product. The complex absorbs strongest at a wavelength between 540 and 580 nm but sill measurable up to 620 nm. When using microplates, the best wavelength for measurement is 595 nm (best signal-to-noise). When samples are added to fixed concentration of H_2O_2, the decrease in the intensity of purple color represents the H_2O_2 scavenging activity of samples. This method could also be used for determining the concentration of H_2O_2 in sample by constructing a standard curve using H_2O_2 standards with known concentration.

Procedures

The hydrogen peroxide scavenging activity of extracts was measured using a commercial kit (PeroXOquant™ Quantitative Peroxide Assay Kits, Pierce, 23280). Working reagent was prepared by mixing 1 volume of Reagent A (25 mM ammonium ferrous (II) sulfate, 2.5 M H_2SO_4) with 100 volumes of Reagent B (100 mM sorbitol, 125 µM xylenol orange in water). Various concentrations of H_2O_2 (0-1000 µM) were prepared as standard from 30 % hydrogen peroxide solution (Wako). Extracts with different concentrations (50-5000 µg/ml for CFAa, 500-5000

μg/ml for others) were first mixed with 0.1 mM (0.1 and 0.2 mM for CfAa) H_2O_2 for 15 min before measurement. Twenty microliters of sample (or standard) was mixed with 200 μl working reagent in a microplate. Then the plate was incubated at room temperature for 15-20 min to allow the reaction to reach the endpoint. The absorbance was measured at 595 nm using a microplate reader (SPECTRA max 250, Gene, US). The concentration of H_2O_2 in sample can be calculated by reference to its assay absorbance compared with the standard curve.

% scavenging activity = [1-(Ab_{595} of sample/Ab_{595} of H_2O_2)] x 100% (2.9)

2.2.3 Total phenolic content

Principle

A spectrophotometric-enzymatic method was established for the determination of total phenolic content in tea and wine by Stevanato *et al.* (2004). This method has been modified by Ma & Cheung (2007) for the determination of total phenolic content in mushrooms. This assay is based on the peroxidase-catalyzed reaction between hydrogen peroxide and phenolic compound in sample, producing phenoxyl radicals that can react with aromatic substrates (e.g. 4-aminophenazone) to form intensely colored complex with a maximum absorption at 500 nm. The absorbance of the reaction is proportional to the amount of phenolic compounds. The reaction is shown in Figure 2.5 Compared with commonly used Folin-Ciocalteau method, this assay is more specific and is not affected by the common interfering substances such as ascorbate, citrate and sulfite (Ma & Cheung, 2007).

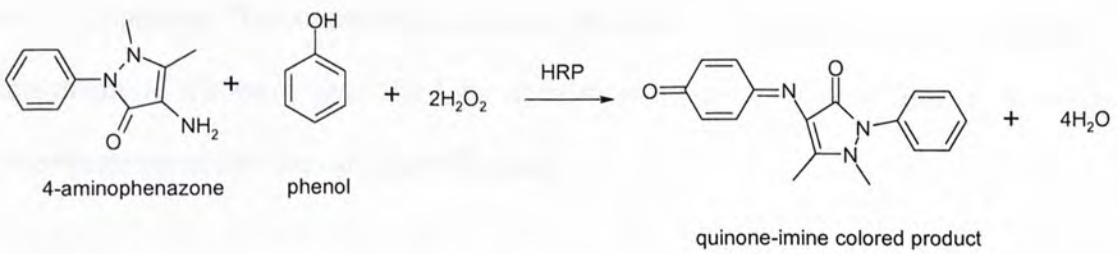

Figure 2.5 Enzymatic reaction for the determination of total phenolic content

HRP: Horseradish peroxidase (HRP).

Procedures

Total phenolic content of the crude extracts was determined by the enzymatic method described by Ma & Cheung (2007). In brief, 3 ml of 30 mM 4-aminophenazone (Sigma), 3 ml of 20 mM hydrogen peroxide, and 1.5 ml of 66 μM horseradish peroxidase (P2088) (Sigma), which were all freshly prepared with 0.1 M potassium buffer solution (pH 8.0), were mixed thoroughly and diluted to a total volume of 30 ml with the same buffer solution to form the reagent solution. They were mixed just before the experiment as the peroxidase was very sensitive and would lose its function after a couple of hours. The experiment was carried out in a 96-well microplate. To each well, 225 μl reagent solution and 25 μl mushroom extracts or phenolic standards (catechin) or blank (water) were added. The plate was then incubated at 37℃ and absorbance was taken at 500 nm at 15 min using a UV-visible microplate kinetics reader (SPECTRA max 250, Gene, US). Mushroom sample extracts of four different concentrations (10, 7.5, 5 and 2.5 mg/ml) were investigated. Each concentration of crude extracts, phenolic standards, and blank was done in triplicate. The concentrations of total phenolic content, expressed as Catechin Equivalent (CE), were quantified by a calibration curve plotted against known concentrations of catechin standard (Sigma).

2.2.4 Cytotoxicity of hydrogen peroxide

To induce oxidative damage, cells were first exposed to different concentrations of H_2O_2 in medium and then several parameters of cell damage were assessed including activity of intracellular dehydrogenases (MTT assay) and extracellular lactate dehydrogenase (LDH assay), DNA damage (Comet assay), lipid peroxidation (TBARS assay) and total protein loss. The results were compared with that of control (cell incubated with medium only without H_2O_2)

For comet assay, cells were treated with H_2O_2 with concentration range from 0.01-0.1 mM for 30 min. While for all the other assays, cells were treated with H_2O_2 with concentration range from 0.1-1 mM for 24 h. Residual H_2O_2 concentration in the culture medium after 24 h was also determined according to procedures in 2.2.2.3, in which 20 µl medium was mixed with 200 µl working reagent followed by measurement of absorbance at 595 nm. The H_2O_2 concentration remained in the culture medium was obtained from the standard curve.

2.2.5 Cytoprotectivity of mushroom extracts

To determine the protective effect of mushroom extracts against H_2O_2-induced oxidative damage, cells were co-incubated with mushroom extracts and H_2O_2. Then the parameters of cell damage mentioned in 2.2.4 except lipid peroxidation were used again to assess the protective effect of extracts. Concentration of H_2O_2 used in comet assay was 0.05 mM while that for other assays was 1 mM. Concentration range of samples under study was 100-1000 µg/ml. Samples were dissolved in medium and filter sterilized before applied to cells. To test whether the extracts themselves would exert any toxic effects, cells were also treated alone with extracts (100-1000 µg/ml), then MTT and LDH assay were used to assess the cytotoxicity.

2.2.6 Colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

Principle

This colorimetric assay was first described by Mosmann (1983). It measures cellular proliferation and survival, therefore, it can be used to assess cytotoxicity of substances. The principle of this assay is based on the use of a tetrazolium salt (MTT), which is a pale yellow substrate that produces a purple formazan product in the mitochondria of living cells but not dead cells. In the reaction, the tetrazolium ring is cleaved as shown in Figure 2.6 This reaction takes place only when the mitochondrial dehydrogenase enzymes are active, and therefore the intenisty of purple formazan produced is directly related to the number of viable cells. The absorbance can be measured at 570 nm after a solubilization solution (usually dimethyl sulfoxide or isopropanol) is added to dissolve the formazan into a colored solution. When the amount of purple formazan produced by cells treated with an agent is compared with that produced by untreated control cells, the effectiveness of the agent in causing cell death can be deduced.

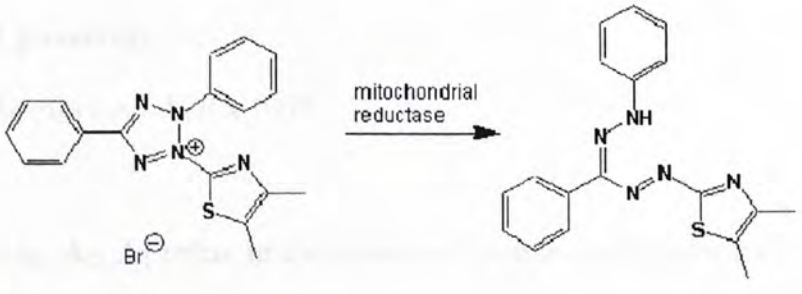

Figure 2.6 Cleavage of tetrazolium ring

Procedures

MTT assay was carried out to assess the viability of cells according to method described by Mosmann (1983) with some modifications. All treatments of cells were carried out 24 h after the cells were seeded in 96-well plates at 1×10^4 cells/well. Each treatment was performed in quintuplicate. After the treatments, 5 mg/ml MTT (Sigma, Cat. # M5655) solution in filtered PBS (pH 7.4) was applied to the each well of cells (10 µl/100 µl medium) and the cells were incubated for an additional 4 h at 37℃. Then, the medium was removed and 150 µl DMSO was added to dissolve the purple formazan crystals. Finally, the absorbance was measured at 570nm with a microplate reader.

Cell viability which reflects the results of cytotoxicity was expressed as % of control

$= (Ab_{570}$ of sample/Ab_{570} of untreated cell) x 100% (2.10)

A graph of cell viability (% of control) against different concentrations of mushroom extracts and H_2O_2 was plotted, respectively.

Results of cytoprotectivity of extracts against H_2O_2-induced damage were expressed as % of protection

$= [1-(A_C-A_S)/(A_C-A_T)] \times 100\%$ (2.11)

Where A_S, A_C, A_T refers to absorbance of sample (cell intoxicated with 1 mM H_2O_2 and treated with extracts), negative control (untreated cells) and positive control (cells intoxicated with 1mM H_2O_2) at 570 nm, respectively.

2.2.7 Lactate dehydrogenase (LDH) assay

Principle

Lactate dehydrogenase (LDH) is a soluble enzyme located in the cytosol. The enzyme is released into the surrounding culture medium following loss of membrane integrity resulting from either apoptosis or necrosis. Therefore, LDH activity can be used as an indicator of cell membrane integrity and thus a measurement of cytotoxicity.

LDH activity present in the culture medium is measured using a coupled two-step reaction (Haslam *et al.*, 2000). In the first step, LDH catalyzes the energetically unfavorable conversion of lactate and NAD^+ to pyruvate and $NADH+H^+$, respectively. In the second step, diaphorase uses the newly-formed $NADH+H^+$ to catalyze the reduction of a tetrazolium salt (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride) (INT) to a highly-colored formazan which absorbs strongly at 490-520 nm. The second step is energetically favorable and so LDH activity is rate-limiting. The amount of formazan formed is proportional to the amount of LDH released into the culture medium as a result of cytotoxicity.

Procedures

Release of LDH into the medium was assayed using a commercial kit (Cayman, Cat. #10008882) to provide a measure of membrane integrity. Cells were seeded in 96-well plate at a density of 1×10^4 cells/well. Treatments were carried out 24 h after and the final volume in each well was 120 µl. Ten milliliters of reaction solution (sufficient for use on one 96-well plate), was prepared by mixing 9.6 ml of the assay buffer and 100 µl of NAD^+ (100X), lactic acid (100X), INT (100X) and

reconstituted diaphorase, respectively. LDH standard with known activity (0-25 mU/ml) were prepared in medium. After treatment of cells, the plate was centrifuged at 400 x *g* for 5 min. Then 100 µl medium (supernatant)/LDH standard was added to another 96-well plate and mixed with 100 µl reaction solution. After incubation for 30 minutes at room temperature, the absorbance was measured at a wavelength of 490 nm using a microplate reader.

Absorbance value of the well containing medium only was subtracted from the absorbance values of all the other wells. Corresponding LDH activity (mU/ml) of samples were determined from standard curve (by plotting of absorbance at 490 nm as a function of LDH concentration). One Unit (U) is the amount of enzyme that catalyzes the reaction of 1 µmol of substrate per minute.

2.2.8 Total cellular protein loss

Protein content of cell lysates after treatments was determined and compared with that of untreated control to determine the total protein loss. This provided another measure of cellular integrity besides LDH activity. Cell lysates were prepared by lysis the cells in 100 µl lysis solution (Cell signaling, Cat # 9803) on ice for 60 min with vortex mix every 15 min. Then the supernatant was collected after centrifugation at 12000 x *g* for 5 min at 4 ℃. The procedures for protein determination can be referred to section 2.2.14.

2.2.9 Comet assay (Single cell gel electrophresis assay)

Principle

Comet assay can be used for evaluating DNA damage in cells as described by Singh *et al.* (1988). It is based upon the ability of denatured, cleaved DNA fragments to migrate out of the nucleus of cell toward the anode under the influence of an electric field, resulting in a "comet-like" appearance that can be visualized microscopically with fluorescent staining of the DNA. Undamaged DNA will remain within the confines of the nucleoid and so the extent of DNA migration will be smaller when a current is applied. Evaluation of the DNA "comet" tail shape and migration pattern provides an assessment of DNA damage. In this assay, cells are suspended in low melting point (LMP) agarose (generally at 37℃) and placed directly on slide precoated with a layer of regular agarose. After a gentle cell lysis, samples are treated with alkali solution to unwind and denature the DNA as well as hydrolyze sites of damage. The samples are then subjected to alkaline electrophoresis to induce DNA migration. Finally, DNA are stained with a fluorescent DNA intercalating dye and visualized by epifluorescence microscopy.

Data may be analyzed qualitatively by scoring the comets according to categories of small-to-large tail lengths. Quantitative and statistical data can also be obtained by analysis of the results using commercially available image analysis software packages which calculate a number of parameters such as tail length and tail moment (a measure of both the migration of the various DNA fragments forming the tail and their relative amounts of DNA).

Procedures

Cellular DNA damage was assessed by Comet assay using a commercial kit from Trevigen (Cat. # 4250-050-K). Cells were seeded in 12-well plate at a density of $1x10^5$ cells/well one day before treatments of cells. After the treatments, cells were harvested with Trypsin/EDTA solution (Hyclone, Cat. # SH30042.01) and washed twice with ice-cold phosphate buffer saline (PBS). Buffers should be chilled to 4℃ or on ice to inhibit endogenous damage occurring during sample preparation and to inhibit repair in the unfixed cells. PBS must be calcium (Ca^{2+}) and magnesium (Mg^{2+}) free to inhibit endonuclease activities. Trypan blue was then used to assess the cell viability. After washing the cells, 20 μl of cell suspension in PBS at $1x10^5$/ml was mixed with 200 μl of low melting point LMAgarose kept at 37℃ (The temperature of agarose is critical or the cells may undergo heat shock). Cell to agarose ratio was 1: 10 (v/v). Then, 75 μl of agarose/cell mixture was spread out onto each sample area on CometSlide™ using side of pipette tip. After that, the slides were placed flat at 4 ℃ in the dark for 10 minutes. A 0.5 mm clear ring could be observed at edge of CometSlide™ area (Increasing gelling time to 30 min improves adherence of samples in high humidity environments). Afterwards, the slides were immersed in prechilled lysis solution (2.5 M sodium chloride, 100 mM EDTA, pH 10, 10 mM Tris base, 1% sodium lauryl sarcosinate, and 1% Triton X-100) and left at 4℃ in the dark. Thirty minutes later, excess solution was tapped off from the slides and the slides were immersed in freshly prepared alkaline solution (pH>13) for 30 min at 4℃ in the dark. The slides were then transferred to a horizontal electrophoresis apparatus surrounding by ice bath. The slides were aligned equidistant from the electrodes and then the alkaline electrophoresis solution (300 mM NaOH, 1 mM EDTA, pH>13) was carefully poured until level just covers sample. The alkaline electrophoresis solution is a non-buffered system, performing the electrophoresis at cooler

temperatures (e.g. 4 ℃) will diminish background damage, increase sample adherence at high pHs and significantly improve reproducibility. To start the electrophoresis, the voltage was set to about 1 Volt/cm. Buffer was added or removed until the current became approximately 300 mA. The electropherosis was performed for 25 min. After electropherosis, the slides were rinsed by dipping several times in distilled water, and then immersed in 70 % ethanol for 5 min. Finally, the slides were air-dried.

To evaluate the DNA damage, the dried agarose/cell was stained with 50 µl SYBR® Green 1 nucleic acid gel staining solution and then visualized at 200x magnification using a fluorescence microscope (Nikon, Eclipse E-600) with an excitation filter of 450 nm to 490 nm. Fifty cells per treatment were selected at random (only cells that did not overlap were selected), their percentage of DNA in tail and the mean Olive Tail Moment were determined using an image analysis system (Komet 3.1 from Kinetics Imaging Ltd., Liverpool) linked to a CCD (Hitachi, KP-MIE/K monochrome CCD camera). Olive Tail Moment was automatically calculated for each cell according to the following equation.

Mean Olive Tail Moment = (Tail mean - Head mean) x % Tail DNA/100 (2.12)

2.2.10 Thiobarbituric Acid Reactive Substances (TBARS) assay

Principle

Measurement of TBARS is a well-established method for screening and monitoring lipid peroxidation, by which many researchers have modified to evaluate different types of samples (Ohkawa *et al.*, 1979; Draper *et al.*, 1993; Armstrong & Browne, 1994; Dawn-Linsley *et al.*, 2005). Lipid peroxidation is one of the indicators of cellular injury due to oxidative stress. Lipid peroxidation results in the formation of highly reactive and unstable lipid hydroperoxides. Decomposition of the unstable peroxides derived from polyunsaturated fatty acids would lead to the formation of a complex series of compounds including malondialdehyde (MDA), which reacts with thiobarbituric acid, forming a red-colored compound absorbing at 532nm. The more the MDA forms, the greater the extent of lipid peroxidation.

Procedures

Products of lipid peroxidation, mainly malondialdehyde (MDA) were measured by TBARS assay using a commercial kit (Cayman, Cat. # 10009055).

One million (1×10^6) cells were cultured in T-25 flask for one day. After treatments of cells, cells were collected and washed with PBS. Then cells were lysed in 120 µl lysis buffer (Cell signaling, Cat # 9803) on ice for 1 h with vortex mix every 15 min. Color reagent (sufficient for 24 samples) was prepared by dissolving 530 mg thiobarbituric acid (TBA) in 50 ml diluted acetic acid and 50 ml diluted sodium hydroxide. MDA standard (0-50 nmol/ml) was prepared for making the standard curve. For measurements, 10 µl of cell lysate was used for protein determination (refer to procedure in 2.2.14), 100 µl of cell lysate/standard was mixed with 100 µl of sodium dodecyl sulfate (SDS) solution, then 4 ml color reagent was added. The

mixture was boiled in 100℃ boiling water bath for 1 h. To stop the reaction, the mixture was placed in ice bath for 10 min. After centrifugation at 1600 x *g* at 4℃ for 10 min, 150 μl of supernatant was loaded into 96-well plate for measurement of absorbance at 532 nm using a microplate reader. Corresponding amount of TBA-reactive product (MDA) formed in sample was obtained from the standard curve and the results were expressed as nmol MDA/mg protein.

2.2.11 Preparation of cell lysate for evaluating cellular antioxidant defense system

In order to investigate whether the protective effect of CfAa on H_2O_2-induced damage involved the boosting of cellular antioxidant defense system, the total glutathione level and enzyme activities of cell lysate after treatment were measured. Cells at a number of 5 x 10^5 were cultured in T-25 flask for one day. Then the cells were co-incubated with CfAa (200-500 μg/ml) and H_2O_2 (0.2 mM/1 mM) for 24 h. Besides, cells were also treated with either CfAa (500 μg/ml) or H_2O_2 (0.2 and 1 mM) alone for 24 h. After treatments, cells were harvested by cell scrapers, cell suspensions were then centrifuged at 180 x *g* for 5 min and washed twice with ice-cold PBS. Supernatants were discarded and cell pelletes were lysed with 100 μl lysis solution (Cell signaling, Cat # 9803) on ice for 60 min with vortex mix every 15 min. After 60 min, cell lysate (supernatant) was obtained by centrifugation at 12000 x *g* for 5 min at 4 ℃. Aliquot of cell lysate was used for protein determination (refer to procedure in 2.2.14) and the remaining were used for determination of total glutathione level and enzyme activities. The cell lysates were stored at -80 ℃ when not in use.

2.2.12 Total Glutathione level

Principle

Glutathione level can be quantified by utilizing a carefully optimized enzymatic recycling method (Owens & Belcher, 1965; Eyer & Podhradský, 1986; Baker *et al.*, 1990) with the use of glutathione reductase. This method involves the reaction between sulfhydryl group (-SH) of GSH and DTNB (5,5'-dithio-bis-2-nitrobenzoic acid, Ellman's reagent), producing a yellow colored 5-thio-2-nitrobenzoic acid (TNB) which can be measured at either 405 or 414 nm. The mixed disulfide, GSTNB (between GSH and TNB) produced concomitantly, is reduced by glutathione reductase to recycle the GSH and produce more TNB. The rate of TNB production is directly proportional to this recycling reaction, hence, directly proportional to the concentration of GSH in the sample. Measurement of the absorbance of TNB provides an estimation of GSH in the sample. GSH is easily oxidized to the disulfide dimer GSSG (e.g. during the reduction of hydroperoxides by glutathione peroxidase). Because of the use of glutathione reductase which turns GSSH to GSH, both GSH and GSSG are measured and this reflects total glutathione. The assay can also be used to measure GSSG only by derivatizing GSH with 2-vinylpyridine before performing the assay (Griffith, 1980). Nearly all samples require deproteination before assay. The reactions are summarized in the Figure 2.7 below.

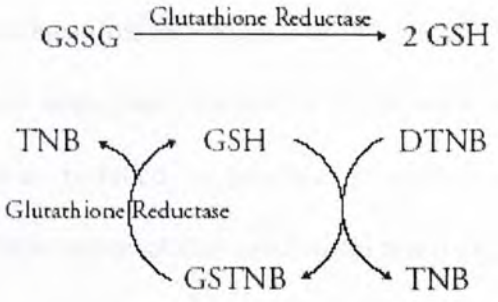

Figure 2.7 GSH recycling

Procedures

Glutathione level was measured using a commercial kit (Cayman, Cat. # 703002). Since almost all biological samples used for GSH measurement contain large amounts of proteins, it is necessary to remove as much protein as possible from the sample to avoid interferences due to particulates and sulfhydryl groups on proteins in the assay. Samples that are low in protein (<1 mg/ml) and are devoid of particulates can be assayed directly.

Cell lysate was deproteinzed by adding an equal volume of freshly prepared 10 % (w/v) metaphosphoric acid (Sigma-Aldrich 23927-5) to the sample. After vortex-mixing, the mixture was allowed to stand at room temperature for 5 min and then centrifuged at 2000 x *g* for 5 min. The supernatant was carefully collected without disturbing the precipitate. The supernatant at this stage can be stored for long periods of time (up to 6 months) at -20℃ without any degradation of GSH or GSSH. Just before the samples were assayed, 50 µl of 4 M solution of triethanolamine (Aldrich, Cat. # T5830-0) was added per ml of the supernatant and vortex mixed immediately. The triethanolamine solution will increase the pH of the sample. The sample was then ready for assay of total GSH (both oxidized and reduced form). Any necessary dilutions of the sample (whenever GSH level beyond the range in standard curve) were done at this stage with MES buffer (0.2 M 2-(N-morpholino)ethanesulphonic acid, 0.05 M phosphate, and 1 mM EDTA, pH 6.0). GSSG standard with concentration 0-8 µM were prepared. Under the assay condition, GSSG was reduced to produce 2 mole-equivalents of GSH, so the equivalent total GSH concentrations used in standard curve were 0-16 µM.

Fifty microliters of standard or sample was added in the designated wells on 96-well

plate. Assay cocktail sufficient for use in one plate was prepared by mixing 11.25 ml MES buffer, 0.45 ml reconstituted cofactor mixture (containing $NADP^+$ and glucose-6-phophate), 2.1 ml reconstituted enzyme mixture (containing glutathione reductase and glucose-6-phophate dehydrogenase), 2.3 ml water, and 0.45 ml reconstituted DTNB (5,5'-dithiobis-2-nitrobenzoic acid, Ellman's reagent). Then, 150 µl of the freshly prepared assay cocktail (within 10 min of preparation) was added to each of the wells containing standards/samples using a multi-channel pipet. The plate was incubated in the dark on an orbital shaker and the absorbance was measured at 405 nm using a plate reader at 25 min.

Total GSH concentration for each sample was determined from the standard curve and the value was multiplied by "2" to account for the addition of metaphosphoric acid in deproteination of samples. If samples were diluted before assaying, the dilution factor should be taken into account. The total GSH concentration was then divided by mg protein in cell lysate.

2.2.13 Enzyme activity

2.2.13.1 Catalase (CAT)

Principle

Catalase activity can be determined using the method described by Johansson & Borg (1988). It is based on the peroxidatic function of CAT, in which low molecular weight alcohols serve as electron donors (equation 1.16). While the aliphatic alcohols serve as specific substrates for CAT, other enzymes with peroxidatic activity do not utilize these substrates. The assay involves the reaction of CAT with methanol in the presence of an optimal concentration of H_2O_2 resulted in the production of formaldehyde which can be measured spectrophotometrically with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald) as the chromogen. Purpald specifically forms a bicyclic heterocycle compound with aldehydes, which changes from colorless to a purple color upon oxidation. The absorbance can be measured at 540 nm.

Procedures

Catalase activity was measured using a commercial kit (Cayman, Cat. # 707002). The experiment was carried out in a 96-well microplate. In each of the well, 100 µl of assay buffer (100 mM potassium phosphate, pH 7.0), 30 µl of methanol, and 20 µl of formaldehyde standard (0-75 µM final concentration in 170 µl reaction)/positive control (bovine liver CAT)/sample were added. Reactions were initiated the by adding 20 µl of hydrogen peroxide (0.035 M) to all the wells being used. Hydrogen peroxide was added as quickly as possible and the precise time that the reaction was initiated was noted. The plate was then incubated on a shaker for 20 min at room temperature. After that, 30 µl of potassium hydroxide solution (10 M) was added to each well to terminate the reaction followed by the addition of 30 µl of

Purpald (chromogen). The plate was incubated for another 10 min at room temperature on the shaker. Lastly, 10 µl of potassium periodate solution was added to each well. After incubation at room temperature on a shaker for 5 min, the absorbance was read at 540 nm using a plate reader.

A standard curve of absorbance of standards as a function of formaldehyde concentration (µM) was plotted. Formaldehyde concentration of samples was calculated using the equation obtained from the linear regression of the standard curve according to the formula below.

$$\text{Formaldehyde concentration } (\mu M) = \frac{(\text{sample absorbance} - \text{y-intercept})}{\text{slope of the curve}} \times \frac{0.17 \text{ ml}}{0.02 \text{ ml}} \quad (2.13)$$

Catalase activity of sample was calculated using the following equation. One unit is defined as the amount of enzyme that will cause the formation of 1.0 nmol of formaldehyde per minute at 25℃. Samples containing CAT activity outside the range in standard curve were diluted and the dilution factor was multiplied.

$$\text{CAT activity (nmol/min/mg protein)} = \left(\frac{\mu M \text{ of formaldehyde in sample}}{20 \text{ min}} \right) \times \frac{\text{Sample dilution}}{\text{mg protein in cell lysate}} \quad (2.14)$$

2.2.13.2 Glutathione peroxidases (GPx)

Principle

Glutathione peroxidase activity can be measured indirectly by a coupled reaction with glutathione reductase (Pagalia & Valentine, 1967). Upon reduction of hydroperoxide by GPx, GSH is oxidized to GSSG (2.15), which is then recycled to its reduced state by GR and $NADPH+H^+$ (2.16). The oxidation of $NADPH+H^+$ to $NADP^+$ is characterized by a decrease in absorbance at 340 nm. Under conditions that the GPx activity is rate-limiting, the rate of decrease in absorbance is directly proportional to the GPx activity in the sample.

$$ROOH + 2GSH \xrightarrow{GPx} ROH + GSSG + H_2O \qquad (2.15)$$

$$GSSG + NADPH + H^+ \xrightarrow{GR} 2GSH + NADP^+ \qquad (2.16)$$

Procedures

Glutathione peroxidase activity was determined using a commercial kit (Cayman, Cat. # 703102). The experiment was carried out in a 96-well microplate. In each of the well, 100 µl of assay buffer (50 mM Tris-HC1, pH 7.6, 5 mM EDTA), 50 µl of co-substrate mixture (containing NADPH, glutathione, and glutathione reductase) and 20 µl of sample/positive control (bovine erythrocyte GPx) were added. Background (non-enzymatic) activity should be subtracted from sample activity, so background well was set by adding 120 µl of assay buffer and 50 µl of co-substrate mixture. Reactions were initiated by adding 20 µl of cumene hydroperoxide solution to all the wells being used. Cumene hydroperoxide was added as quickly as possible and the precise time that the reaction was initiated was noted. The plate was carefully shaken

for a few seconds to mix. Then, the absorbance was read once every minute at 340 nm using a plate reader to obtain at least 5 time points (The initial absorbance of the sample wells should not be above 1.2 or below 0.51). The amount of GPx added to the well should cause an absorbance decrease between 0.02 and 0.135/min, when necessary, samples should be diluted or concentrated.

The change (decrease) in absorbance (ΔA_{340}) per minute was determined by plotting the absorbance values as a function of time to obtain the slope (rate) of the linear portion of the curve. The rate of ΔA_{340}/min for the background or non-enzymatic well was subtracted from that of the sample wells.

GPx activity of samples was calculated using the following formula (2.17). The reaction rate at 340 nm can be determined using the NADPH extinction coefficient of 0.00373 μM^{-1} (the actual extinction coefficient for NADPH at 340 nm is 0.00622 $\mu M^{-1}cm^{-1}$, the value used has been adjusted for the pathlength of the solution in the well (0.6 cm)). One unit is defined as the amount of enzyme that will cause the oxidation of 1.0 nmol of NADPH to $NADP^+$ per minute at 25℃.

$$\text{GPx activity (nmol/min/mg protein)} = \frac{\Delta A_{340}/\text{min}}{0.00373\ \mu M^{-1}} \times \frac{0.19\ \text{ml}}{0.02\ \text{ml}} \times \frac{\text{Sample dilution}}{\text{mg protein of cell lysate}} \quad (2.17)$$

2.2.13.3 Glutathione Reductase (GR)

Principle

Glutathione Reductase activity can also be determined according to the reaction above (2.16) by measuring the rate of decrease in absorbance at 340 nm, which is directly proportional to the GR activity of sample as GR is present at rate-limiting concentration.

Procedures

GR activity in cell lysate was determined using a commercial kit (Cayman, Cat. # 703202). In each of the well, 100 µl of assay buffer (50 mM potassium phosphate, pH 7.5, 1 mM EDTA), 20 µl of GSSG solution (9.5 mM) and 20 µl of sample/positive control (Baker's yeast GR) were added. For background or non-enzymatic well, 120 µl of assay buffer and 20 µl of GSSG solution were added. Reaction was initiated by adding 50 µl of NADPH to all of the wells being used. NADPH was added as quickly as possible and the precise time that the reaction started was noted. The plate was carefully shaken for a few seconds to mix. Then, the absorbance was read once every minute at 340 nm using a plate reader to obtain at least 5 time points. The initial absorbance of the sample wells should not be above 1.2 or below 0.5. To obtain reproducible results, the amount of GR added to the well should cause an absorbance decrease between 0.008 and 0.1/min. When necessary, samples should be diluted or concentrated.

GR activity of samples was calculated in the same way as GPx activity (2.17).

2.2.13.4 Superoxide dismutase (SOD)

Principle

Activity of all three types of SOD (Cu/Zn-, Mn-, and Fe-SOD) is assessed by using a tetrazolium salt for the detection of the dismutation of superoxide radicals generated by xanthine oxidase and hypoxanthine (Nishikimi *et al.*, 1972). Tetrazolium salt reacts with $O_2^{\cdot-}$ to produce formazan dye which can be measured at 450 nm. Linearized rate was calculated by dividing sample absorbance from control absorbance. SOD activity in sample is obtained from a standard curve (A plot of linearized SOD standard rate as a function of final SOD activity) prepared by using SOD standard with known SOD activity. One unit of SOD is defined as the amount of enzyme needed to exhibit 50 % dismutation of the superoxide radical.

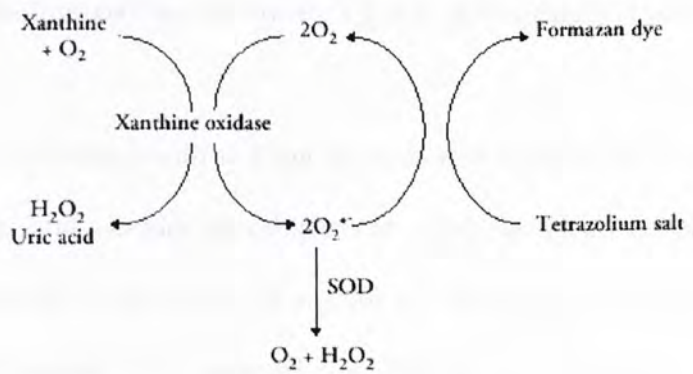

Figure 2.8 Reactions for detection of SOD activity

Procedures

Total superoxide dismutase activity (cytosolic and mitochondrial) in cell lysate was determined using a commercial kit (Cayman, Cat. # 706002). SOD standards (bovine erythrocyte SOD (Cu/Zn)) with final activity 0-0.25 U/ml were prepared. For experiments, 200 µl of the diluted radical detector (tetrazolium salt solution) and 10

µl of sample/standard were added per well on 96-well plate. Each standard and sample was run in triplicated. Reaction was initiated by adding 20 µl of diluted xanthine oxidase to all of the wells being used. Xanthine oxidase was added as quickly as possible. For assaying sample background, 20 µl of sample buffer (50 mM Tris-HC1, pH 8.0) instead of xanthine oxidase was added. The plate was carefully shaken for a few seconds to mix. After incubation on a shaker for 20 minutes at room temperature, the absorbance was measured at 450 nm using a plate reader. Sample should be diluted or concentrated if its enzymatic activity did not fall within the standard curve range.

Average absorbance of each standard and sample was calculated. Sample background absorbance was subtracted from the sample absorbance generated in the presence of xanthine oxidase for correcting non-SOD generated absorbance.

Absorbance of standard with 0 U/ml activity was divided by itself and by all the other standards and samples absorbances to yield the linearized rate (LR) (i.e., LR for Std 0 = Abs Std 0/Abs Std 0; LR for Std 1 = Abs Std 0/Abs Std 1).

A graph of linearized SOD standard rate (LR) as a function of final SOD activity (U/ml) was plotted. SOD activity of the samples was calculated using the equation obtained from the linear regression of the standard curve substituting the linearized rate (LR) for each sample. One unit is defined as the amount of enzyme needed to exhibit 50 % dismutation of the superoxide radical.

$$\text{SOD activity (U/mg protein)} = \frac{\text{sample LR - y-intercept}}{\text{slope}} \times \frac{0.23\ \text{ml}}{0.01\ \text{ml}} \times \frac{\text{Sample dilution}}{\text{mg protein in cell lysate}} \quad (2.18)$$

2.2.14 Determination of protein

Protein content of cell lysate was quantified by BCA™ protein assay kit (Pierce, Cat. # 23225). A set of bovine serum albumin (BSA) standards at concentration of 0 (blank), 25, 50, 100, 200, 400 and 1000 μg/ml was prepared in distilled water. Working reagent (WR) was prepared by mixing 50 parts of BCA™ Reagent A (containing sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1 M sodium hydroxide) with 1 part of BCA™ Reagent B (containing 4% cupric sulfate). Ten microliters of sample or standard (duplicate) was added into a microplate well and mixed with 200 μl working reagent (sample to WR ratio = 1:20). The microplate was then incubated at 37℃ for 30 min and the absorbance was measured at 562 nm. Absorbance of blank standard was subtracted from that of all the other standards and samples. Standard curve was constructed by plotting the average Blank-corrected absorbance for each BSA standard against its concentration in μg/ml. The corresponding protein concentration in samples was obtained from the standard curve. The sample was diluted with distilled water whenever its absorbance was outside the range of standard curve.

2.2.15 Statistical analysis

The data of the experiments were presented as means ± standard deviation (SD). The variability between controls and samples were compared using Student's t-test while multiple comparisons between various treatments were investigated by one-way ANOVA together with Tukey's method using SPSS software. Differences with $p<0.05$ were considered statistically significant.

Chapter 3 Results and discussions

3.1 Extraction yield

The yield of four extracts from *A. aegerita* was from 34.6 to 55.6 % as shown in Table 3.1 following the two extractions with hot water (70℃) and cold water (ambient, 25℃). The high extraction yield (> 30 %) suggested that most of the compounds in *A. aegerita* were water-soluble. The results also reflected that using hot water as an extraction medium generally resulted in higher yield of extracts than using cold water, which implied that there were more hot water-soluble components in the mushroom. For fresh *A. aegerita*, yield of hot water extract (HfAa) was 21 % higher than that of cold water extract (CfAa), while for dried *A. aegerita*, yield of hot water extract (HdAa) was 15.1 % higher that of cold water extract (CdAa).

The moisture content of fresh *A. aegerita* was found to be 90.8 %, which was comparable with that (89.4 %) obtained by Lin *et al.*, (2006). The high moisture content indicated that water contributed most of the weight of the fresh mushroom, and the dried matter in the mushroom was just about 10 %. This implied that the amount of dry matter in the fresh *A. aegerita* extracts (both HfAa and CfAa) obtained from 30 g fresh *A*a was small (< 2 g). Therefore, it was more representative to express the extraction yield on a dry weight basis (i.e. weight of extract/weight of dry matter of mushroom). In this way, it was found that the yield of cold and hot water extract in terms of percentage dry matter for fresh *A. aegerita* (34.6 and 55.6 %, respectively) was similar to those for dried *A. aegerita* (38.0 and 53.1 % respectively) (Table 3.1).

According to a study by Tsai *et al.* (2006), extraction yield of hot water extract from fresh *A. aegerita* fruiting bodies was found to be 42.2 % (w/w) on dry weight basis, which was lower than that (HfAa) obtained in this study (55.6 %). In the study by Lin

et al., (2006), extraction yield of cold water (ambient temperature) extract for fresh *A. aegerita* fruiting bodies was 47.7 % (w/w) on dry weight basis, which was higher than that (CfAa) obtained in this study (34.6 %). The difference in extraction yield between the previous studies and the present one may be due to the difference in the source of mushroom, sample preparation and extraction procedures. For the source of mushrooms, in both of the above studies, mushrooms were from Taiwan while the fresh mushrooms used in this study were purchased in local market produced from Mainland China. For the sample preparation and extraction procedures, in the study by Tsai *et al.* (2006), hot water extract of fresh *A. aegerita* was obtained by first lyophilizing the sample, followed by milling into fine powder before extracted by deionised water (10 g sample in 200 ml water) at reflux for 3 hours, and the residue was refluxed with two additional 100 ml portions of deionised water for 3 hours. In the study by Lin *et al.* (2006), 200 g of fresh mushroom was ground in a Waring blender for 3 min with 400 ml water and the resulting mixture was stirred for 30 min at ambient temperature to obtain the water extract. In the present study, 30 g of fresh *A. aegerita* was extracted by first homogenizing in a Waring blender and then suspended in 600 ml distilled water with continuous stirring at 70℃ and ambient temperature (~25℃) for 3 hours to obtain the HfAa and CfAa, respectively. The differences in extraction ratio (sample to extraction medium) and extraction time when comparing the previous studies with the present study could probably contribute to the difference in extraction yields.

One of the advantages for studying water extracts is that they can be conveniently applied to cell culture study. The purpose of using both cold and hot water for extraction was to evaluate the effect of high temperature on antioxidant properties of extracts based on the assumption that some heat-labile bioactive components may be

destroyed by heat. The use of hot water to extract soluble components simulated the making of Chinese medicine and the brewing of herbal tea. Therefore, comparing to other solvent extracts, the information obtained by using water extracts would be more valuable for these products to be used in human diets. Since both dried and fresh *A. aegerita* were commonly used as food, it was worth to compare their antioxidant properties in terms of difference in the antioxidant components in dried and fresh *A. aegerita*.

Table 3.1 Extraction yield of different crude water extracts from *Agrocybe aegerita*

Sample	Yield (% dry matter)
Cold water extract of fresh Aa (CfAa)	34.6 %
Hot water extract of fresh Aa (HfAa)	55.6 %
Cold water extract of dried Aa (CdAa)	38.0 %
Hot water extract of dried Aa (HdAa)	53.1 %

Data are mean values obtained from duplicate extractions

3.2 Chemical assays for *in vitro* antioxidative properties of mushroom extracts

3.2.1 $ABTS^{\cdot+}$ scavenging activity

This assay has been adopted by many research laboratories for studying antioxidant capacity of a wide variety of food samples due to its operational simplicity and high sensitivity (Huang *et al.*, 2005). The $ABTS^{\cdot+}$ could be specifically detected at 734 nm, a wavelength far from the visible region so that less interferences such as sample color would affect the result. The assay aims at determining the antioxidant activity of hydrogen-donating antioxidants (scavengers of aqueous phase radicals) and chain-breaking antioxidants. One of the drawbacks of this assay is that this radical would not be generated physiologically.

Figure 3.1 shows the scavenging activity of ABTS radical cation of the four water crude extracts of *A. aegerita* in terms of TEAC, represented by mM Trolox having the same scavenging ability of 1 mg of dried sample. A higher TEAC value indicates a greater scavenging activity of ABTS radical cation. TEAC for CfAa, HfAa, CdAa and HdAa was 12.4, 13.4, 12.9 and 12.9 mM Trolox/mg sample, respectively. Although HfAa showed slightly higher TEAC value, there was no significant difference ($p>0.05$) in scavenging activity of ABTS radical cation between the four extracts.

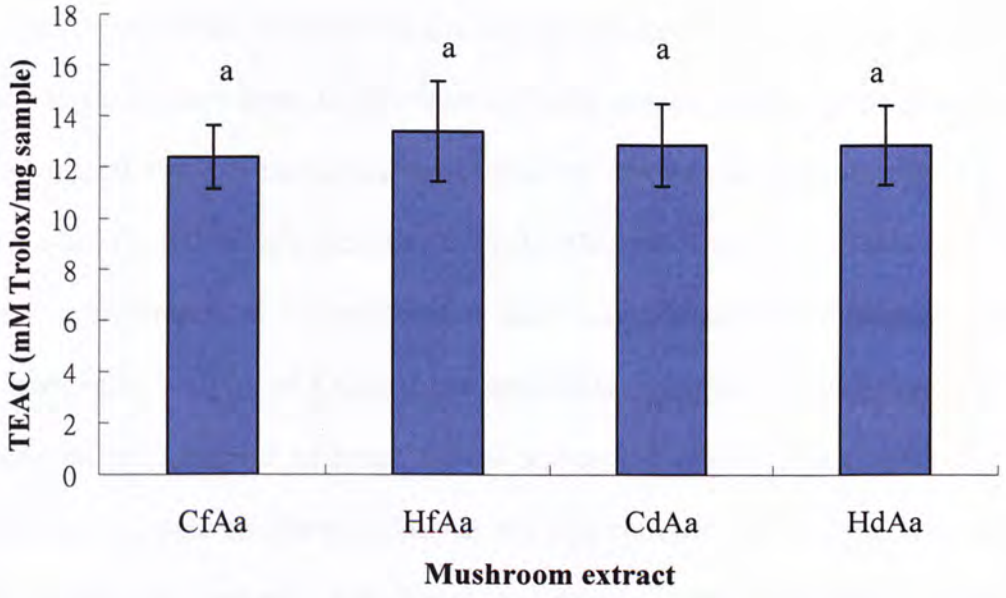

Figure 3.1 ABTS radical cation scavenging activity of different water extracts of Aa. Results are expressed as Trolox Equivalent Antioxidant Capacity (TEAC). Data are means ± SD (n=12). Same letter indicates that there is no significant difference at $p>0.05$ between different extracts (One-way ANOVA, Tukey's multiple comparison).

3.2.2 Hydroxyl radicals scavenging activity

Hydroxyl radical is the most active radical which can attack many macromolecules such as DNA and proteins, resulting in mutagenesis and carcinogenesis of cells (Lee & Jang, 2004). Therefore, evaluation of the scavenging activity of hydroxyl radical is essential in the determination of the antioxidant activity of a dietary antioxidant. This assay aims at measuring the hydroxyl radical scavenging activity based on the inhibition of the hydroxyl radical generated in Fenton reaction to degrade the deoxyribose which would yield a pink chromogen upon heating with thiobarbituric acid (TBA) at low pH. Hydroxyl radical scavengers react with hydroxyl radicals prior to their attack to deoxyribose and diminish the chromogen formation. An antioxidant that reduces the absorbance in the reaction mixture indicates that it is a strong hydroxyl radical scavenger.

Figure 3.2 and Table 3.2 show the scavenging activity of hydroxyl radical of the four water crude extracts from *A. aegerita* at different concentrations. At the concentration of 10 mg/ml, the scavenging activity of four extracts was significantly different from each other ($p<0.05$), with the order of HfAa>HdAa>CdAa>CfAa (Table 3.2). While at the concentration of 2.5 and 5mg/ml, there was no significant difference ($p>0.05$) in scavenging activity of CfAa, CdAa and HdAa. It could be concluded that HfAa possessed the strongest hydroxyl radical scavenging activity, but that for the other three extracts were similar especially at low concentration. Scavenging activity of all four mushroom extracts was found to increase with concentration, with the correlation coefficient (R^2) ranging from 0.84-0.95 (Figure 3.2), suggesting that the amount of antioxidant compounds which scavenge the hydroxyl radical increased with concentration. Hydroxyl radical scavenging activity of methanol extract from *A. aegerita* reported by Huang *et al.* (2002) was 3.82 % at 5 mg/ml, which was much lower than that of all the four water extracts (42.29-51.93%) used in this study at the same concentration. Therefore, the water extracts of Aa may be more effective than methanol extract of Aa with regard to scavenging ability on hydroxyl radical. In addition, it was reported that methanol extracts from some ear mushrooms were not good scavengers for hydroxyl radicals (Mau *et al.*, 2001).

When comparing the positive control (DMSO) with the water crude extracts from *A. aegerita*, the hydroxyl radical scavenging activity of DMSO (88.5 %) (Table 3.2) was almost two times higher than that of all the extracts. However, DMSO was not a common antioxidant and some common antioxidants such as Trolox (250 μM) also showed low hydroxyl radical scavenging activity (40%) (Racchi, *et al,* 2002).

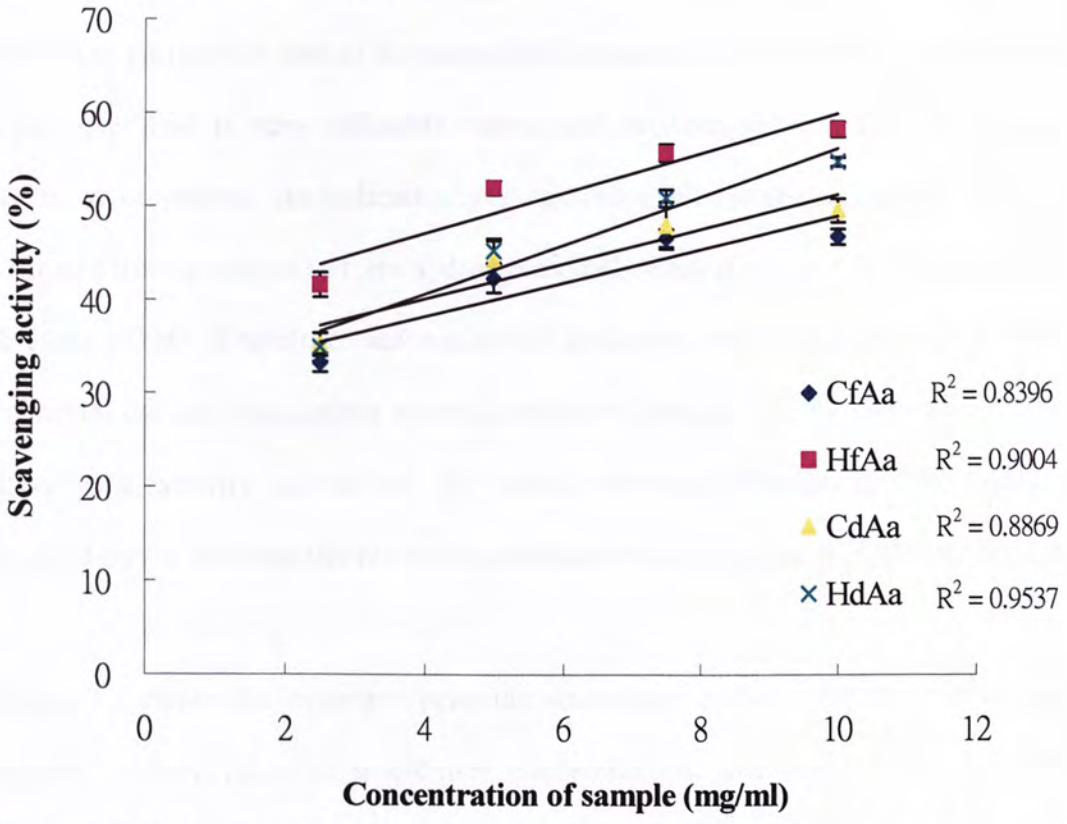

Figure 3.2. Hydroxyl radicals scavenging activity (%) of different water extracts of Aa at different concentrations. Data points are means ± SD (n=3).

Table 3.2 Hydroxyl radicals scavenging activity (%) of different water extracts from Aa at different concentrations and DMSO

Concentration of extract (mg/ml)	%			
	2.5	5	7.5	10
CfAa	33.2 ± 1.00^{a}	42.3 ± 1.54^{a}	46.7 ± 0.95^{a}	46.9 ± 0.83^{a}
HfAa	41.6 ± 1.32^{b}	51.9 ± 0.76^{b}	55.8 ± 0.95^{b}	58.4 ± 0.82^{b}
CdAa	35.2 ± 1.26^{a}	44.3 ± 2.03^{a}	48.0 ± 2.53^{ac}	49.7 ± 1.25^{c}
HdAa	34.9 ± 1.54^{a}	45.2 ± 1.30^{a}	50.9 ± 0.94^{c}	54.9 ± 0.48^{d}
DMSO (3 mg/ml)	88.5 ± 0.933			

Data are means ± SD (n=3). Different superscripts within the same concentration (column) represents significant difference between samples ($p<0.05$) (One-way ANOVA, Tukey's multiple comparison).

3.2.3 Hydrogen peroxide scavenging activity

Hydrogen peroxide is one of the major ROS generated in our bodies, it mixes easily with water and is very diffusible within and between cells *in vivo*. It is also a precursor of various free radicals (e.g. it can cross cell membranes rapidly and react with free iron or copper to form hydroxyl radical which is much more damaging (Lee & Jang, 2004). Therefore, scavenging of hydrogen peroxide may be one of the important defense mechanism to avoid oxidative damage. The measurement of H_2O_2 scavenging activity is one of the useful methods determining the ability of antioxidants to decrease the level of prooxidants H_2O_2 (Czochra & Widensk, 2002).

Figure 3.3 shows the hydrogen peroxide scavenging activity (%) of the four water extracts from *A. aegerita* at different concentrations. The results show that CfAa exhibited the strongest scavenging activity against 0.1 mM H_2O_2, as compared with the other three extracts. There is no significant difference ($p>0.05$) in H_2O_2 scavenging activity for CfAa at a concentration range of 0.5-5 mg/ml, indicating that the scavenging activity of CfAa in this concentration range was not dose-dependent. The incubation of 0.1 mM H_2O_2 with CfAa at the minimum concentration applied (0.5 mg/ml) for 15 minutes could already result in nearly 100 % scavenging activity (Figure 3.3).

Based on this finding, smaller concentrations of CfAa were used to see if the scavenging activity was dose-dependent and the results were shown as Figure 3.4. The results indicated that the scavenging activity of CfAa increased with concentration in the range of 50-500 µg/ml. When comparing the scavenging activity of CfAa against 0.1 and 0.2 mM H_2O_2, higher concentration of CfAa was required to exhibit the same percentage scavenging activity for 0.2 mM H_2O_2 (Figure 3.4). For

the curve of 0.1 mM H_2O_2, although the curve appeared to be parabola, it was believed to be a straight line if the last data point was not taken into account. As the maximum scavenging activity had been reached at a concentration smaller than 500 µg/ml, so the activity was saturated at 500 µg/ml, leading to the leveling of the curve. However, for 0.2 mM H_2O_2, the maximum scavenging activity of CfAa was reached at concentration of 500 µg/ml or larger, so the curve was a straight line.

As the strong scavenging activity of H_2O_2 was seen only in CfAa, this suggested that the component in this extract responsible for scavenging the H_2O_2 is heat-labile, and might not be present in dried *A. aegerita*.

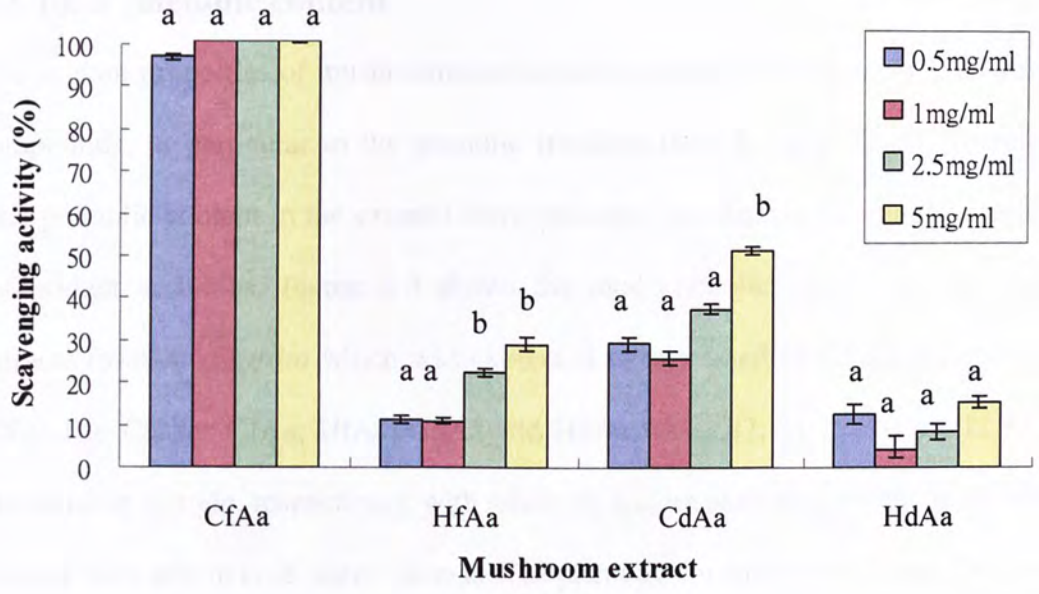

Figure 3.3 Hydrogen peroxide scavenging activity (%) of different water extracts of Aa at different concentrations. 0.1 mM H_2O_2 (final concentration) was mixed with different concentrations of Aa extracts for 15 min, then scavenging activity of extracts was measured. Data are means ± SD (n=3). Different letters represent significant difference between concentrations for the same extract (One-way ANOVA, Tukey's multiple comparison, $p<0.05$).

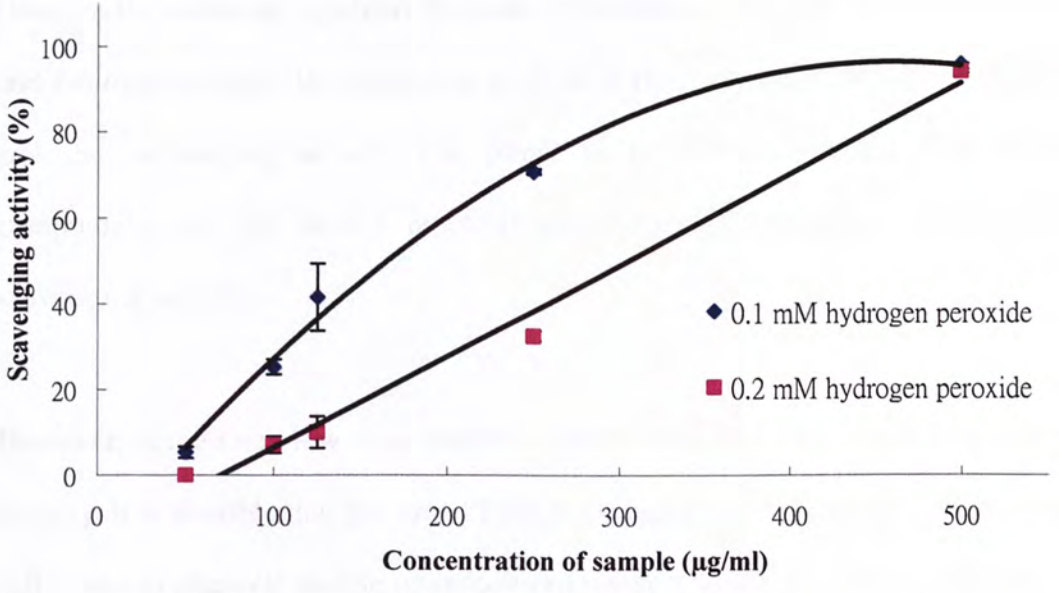

Figure 3.4 Scavenging activity (%) of CfAa at different concentrations against 0.1 and 0.2 mM hydrogen peroxide. Data are means ± SD (n=3).

3.3 Total phenolic content

Antioxidant properties of mushrooms are usually correlated to low-molecular-weight compounds, in particular to the phenolic fractions (Lee & Jang, 2004). Therefore, total phenolic content in the extracts were measured to estimate their involvement in antioxidant activities. Figure 3.5 shows the total phenolic content in four water extracts from *A. aegerita* which was expressed in expressed in Catechin Equivalent (CE). The CE for CfAa, HfAa, CdAa and HdAa was 8.97, 11.79, 10.95, 12.98 μg catechin/mg sample, respectively, with relatively higher phenolic content in hot water extracts than that in cold water extracts. The phenolic content of HfAa and CdAa was not significantly different ($p>0.05$).

As the total phenolic content of CfAa was the lowest among the four extracts, it might reflect that the strong H_2O_2 scavenging activity demonstrated in CfAa was not attributed to its phenolic fraction. Lee & Jang, (2004) have studied the hydrogen peroxide scavenging activity of water extracts from five edible mushrooms (*Volvariella volvacea, Agaricus bisporus, Flanvnulina velutipes, Pleurotus ostreatus* and *Lentinus edodes*), the regression coefficient (R^2) between total soluble phenolics and the scavenging activity was found to be 0.49, suggesting that phenolic compounds may not be the potential antioxidative components involved in the scavenging activity.

However, sometimes low total phenolic content may not necessarily result in low activity. It is possible that the strong H_2O_2 scavenging activity of CfAa was due to the difference in phenolic profile (composition) rather than the quantity of total phenolic compounds, so further investigation was needed to confirm this.

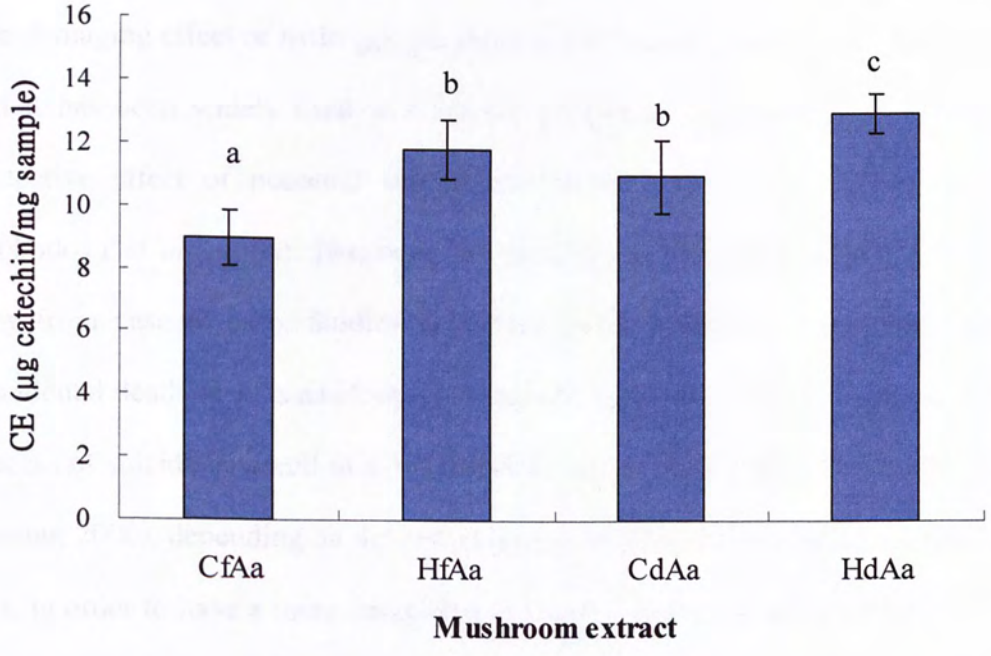

Figure 3.5 Total phenolic content (CE) of cold and hot water extracts of dried Aa and fresh Aa. Data are means ± SD (n=12). Different letters represent significant difference between different extracts ($p<0.05$). (One-way ANOVA, Tukey's multiple comparison).

3.4 Cytotoxicity of hydrogen peroxide

The damaging effect of hydrogen peroxide is well-known as described in chapter one and it has been widely used as a model to generate oxidative stress for studying protective effect of potential antioxidants (Roig *et al.*, 2002; Liu *et al.*, 2007; Svobodova *et al.*, 2006). However, the toxicity and damaging effect of H_2O_2 may vary from case to case. Studies have shown that H_2O_2 can induce both necrosis (accidental death of cells and living tissues) and apoptosis (programmed cell death - a process of suicide by a cell in a multicellular organism) (Chandra *et al.*, 2000; Lee & Shacter, 2000), depending on the concentration and the cell type being studied. In this part, in order to have a more completed evaluation on the cytotoxic effect of H_2O_2 on normal cell, human adult skin dermal fibroblast (HDFa) cells were exposed to various concentrations of H_2O_2. Their cell viability and cellular damage were then assessed and the intoxication conditions were optimized for studying the protective effects of mushroom extracts.

There are various methods for estimating the numbers of viable cells in culture medium, each with advantages and disadvantages. Some methods are suitable for large number of cultures; some for adherent but not for suspension cells; some are inaccurate with clumped cells and some are more sensitive than others (Phillips, 1973; Cook & Mitchell, 1989; Morris *et al.*, 1997; Doyle & Griffiths, 1998; Falkenhain *et al.*, 1998). For example, direct counting by haemocytometer with the use of a vital stain such as trypan blue provides information about both cell number and viability, but it is labourious and subject to human errors for large numbers of samples. Besides, this method is difficult to be applied to adherent cells like HDFa as typsinization is required.

In this study, cell viability was measured based on indirect estimation of cell numbers which involves measurement of a cell-associated characteristic that correlates with the cell population. Three cell characteristics associated with viable cells were chosen: mitochondrial dehydrogenase activity (MTT assay), cellular lactate dehydrogenase (LDH) released in culture medium as an indicator of cell membrane integrity (permeability) and the amount of cellular protein in a culture which is generally a function of the cell numbers. MTT and LDH assays are useful for both adherent and suspension cells and can be completed in a short time, which are convenient for large number of samples as they are carried out in multi-well mircroplates.

Evaluation of the antioxidant status in cells can be carried out by measurements of biomarkers. Biomarkers are biological molecules having their chemical structures being modified as a result of an attack by free radicals or other reactive species and thus can be used as indicators to reliably assess the oxidative stress status in animals models and in humans (Mello & Kubota, 2007). A valid biomarker should be a major product of oxidative modification that may be implicated directly in the development of disease. Macromolecules such as DNA, lipids, and proteins are major targets of ROS and their oxidative modification often resulted in the development of various diseases. Therefore, these products of damage are commonly used as biomarkers and their measurements are the most common approach for studying ROS. The assessment of these damage products is important in understanding the role of ROS and subsequently help in devising proper intervention strategies (Hwang & Kim, 2007). In this project, H_2O_2-induced DNA damage and lipid peroxidation were studied.

3.4.1 Colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

MTT is reduced to purple formazan by mitochondrial dehydrogenase. This reduction takes place only when mitochondrial dehydrogenase is active, and therefore conversion is directly related to the number of viable cells (Mosmann, 1983). In the present study, untreated cells (incubated with medium only) were regarded as having 100 % viability, and the cell viability of treated cells were calculated by comparing their mitochondrial dehydrogenase activity (color intensity of purple formazan formed) with that of the untreated control cells. As shown in Figure 3.6, H_2O_2 induced a dose-dependent disruption of mitochondrial dehydrogenase activity in HDFa, representing a decrease in cell viability when H_2O_2 concentration increases from 0.1-1 mM.

Exposure of HDFa to 0.1 mM H_2O_2 (the lowest concentration applied) resulted in a slightly decrease in cell viability (78.9 ± 3.87 % of control) indicating that the cells might under mild oxidative stress. While at a H_2O_2 concentration of 1 mM (highest concentration applied), cell viability was reduced to just 8.45 ± 1.33 % of control, revealing that the cells were under severe oxidative stress and most of the cells could not survive in this concentration of H_2O_2. The graph also shows that the decrease in cell viability was less dramatic when H_2O_2 concentration was over 0.5 mM (Figure 3.6). The H_2O_2 concentration causing damage (cell death) to about 50 % of the exposed cells (LC_{50}) was approximately 0.32 mM obtained from the graph. The LC_{50} obtained by MTT assay for HaCaT (human keratinocyte) reported by Svobodova *et al.* (2006) was 0.5 mM. Although both HaCat and HDFa are human skin cells, the former was spontaneously immortalized cell line, so there may be difference in their

resistance (tolerance) to H_2O_2. The results implied that HDFa used in this experiment was more sensitive to H_2O_2.

3.4.2 Lactate dehydrogenase (LDH) assay

LDH is located in cytosol, but is released into the surrounding medium by dead and dying cells upon cell damage/cell lysis. The more LDH released into the surrounding medium, the higher the LDH activity and thus the more severe the cell damage/cell lysis, which is corresponding to the number of dead cells. Measurement of LDH activity in the medium can be used for estimating the cell viability as well as characterizing the stress-induced membrane damage (Haslam *et al.*, 2000).

Figure 3.7 shows the extracellular LDH activity against different H_2O_2 concentration for HDFa. The results indicated that exposure of HDFa to H_2O_2 would lead to a dose-dependent increase in extracellular LDH activity. This implied that the HDFa cell viability decreased when H_2O_2 concentration increased from 0.1-1 mM. The LDH activity at H_2O_2 concentration greater than 0.5 mM began to level off, which was similar to that obtained from MTT assay (Figure 3.6). LDH activity in the culture medium of cells treated with 1 mM H_2O_2 (6.46 ± 0.61 mU/ml) was 12.8 times higher than that of the control (0.504 ± 0.22 mU/ml), so the membrane damage in cells exposed to 1 mM H_2O_2 was severe. This implies that H_2O_2 cytotoxicity might be mediated by its damaging effect on cell membrane.

3.4.3 Total cellular protein loss

Upon cell lysis and cell death, cellular proteins may be degraded and lost. Therefore, loss of total cellular proteins can be used as another measure of cellular integrity. By comparing the total protein content of cells treated with various concentration of H_2O_2 with untreated control cells which are assigned to be 100 % viability, the effect of different H_2O_2 concentration on cell viability can be estimated. After treatment of H_2O_2, cell pellet was collected by centrifugation and was lysed for the determination of protein content. Protein remained in cell debris and protein that was lost to the culture medium would be removed upon aspiration of the supernatant, and therefore would not be measured. The protein remained in the cell pellet that was subsequently measured corresponded to the quantity of the intact (viable) cells. Figure 3.8 shows that there was no significant difference ($p>0.05$) in total protein content of cells treated with 0.1 mM H_2O_2, 0.2 mM H_2O_2 and untreated cells. However, at a H_2O_2 concentration of 0.5 mM or above, less than 40 % of total cellular protein compared with control ($p < 0.05$) was left, indicating that most of the protein was lost due to H_2O_2-induced cell dead. Again, like the trend observed in MTT and LDH assay, when H_2O_2 concentration increased from 0.5 mM to 1 mM, the decrease in protein content was small and not significantly different ($p>0.05$) (Figure 3.8).

At a H_2O_2 concentration of 1 mM, cell viability in terms of total cellular protein was 31.6 % of control while in MTT assay, the cell viability in terms of mitochondrial dehydrogenase activity was 8.45 % of control. The higher viability obtained in terms of total cellular protein could be explained by the possibility that some dead cell debris might be collected in the cell pellet during centrifugation, resulted in overestimation of protein content. The high amount of protein retained in the dead cells could be due to the presence of membrane-bound proteins.

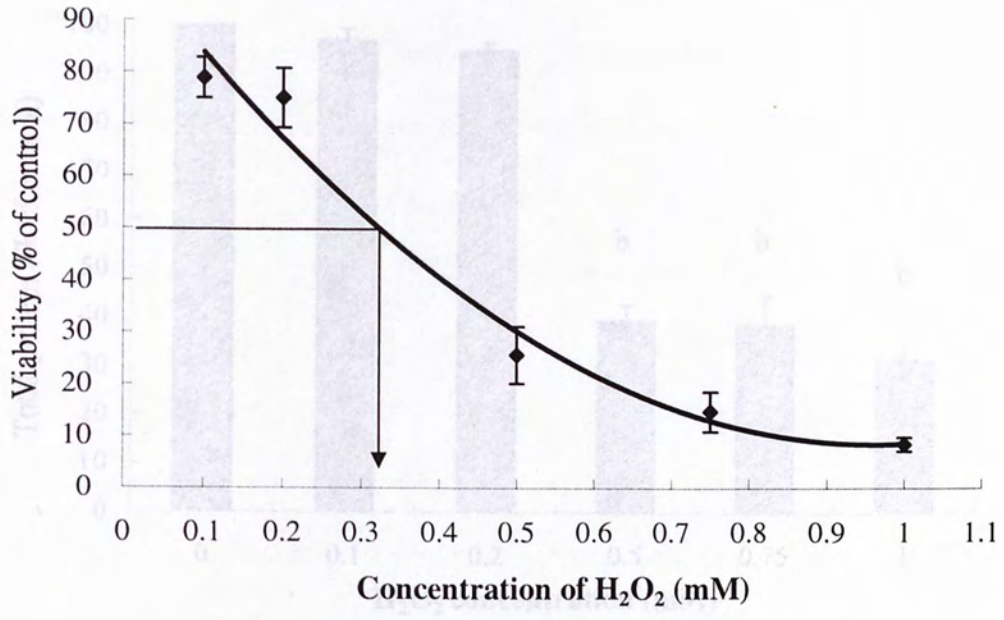

Figure 3.6 Toxic effect of H_2O_2 on HDFa estimated by MTT assay. Data are means ± SD (n=5).

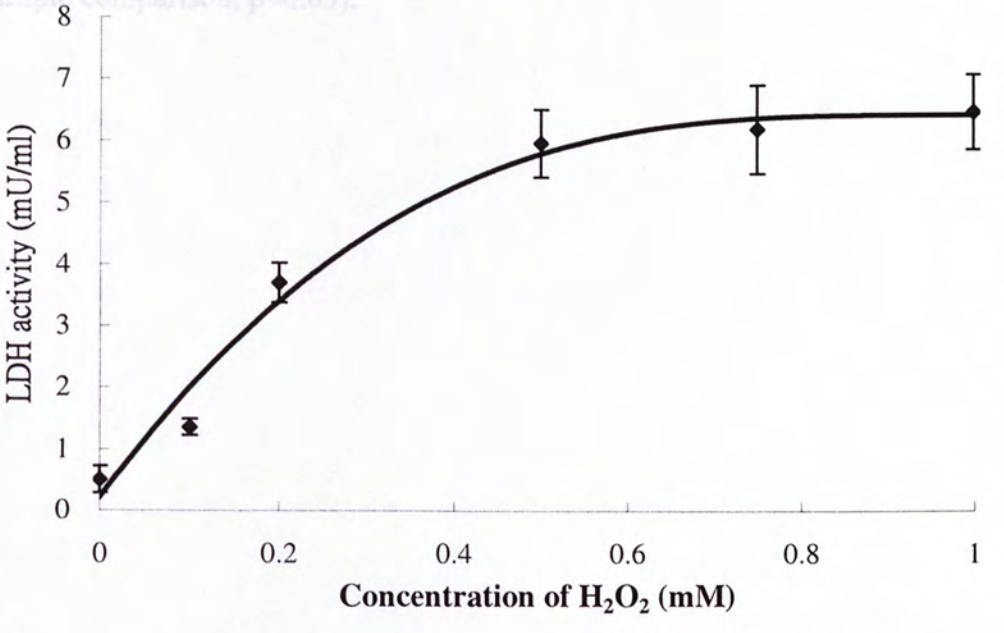

Figure 3.7 Toxic effect of H_2O_2 on HDFa assessed by LDH assay. Data are means ± SD (n=5).

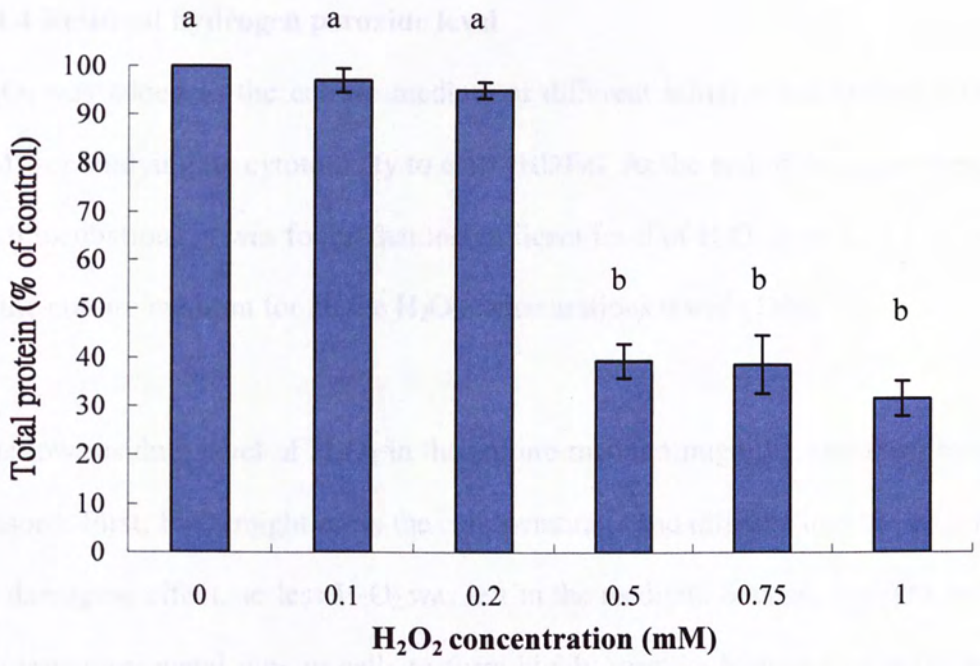

Figure 3.8 Total cellular protein content (% of control) of HDFa after treatment of H_2O_2 at different concentrations. Data are means ± SD (n=3). Different letters represent significant difference between treatments (One-way ANOVA, Tukey's multiple comparison, $p<0.05$).

3.4.4 Residual hydrogen peroxide level

H_2O_2 was added to the culture medium at different initial concentration (100-1000 μM) for studying its cytotoxicity to cells (HDFa). At the end of the experiment (after 24 h incubation), it was found that insignificant level of H_2O_2 (less than 1 %) was left in the culture medium for all the H_2O_2 concentrations tested (Table 3.3).

The low residual level of H_2O_2 in the culture medium might be explained by several reasons. First, H_2O_2 might cross the cell membrane and diffused into the cells to exert its damaging effect, so less H_2O_2 was left in the medium. Second, it might react with the transition metal ions in cells to form highly reactive hydroxyl radicals, so H_2O_2 could not be detected. In fact, cytotoxicity of H_2O_2 is thought to be mediated primarily by the generation of hydroxyl radicals via Fenton reaction (Starke & Farber, 1985; Lloyd *et al.*, 1997). Third, it is also possible that H_2O_2 was decomposed into water and oxygen gas spontaneously after exerting its damaging effect.

Table 3.3 Residual levels of H_2O_2 in culture medium for different initial H_2O_2 concentration after 24-hour incubation

Initial H_2O_2 concentration (μM)	Residual H_2O_2 concentration (μM)
100	0.816 (0.816%)
200	0.867 (0.434%)
500	0.816 (0.163%)
750	4.133 (0.551%)
1000	5.816 (0.582%)

Figures in parenthesis represent the percentage of initial H_2O_2 concentrations left

3.4.5 Lipid peroxidation

The oxidation of membrane lipids, one of the primary events in oxidative cellular damage, can be assessed by measurement of MDA (TBARS) which is a breakdown product of lipid peroxides. The level of MDA after 24-hour exposure of HDFa cells to different concentration of H_2O_2 is shown in Table 3.4 The results show that MDA level after treatment of different concentration of H_2O_2 was similar to that of control, indicating that there was not much lipid peroxidation induced by H_2O_2. However, H_2O_2 was well-known to induce lipid peroxidation in a number of cells. For example, treatment of PC12 cells with 150 μM H_2O_2 caused the increase in the intracellular MDA level by 90.2 % (Liu *et al.*, 2007). Therefore, it was rather unusual that there was not much increase in MDA level even for 24-hour treatment of 1 mM H_2O_2 to HDFa cells. One of the possible explanations for these results was that the cell density used in this study was not high enough so that even there was increase in MDA level, it could not be detected. However, the cell intensity (1×10^6) could hardly be increased, as the cells grew slowly and was rather large in size (one T-175 culture flask contained about 1×10^6 cell only). Therefore, this assay was not used for studying the protective effect of mushroom extracts.

Some studies have shown that lipid peroxidation correlates with LDH leakage due to cell injury (Joyeux *et al.*, 1990). Lipid peroxidation in the cell membrane can result in damage of cell membrane integrity, defective membrane transport mechanisms and increased permeability (Wijeratne *et al.*, 2005), so that cellular components like LDH can leak into the culture medium. However, others have suggested that the two parameters are not related: MDA is due to disorders in the inner membranes and the plasma membrane while LDH release is mainly due to damage to plasma membrane (Morel *et al.*, 1990). According to the results in section 3.4.2, an increase in LDH

leakage into the culture medium after exposure of HDFa to H_2O_2 indicated that the cell membrane was damaged by H_2O_2, however, since MDA level after treatment of H_2O_2 remained comparable to the untreated control level, whether the membrane damage was mediated by lipid peroxidation was unclear.

Table 3.4 Level of lipid peroxidation induced by different concentration of H_2O_2

H_2O_2 concentration (mM)	MDA (nmol/mg protein)
0 (Control without H_2O_2)	3.25 ± 0.482
0.1	3.27 ± 0.614
0.2	3.08 ± 0.355
0.5	3.66 ± 0.531
0.75	4.01 ± 0.964
1	3.92 ± 0.768

Data are means ± SD (n=3)

3.4.6 DNA damage

DNA damage is known to be one of the most sensitive biological markers for evaluating oxidative stress representing the imbalance between free radical generation and efficiencies of the antioxidant system (Gutteridge, 1995; Kassie *et al.*, 2000).

Comet assay (which is also called single-cell gel electrophoresis) used in this experiment is a rapid and sensitive fluorescence microscopic method for the detection of DNA damage on individual cell level and is extensively used for routine screening of potential genotoxic agents. The alkaline version (pH < 13) of the comet assay mainly detects primary (repairable) DNA single- and double-strand breaks and alkali-labile sites (Rosa *et al.*, 2007).

For the detection of H_2O_2-induced DNA damage, a lower concentration range of H_2O_2 (0.01-0.1 mM), compared to 0.1-1 mM used in cell viability tests was chosen. It is because false positives may occur when high doses of cytotoxic agents are used. So, prior to performing the comet assay, a cell viability test should be performed to ensure that at least 75 % cell viability is achieved. From MTT assay, cell viability for cells exposed to 0.1 mM H_2O_2 was about 80 %, so it was chosen to be the highest concentration being studied. In addition, when trypan blue exclusion method was carried out before performing the comet assay, cell viability for all H_2O_2 treatments (concentrations) was greater than 75 %.

The pattern of DNA migration shown in the comet images provides an estimation of the extent of DNA damage due to the ability of denatured or cleaved DNA fragments to migrate out of the nucleus of cell towards the anode under the influence of an

electric field. The greater the DNA migration, the more severe the DNA damage is. Comet images of HDFa are shown in Figure 3.9. For untreated control cells, the DNA was rather intact and remained within the confines of the nucleoid, only little migration of DNA (short comet tail) was observed (Figure 3.9a). This small migration of DNA indicated that the endogenous level of damage within cells and damage that might occur during sample preparation were low. When H_2O_2 concentration increased from 0.01 mM to 0.1 mM, an increase in DNA migration distance was observed and the comet tail became longer and brighter, revealing a greater DNA damage (Figure 3.9b-f). For 0.1 mM H_2O_2 treatment, DNA damage was severe, with almost all DNA being present in the tail and there was no distinct head (Figure 3.9f). These results also indicated that exposure of HDFa to H_2O_2 (0.01 mM or above) for 30 minutes was sufficient to produce detectable DNA damage.

A number of parameters can be used for the quantitation of DNA damage from the comet assay results, in which tail length (migration distance), percentage of DNA in tail (tail DNA density) and Olive tail moment (integrated value of DNA density of the comet tail multiplied by the migration distance) are commonly used. Although tail moment combine the information of both the tail length and percentage tail DNA, it has been pointed out that the tail moment should be addressed carefully and always used in addition to the percentage tail DNA and/or the tail length, due to its masking effect in some cases (De Boeck *et al.*, 2000). It has been reported that using percentage tail DNA as parameter for expression of DNA damage had lower inter-assay and inter-experimental variation than those of tail length (De Boeck *et al.*, 2000).

In this study, the level of H_2O_2-induced DNA damage was expressed as the average

% tail DNA and the mean Olive tail moment obtained from 50 randomly selected cells in each treatment. Figure 3.10a shows that the average background % tail DNA for untreated cells was 22.3 ± 9.22 %. Increasing H_2O_2 concentration increased the % tail DNA linearly and there was a more than two-fold increase in % tail DNA for cells treated with 0.05 mM H_2O_2 when compared with that of untreated control (Figure 3.10a). Results expressed as mean Olive tail moment as shown in Figure 3.10b agreed well with those expressed as % tail DNA (Figure 3.10a), both of which showing a linear relationship between DNA damage and H_2O_2 concentration. For treatment of 0.1 mM H_2O_2, the DNA damage was severe as reflected in the comet image (Figure 3.9f) in which there was no distinct head with nearly all the DNA being present in the tail. However, the value of the % tail DNA and the mean Olive tail moment calculated by the software were low, indicating that the DNA damage was minor. It seems that there was contradiction between the results obtained from the comet image and the calculated values. In fact, it was because the whole comet image was mistakenly regarded as head by the software, leading to low value of the % tail DNA and the mean Olive tail moment. It was unreliable to use these two values to represent the DNA damage. Therefore, the results for treatment of 0.1 mM H_2O_2 were ignored and did not appear in the curves in Figure 3.10a and b.

It was found that the standard deviations (SD) of the % tail DNA and the mean Olive tail moment were relatively large for all H_2O_2 treatments. It might be due to the difference in the level of DNA damage for individual cells, with some cells being more sensitive or resistant to H_2O_2. As with any cell counting assay, variability is intrinsic to the comet assay leading to a larger SD value. However, there was still significant difference ($p<0.05$) between all H_2O_2 treatments and negative untreated control for both results expressed as the % tail DNA and the mean Olive tail moment

(Figure 3.10a and b).

Analyzing the DNA damage of cells by visual scoring in parallel with the computer software may solve the problem of the large SD. Comet images can be scored visually by naked eyes based on 5 recognizable classes of comet, from class 0 (undamaged, no discernible tail) to class 4 (maximum DNA damage, almost all DNA in tail, insignificant head). Each cell (comet) is given a value according to the class it is assigned to, so an overall score (damage index) can be derived for each treatment. For 100 comets scoring, the overall score should be ranging from 0 (completely undamaged: 100 cells x 0) to 400 (with maximum damage: 100 cells x 4) arbitrary units (Rosa *et al.*, 2007). The higher the overall score, the greater the DNA damage is. However, visual scoring may be subjective especially when the level of DNA damage is in between two classes.

It was reported that the % Tail DNA for 30-min treatment of 0.05 mM H_2O_2 in human intestine 407 cells was about 43 % (Yen *et al.*, 2003), which was similar to the result (48.7 %) in the present study (Figure 3.10a). While the DNA damage of Caco-2 (cancer) cells treated with 0.05-0.1 mM H_2O_2 was not significantly different from that of control cells. DNA damage in Caco-2 cells increased at H_2O_2 concentrations greater than 0.15 mM (Wijeratne *et al.*, 2005). This implied that DNA in cancer cells may be more resistant to H_2O_2 damage.

DNA damage, if not repair will result in mutations in DNA. Also, if damaged DNA replicates before it is repaired, a permanent DNA alteration could occur, again leading to carcinogenesis (Jackson, 2002).

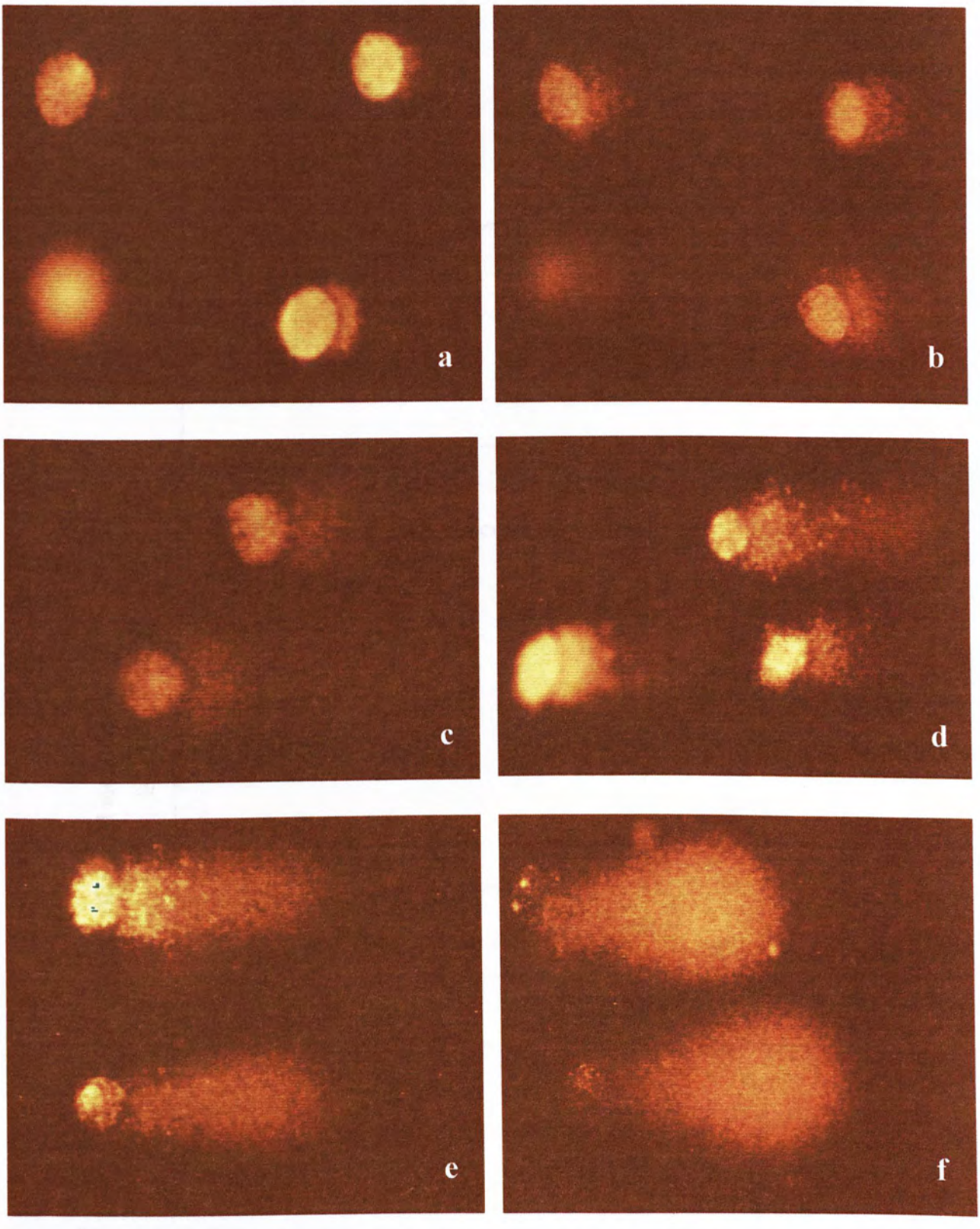

Figure 3.9 Comet images of HDFa for (a) negative control without H_2O_2 treatment; treatment of (b) 0.01, (c) 0.02, (d) 0.04, (e) 0.05, (f) 0.1 mM H_2O_2.

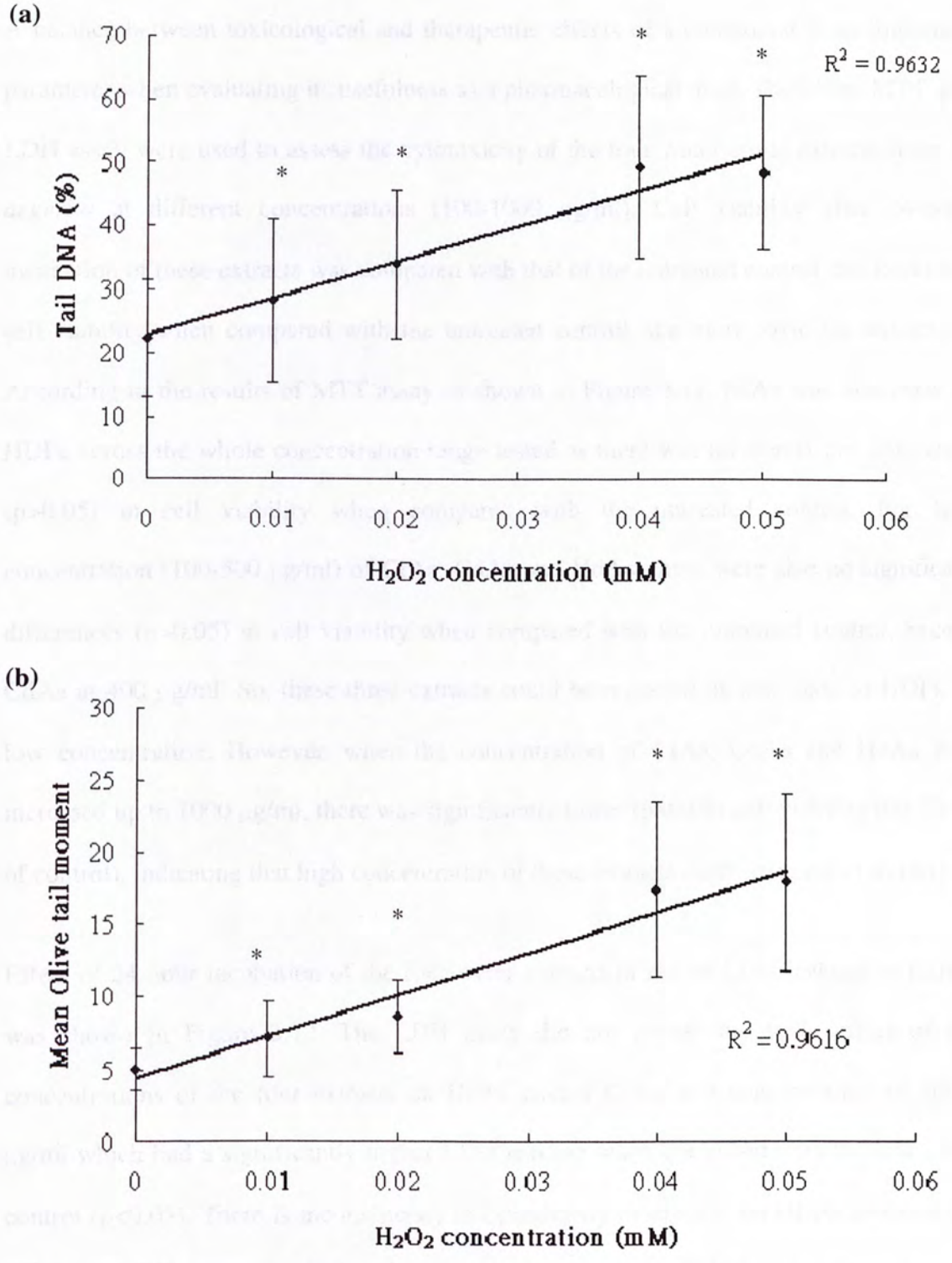

Figure 3.10 Relationship between H_2O_2 concentration and DNA damage on HDFa expressed as (a) % tail DNA; (b) mean Olive tail moment. Data are means ± SD (n=50). * indicates significant difference between the means of treatment with untreated control (Student's t tests, $p<0.05$).

3.5 Cytotoxicity of extracts

A balance between toxicological and therapeutic effects of a compound is an important parameter when evaluating its usefulness as a pharmacological drug. Therefore, MTT and LDH assay were used to assess the cytotoxicity of the four water crude extracts from *A. aegerita* at different concentrations (100-1000 μg/ml). Cell viability after 24-hour incubation of these extracts was compared with that of the untreated control, the lower the cell viability when compared with the untreated control, the more toxic the extract is. According to the results of MTT assay as shown in Figure 3.11, HfAa was non-toxic to HDFa across the whole concentration range tested as there was no significant difference ($p>0.05$) in cell viability when compared with the untreated control. For low concentration (100-500 μg/ml) of CfAa, CdAa and HdAa, there were also no significant differences ($p>0.05$) in cell viability when compared with the untreated control, except CdAa at 400 μg/ml. So, these three extracts could be regarded as non-toxic to HDFa at low concentration. However, when the concentration of CfAa, CdAa and HdAa was increased up to 1000 μg/ml, there was significantly lower ($p<0.05$) cell viability (60-70 % of control), indicating that high concentration of these extracts exert toxic effect to HDFa.

Effect of 24-hour incubation of the four water extracts of Aa on LDH leakage in HDFa was shown in Figure 3.12. The LDH assay did not reveal any toxic effect of all concentrations of the four extracts on HDFa except CfAa at a concentration of 1000 μg/ml which had a significantly higher LDH leakage when compared with the untreated control ($p<0.05$). There is inconsistency in cytotoxicity of extracts on HDFa revealed by MTT and LDH assay, in which only CfAa at concentration of 1000 μg/ml showed toxic effect in both assays. At a concentration of 1000 μg/ml, CdAa and HdAa did not show toxic effect in LDH assay implying that the toxic effect of these extracts is not mediated through membrane damage. The cytotoxic effect of these extracts should be taken into account when high concentration was used.

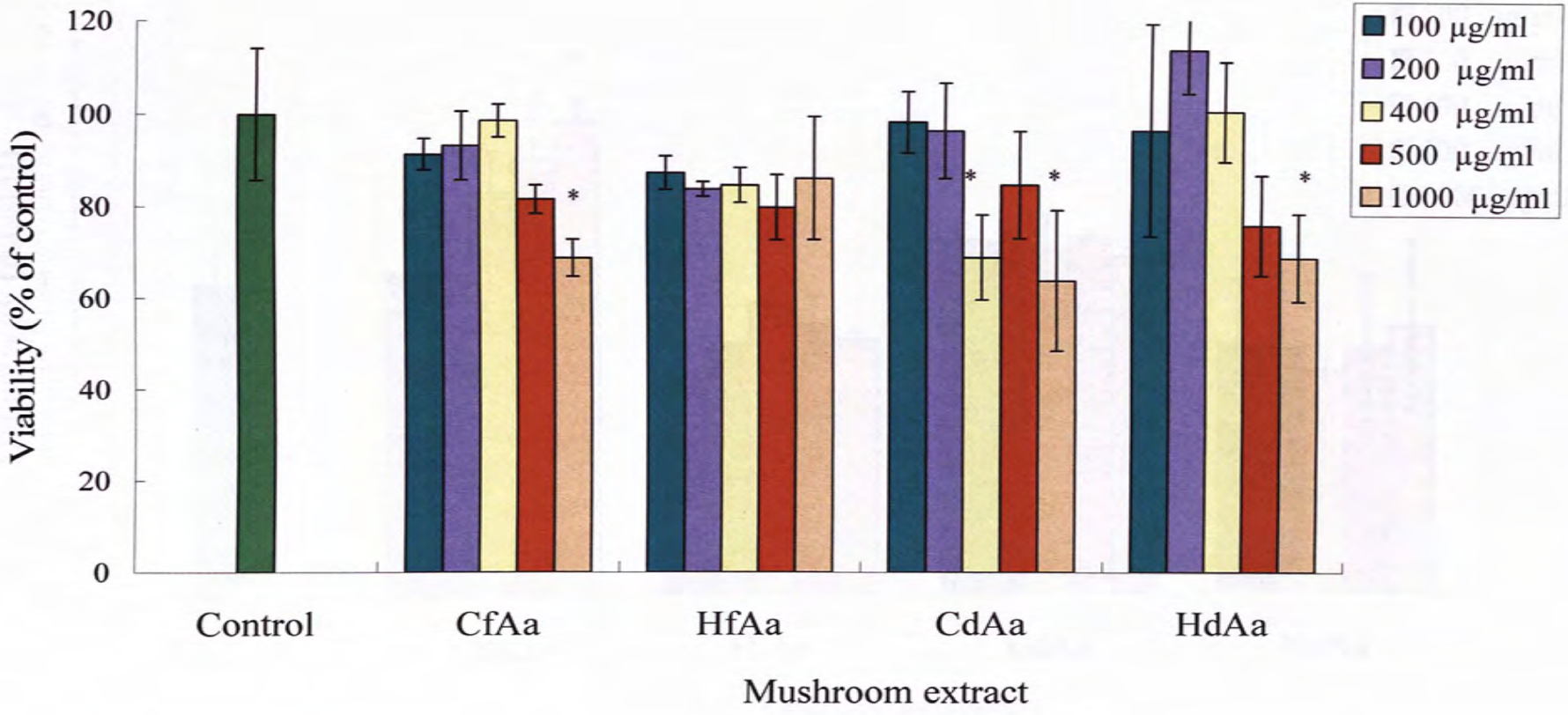

Figure 3.11 Toxic effect of different water extracts from Aa on adult human skin dermal fibroblast cells measured by MTT assay. Data are means ± SD (n=3). * represent significant difference of the means between treatment and untreated control (Student's *t*-tests, $p<0.05$).

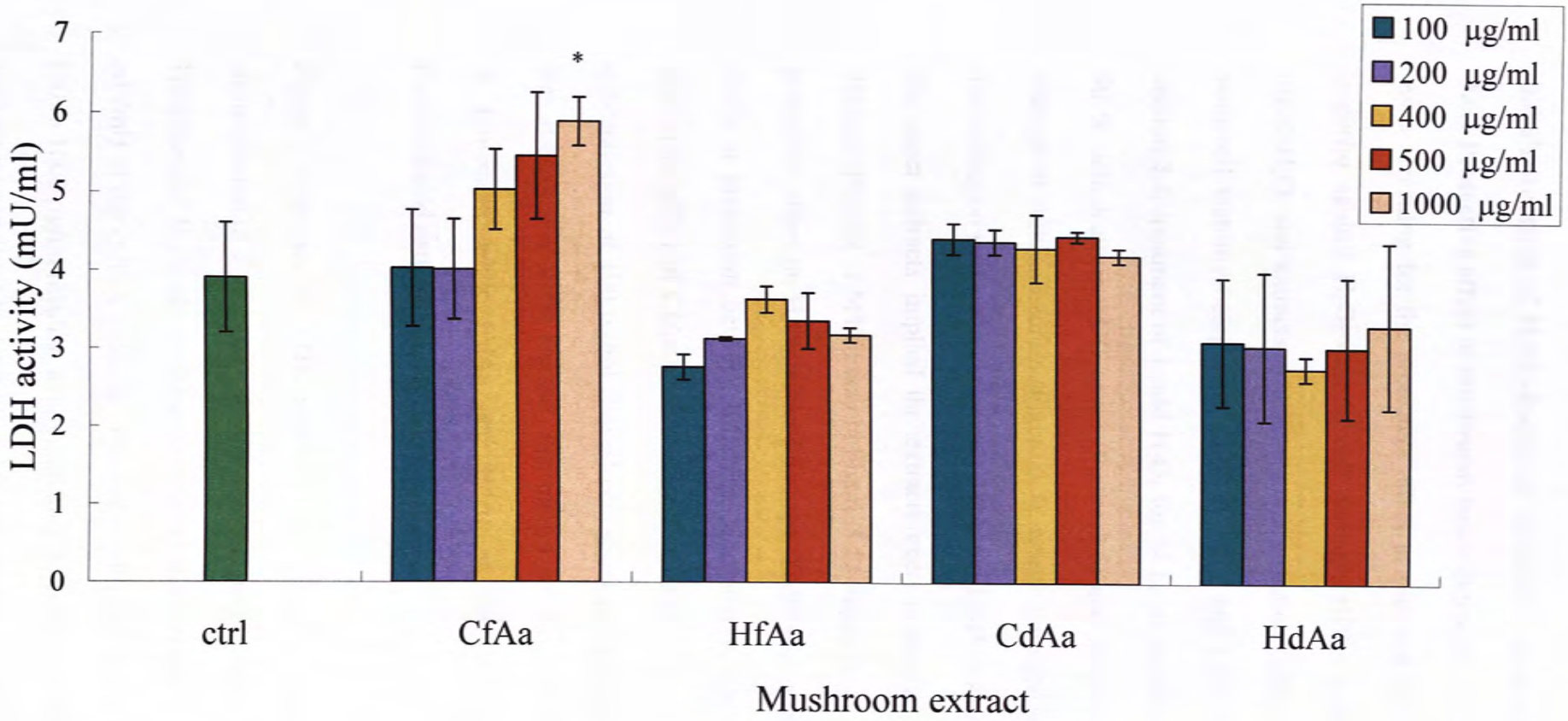

Figure 3.12 Toxic effect of different water extracts from Aa on adult human skin dermal fibroblast cells measured by LDH assay. Data are means ± SD (n=3). * represent significant difference of the means between treatment and untreated control (Student's *t*-tests, $p < 0.05$).

3.6 Protection of H_2O_2-induced oxidative damage in HDFa cells

3.6.1 Protective effect of mushroom water extracts

As a screening for the protective effect of the four water crude extracts from *A. aegerita* against H_2O_2-induced cell damage, HDFa cells were co-incubated with 1mM H_2O_2 and extracts at different concentration (100-1000 µg/ml) for 24 hours, with cell viability being determined by MTT and LDH assay. From the results in section 3.4, treatment of 1 mM H_2O_2 for 24 hours in HDFa could induce more than 90 % cell death in MTT assay (Figure 3.6) and around 13-fold increase in LDH leakage in culture medium (Figure 3.7), when compared with the untreated control. The reduction in the percentage cell death and LDH leakage after co-incubation with the water extracts implied the extracts exert protective effects on H_2O_2-induced damage. Results of MTT assay in Figure 3.13 shows that CfAa exerted a very strong protective effect on H_2O_2-induced damage. There was no significant difference ($p > 0.05$) in protection between different concentrations of CfAa, implying that the protective effect of CfAa was not dose-dependent in this concentration range. At a concentration of 100 µg/ml (lowest concentration applied), the protection of CfAa was already over 80 %. For the other three water extracts, HfAa showed less than 20 % protection while CdAa and HdAa showed nearly no protection against H_2O_2-induced damage (Figure 3.13).

Figure 3.14 shows the LDH activity in the culture medium of HDFa after 24 hour co-incubation of 1 mM H_2O_2 with different concentrations of the four water extracts. Treatment of H_2O_2 alone (0 µg/ml extract) showed the highest LDH activity (6.46 mU/ml) in the culture medium. Treatment with CfAa at a concentration range from 100 to 1000 µg/ml resulted in a remarkable decrease in LDH leakage into the culture medium on exposure to 1 mM H_2O_2 (LDH activity dropped to less than 2.5 mU/ml)

(Figure 3.14). At a concentration over 500 µg/ml, CfAa caused a slight rise in LDH activity, probably due to the toxic effect to HDFa caused by its high concentration as reported in section 3.5. The ability of extracts to decrease the LDH activity in the culture medium was also expressed as % protection as shown in Figure 3.15. CfAa shows more than 70 % protection in the concentration range studied. These results indicate that CfAa protected the HDFa cells by the maintenance of membrane integrity, thus prevent them from leaking LDH into the culture medium. It has been reported that the molecular structure of flavonoids allow them to accumulate at water-lipid interfaces and/or penetrate into membrane, therefore, protecting cellular membranes from oxidative damage by acting as reducing agents, and/or modifying the fluidity of membrane components (Morand *et al.*, 1998; Arora *et al.*, 2000; Roig *et al.*, 2002). This implies that the components in CfAa that contributed to the protective effect against H_2O_2-induced LDH leakage might be flavonoids or structure similar to flavonoids.

For the other three water extracts, only a slight decrease in LDH activity in the culture medium was observed (Figure 3.14) and the percentage protection was much lower (less than 40 %) than that of CfAa (over 70 %) (Figure 3.15). Since the protective effect of HfAa, CdAa and HdAa examined by both MTT and LDH assay was not prominent, only CfAa was selected for further investigation.

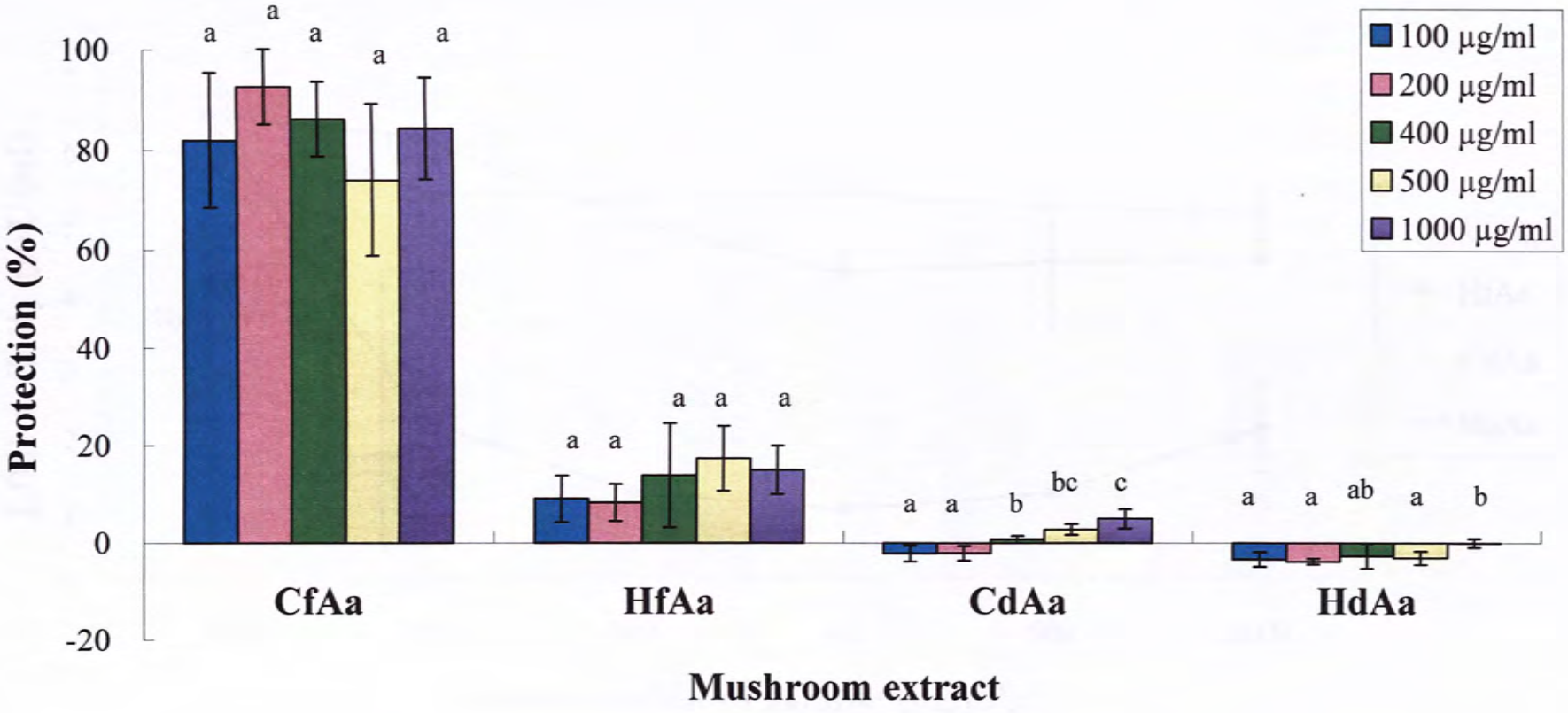

Figure 3.13 Protective effect of different water crude extracts from Aa against 1 mM H_2O_2-induced damage in HDFa assessed by MTT assay. Data are means ± SD (n=5). Different letters represent significant difference between concentrations for the same extract (One-way ANOVA, Tukey's multiple comparison, $p<0.05$).

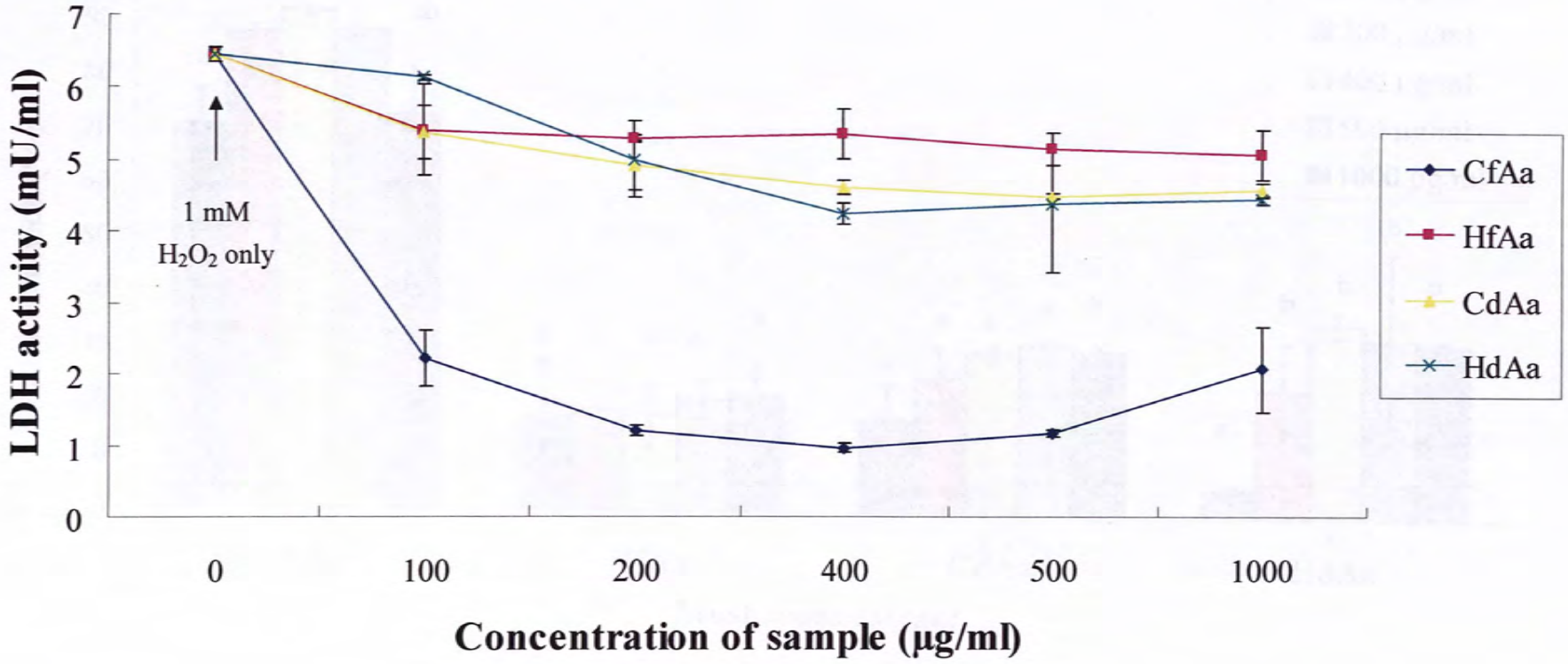

Figure 3.14 LDH activity in the culture medium of cells exposed to H_2O_2 and different concentration of the four water crude extracts from Aa. Data are means ± SD (n=3)

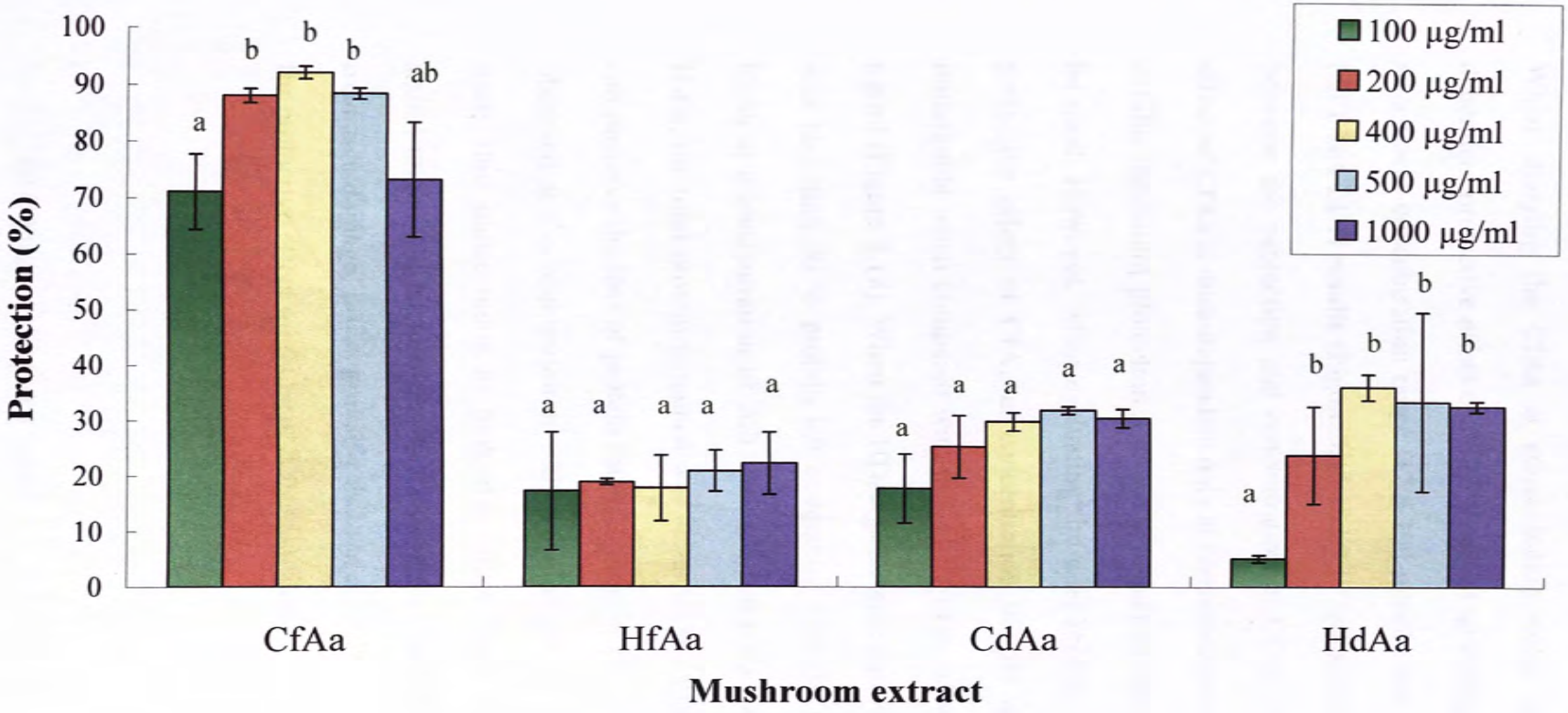

Figure 3.15 Protective effect of different water crude extracts from Aa against H_2O_2-induced damage in HDFa assessed by LDH assay. Data are means ± SD (n=3). Different letters represent significant difference between concentrations for the same extract (One-way ANOVA, Tukey's multiple comparison, $p<0.05$).

3.6.2 Protective effect of CfAa on H_2O_2-induced damage to HDFa

When studying the CfAa at concentration range of 100-1000 μg/ml, no dose-dependent protective effect could be observed according to the results in section 3.6.1, so a lower concentration range (12.5-100 μg/ml) was also studied in here. Both the MTT and LDH results (Figure 3.16 and 3.17, respectively) show a linear relationship between the protection and concentration of CfAa. This means that the protective effect of CfAa is dose-dependent only at concentration range lower than 100 μg/ml. To exhibit maximum protection, CfAa at a concentration greater than 100 μg/ml should be used. However, when evaluating the total protein content, it was found that the protective effect of CfAa at concentration of 100 and 1000 μg/ml was not very remarkable when compared with that of CfAa at concentration of 200, 400 and 500 μg/ml (Figure 3.18). When the HDAa cells were treated with 1 mM H_2O_2 alone, there was less than 30 % protein left comparing with the untreated control. However, if CfAa at a concentration of 200, 400 and 500 μg/ml was added simultaneously with H_2O_2, the total protein remained was about 90 % of the control, indicating that CfAa can preserve the loss of protein caused by H_2O_2. The relatively lower protective effect observed at a concentration of 1000 μg/ml might be due to the cytotoxicity of CfAa itself, thus undermining its protective effect. Since CfAa at a concentration of 100 μg/ml might still be too low for exerting the maximum protection of HDFa against oxidative damage, it was probable that the most effective concentration range of CfAa for protective effect might be at 200-500 μg/ml.

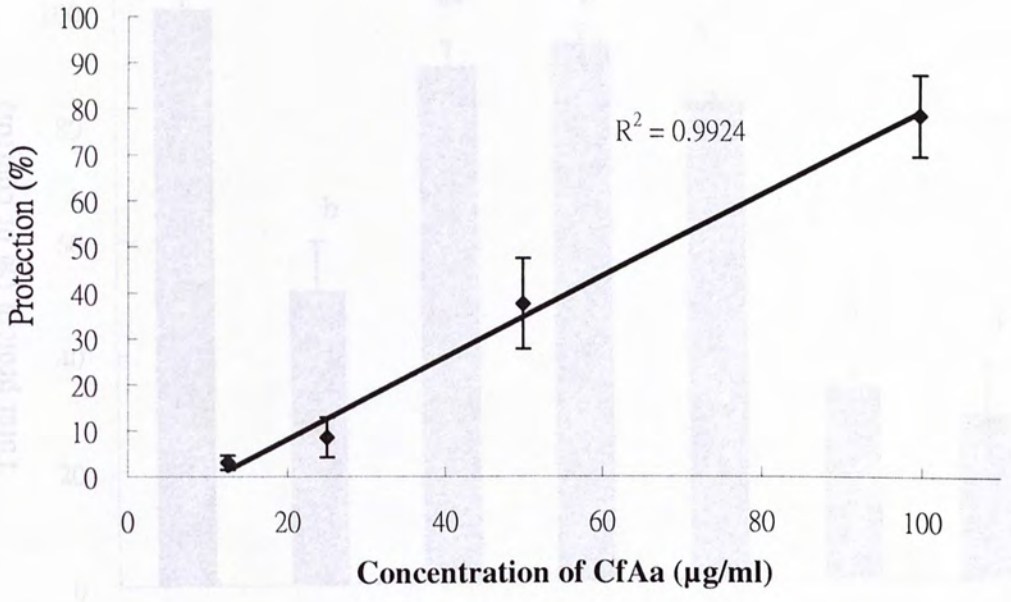

Figure 3.16 Protective effect of CfAa on H_2O_2-induced damage at concentration range of 12.5-100 µg/ml examined by MTT assay. Data are means ± SD (n=3)

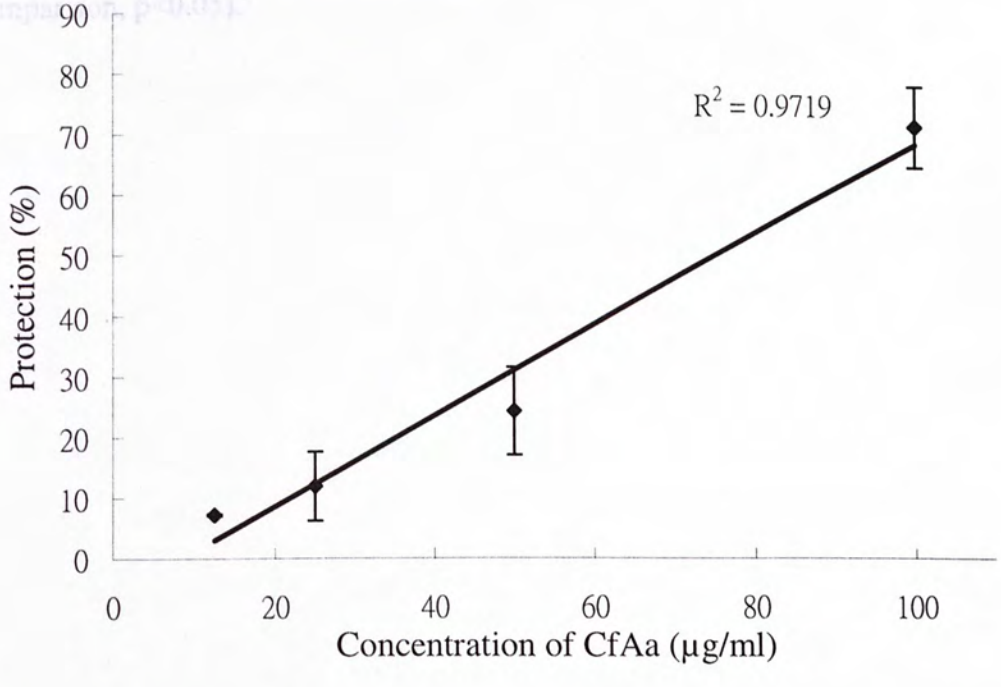

Figure 3.17 Protective effect of CfAa on H_2O_2-induced damage at concentration range of 12.5-100 µg/ml examined by LDH assay. Data are means ± SD (n=3).

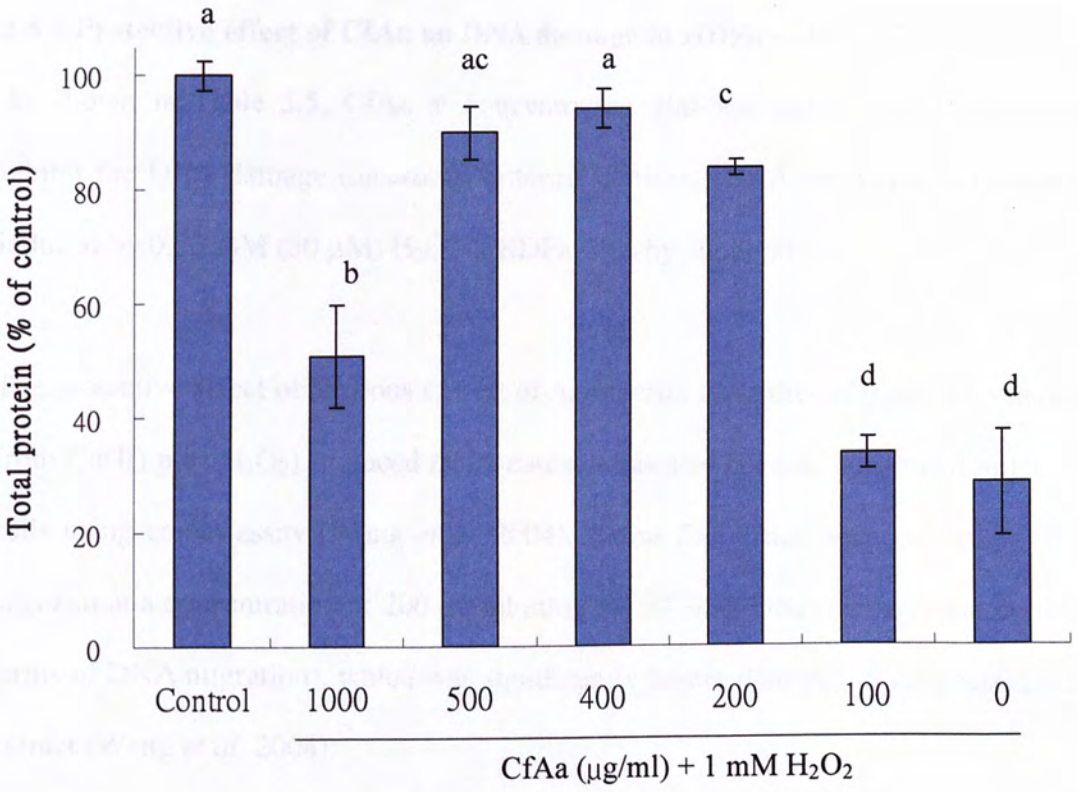

Figure 3.18 Total protein content of cells after treatment with different concentration of CfAa and 1 mM H_2O_2. Data are means ± SD (n=3). Different letters represent significant difference between treatments (One-way ANOVA, Tukey's multiple comparison, $p<0.05$).

3.6.3 Protective effect of CfAa on DNA damage in HDFa cells

As shown in Table 3.5, CfAa at concentration 200-500 μg/ml could significantly inhibit the DNA damage (measured in terms of % tail DNA and Olive tail moment) induced by 0.05 mM (50 μM) H_2O_2 in HDFa cells by about 50 %.

The protective effect of aqueous extract of *A. aegerita* on hydroxyl radicals (generated from Cu(II) plus H_2O_2) -induced DNA damage has also been demonstrated in HepG2 cells using comet assay (Wang *et al.* 2004). It was found that aqueous extract of *A. aegerita* at a concentration of 200 μg/ml inhibited 67 % of DNA damage (measured in terms of DNA migration), which was significantly higher than that of *Lentinus edodes* extract (Wang *et al.* 2004).

It has been found that some mushrooms showed protection on DNA damage only in cold-water crude extract. For example, cold water (20℃) extract of *Agaricus bisporus* at a concentration of 0.5 mg/ml showed a virtually complete protection against H_2O_2-induced (10 μM) DNA damage to Raji cells (a human lymphoma cell line), but the protection was not observed in its hot water extract (Shi *et al.*, 2002a). This indicated that the temperature used in the extraction is crucial and the active components could be denatured in high temperature. Later, the genoprotective effect of *A. bisporus* was found to be associated with a heat-labile protein, identified as tyrosinase and present in the fruit body. The genoprotective effect of this tyrosinase is dependent upon the enzymic hydroxylation of tyrosine to 3,4-dihydroxy-L-phenylalanine (L-DOPA) and subsequent conversion of this metabolite to dopaquinone (Shi *et al.*, 2002b).

Oxidative DNA damage has been recognized as major cause of cell death and mutations in all aerobic organisms. In humans, oxidative DNA damage is also considered an important promoter of cancer (Slupphaug *et al.*, 2003). So, the protective effect of CfAa on DNA damage may help preventing the development of cancer.

Table 3.5 Protective effect of CfAa on H_2O_2-induced DNA damage in HDFa cells

Teatment	DNA damage		Protection (%)	
	% tail DNA	Olive tail moment	% tail DNA*	Olive tail moment**
Control (untreated)	20.2 ± 10.2^{a}	4.28 ± 1.35^{a}	--	--
0.05 mM H_2O_2	65.8 ± 10.9^{b}	20.8 ± 5.68^{b}	--	--
CfAa 500 µg/ml + 0.05 mM H_2O_2	41.7 ± 10.9^{c}	11.2 ± 2.11^{c}	52.8	58.3
CfAa 400 µg/ml + 0.05 mM H_2O_2	42.9 ± 9.54^{cd}	12.2 ± 2.49^{c}	50.2	52.2
CfAa 200 µg/ml + 0.05 mM H_2O_2	44.3 ± 8.73^{d}	11.9 ± 3.45^{c}	47.2	53.9

Different superscripts within the same column represent significant difference between treatments (One-way ANOVA, Tukey's multiple comparison, $p<0.05$).

*The percent of protection

= [1- (%TD of CfAa - %TD of H_2O_2) / (% TD of control - %TD of H_2O_2)] x 100 %

**The percent of protection

= [1- (TM of CfAa - TM of H_2O_2) / (TM of control - TM of H_2O_2)] x 100 %

Where TD is tail DNA and TM is Olive tail moment

3.7 Modulation of cellular antioxidant defense system by CfAa

It is recognized that most cell types exhibit a complex antioxidant response as part of their oxidative stress defenses. The antioxidant enzymes play an important role in the defense of cells against oxidative insults. Higher levels of the antioxidant enzymes have been correlated with decreased susceptibility to cell damage (Werts & Gould, 1986). Growing evidence has indicated that the ability of cells to maintain their redox balance in the setting of stress plays an essential role in cell survival (Weber *et al.*, 2007). In order to investigate whether the protective effect of CfAa on H_2O_2-induced damage were mediated by enhancing the cellular antioxidant defense system, glutathione cellular redox status and the concerted action of several main detoxifying enzymes related to H_2O_2 (superoxide dismutase, catalase, glutathione peroxidase and glutathione reducatase) were assayed to provide data on major pathways involved in detoxifying H_2O_2. Since there may be difference in cellular antioxidant defense system for different degree of oxidative stress, two concentration of H_2O_2 (0.2 mM and 1 mM) were studied. An understanding of cellular responses to oxidative stress may also lead to new insights into the pathogenesis of oxidative stress-related diseases and to new therapeutic strategies for these pathologic conditions.

3.7.1 Intracellular total glutathione

Glutathione (exists in both the reduced (GSH) and the oxidized (GSSG) forms) is an important skin antioxidant, which is involved in maintaining the intracellular redox balance (Svobodova *et al.*, 2006). It has been known to play a key role in protecting human skin cells from UV-induced damage (Tyrrell & Pidoux, 1986). GSH depletion generates a pro-oxidant status in cells, rendering them sensitive to oxidative agents and ROS (Estrela *et al.*, 2006), so the depletion of GSH has been found to promote oxidative stress in various model systems and it could be considered as markers of oxidative stress (Bhattacharya & Bhattacharya, 2007).

Results in Table 3.6 shows that HDFa cells treated with both 0.2 mM and 1 mM H_2O_2 significantly led to a decrease in total glutathione level (3.70 ± 0.101 and 2.24 ± 0.865 nmol/mg protein, respectively) when compared with that of the untreated control cells (6.54 ± 0.142 nmol/mg protein). The decrease in total glutathione level in 1 mM H_2O_2 treatment was more severe than that in 0.2 mM H_2O_2 treatment, which was consistent with the result that cell viability for 1 mM H_2O_2 treatment was much lower than that for 0.2 mM H_2O_2 treatment (Section 3.4). This suggests that H_2O_2-induced oxidative stress may be caused by GSH depletion, leading to cell injury and loss of viability. It has been reported that a loss of GSH is associated with impairment of the electron transport chain and breakdown of ATP synthesis that lead to further decrease in GSH content in cells (Svobodova *et al.*, 2006). In some other cases, increase in GSH level had been observed in response to oxidative stress in acute toxicity studies (Schuliga *et al.*, 2002; Yeh *et al.*, 2002), but the dosage used was sub-toxic or non-lethal. According to results in section 3.4, 1 mM H_2O_2 was lethal dosage, so it is rational that results in this study shows GSH depletion but not GSH elevation in response to H_2O_2 treatment.

Treatment of compounds that stimulate an increase in cellular GSH level usually can lower or abolish the oxidative damage (Rosa *et al.*, 2007). In this study, results shows that cells treated with CfAa alone at a concentration of 500 μg/ml did not lead to an increase in total glutathione level when compared with that of untreated control (Table 3.6). However, co-incubation of CfAa at all the concentrations tested (200, 400 and 500 μg/ml) with 0.2 or 1 mM H_2O_2 kept the total glutathione level between that of the stressed (H_2O_2 treated only) and control (untreated) cells (Table 3.6). This suggests that treatment of CfAa alone could not enhance the GSH level, but it could attenuate the decrease in GSH level induced by H_2O_2. The protective effect of CfAa might be due to other mechanism instead of enhancing GSH level, but it is also possible that CfAa could enhance GSH level in stress environment. To further investigate the role of GSH in the protective effect of CfAa, buthionine sulfoximine (BSO) could be used to inhibit GSH synthesis by inactivating the key enzyme (γ -glutamyl-cysteine synthetase) in GSH synthesis (Griffith & Meister, 1979). Elimination/diminution of the protective effect of CfAa upon BSO treatment would indicate the requirement of GSH synthesis for the protection of CfAa.

As shown in Table 3.6, there is no significant difference in total glutathione level between different concentrations of CfAa, suggesting that there was no dose-dependent response in this concentration range. This was consistent with the protective effects of CfAa observed in section 3.6.

3.7.2 Enzyme activities

Superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) are considered to be the most important enzymes in protecting oxidative challenge (Wijeratne et al., 2005). SOD destroys the superoxide anion by converting it to hydrogen peroxide, which in turn is destroyed by CAT or GPx reactions. Antioxidant enzymes also exhibit synergistic interactions by protecting each other from specific free radical attacks. SOD protects CAT and GPx from inactivation by superoxide anion, while CAT and GPx protect SOD from inactivation by hydroperoxides (Bray et al., 1974; Sinet & Garber, 1981; Blum & Fridovich, 1985). The protective effects of CAT and GPx may have been responsible for the activity of SOD that was present in cells treated with higher concentrations of H_2O_2. In a biological system the observed effect of an enzyme would be the net effect all synergistic and antagonistic effects of other enzymes and compounds present in the cellular environment (Wijeratne *et al.*, 2005), so enzyme activities may be inter-related and it is more meaningful to study the activity of several enzymes together.

As shown in Table 3.6, SOD activity remained unchanged for all treatments except that for treatment of 1 mM H_2O_2 alone (0.305 ± 0.017 U/mg protein) which was slightly lower than the untreated control level (0.344 ± 0.015 U/mg protein). It was reported that pure SOD in a noncellular system showed an increase in oxidation-reduction capacity when exposed to 250-1000 μM H_2O_2 for 15 min. However, SOD activity decreased dramatically with longer exposure durations to H_2O_2 (Jhonson & Macdonald, 2004). Inhibition of SOD by H_2O_2 could be associated with the reduction of the active site (Cu^{2+}) in SOD to Cu^{+} (Symonyan & Nalbandyan, 1972), the destruction of histidine that is close to Cu^{2+} (Bray et al., 1974), and/or the structure alteration of SOD that restricts access to Cu^{2+} (Jhonson & Macdonald, 2004).

For a treatment of 0.2 mM H_2O_2, there was no significant change ($p>0.05$) in CAT activity but a decrease in GPx activity (by 20.3 %) when compared with that of untreated control. While for a treatment of 1 mM H_2O_2, there was a significant decrease ($p<0.05$) in both the CAT and GPx activity by 32.5 % and 44.4 %, respectively when compared with that of the untreated control. As GSH is the principle substrate of GPx for the decomposition of H_2O_2 into H_2O, GSH availability may limit the activity of GPx, so the depletion in total glutathione for the treatment of 0.2 and 1 mM H_2O_2 might be responsible for the decrease in activity of GPx.

Since SOD is a peroxide-generating enzyme while GPx and CAT are peroxide-removing enzymes, changes in CAT/SOD and GPx/SOD ratios are more indicative of antioxidant cellular response than individual enzyme activities (Roig *et al.*, 2002). Sometimes, even there is a decrease in CAT or GPx activities, there can still be an increase in CAT/SOD or GPx/SOD ratio if SOD activity decreases. Increase in CAT/SOD or GPx/SOD ratio indicates that the ability of cells to act against H_2O_2 increases. The results of the present study indicated that both the CAT/SOD and GPx/SOD ratio for the treatment of 0.2 and 1 mM H_2O_2 were lower than that of the untreated control cells, with more dramatic decrease in the case of 1 mM H_2O_2 treatment (Table 3.6).

In some cases, cells response to H_2O_2 challenge (especially for mild stress) by enhancing the activity of either CAT or GPx, or both to prevent oxidative damage. For example, exposure of Caco-2 (human colorectal adenocarcinoma) cells to 50-250 μM H_2O_2 for 30 min significantly increased the CAT and GPx activity (Wijeratne et al., 2005). It was also found that exposure of Chinese hamster V79

cells to repeated low doses of H_2O_2 (30 μM) induced resistance of cell to subsequent greater oxidative stress (0.1 - 0.9 mM H_2O_2) by enhancing antioxidant defense through higher GSH content and greater activity of enzymes like Cu-Zn SOD, CAT and GPx (Bose et al.,2005). Although GPx and CAT share the same substrate (H_2O_2), it had been reported that CAT was more significant at protecting against severe oxidant stress (Roig *et al.*, 2002) while there was other report suggested that GPx was more active than CAT in removing H_2O_2 (Wijeratne *et al.*, 2005). Hence, the effect of H_2O_2 challenge may be different from condition-to-condition (e.g. different cell lines, concentrations of H_2O_2 and incubation times). To determine which enzyme (CAT or GPx) is responsible for the majority of H_2O_2-scavenging activity, CAT inhibitor (aminotriazole) was used in some studies (Ohta *et al.*, 2006), if incubation with CAT inhibitor resulted in a significant loss of H_2O_2-scavenging activity, that means CAT is the major enzyme acting against H_2O_2.

On the other hand, in some other cases, antioxidant enzymes could be inhibited by H_2O_2 treatment. A study by Liu *et al.* (2007) found that treatment of PC12 cells (rat pheochromocytoma) with 150 μM H_2O_2 for 12 hours caused the decrease in the activities of SOD, CAT and GPx by 77.6 %, 66.6 % and 69.5 %, respectively. The results in this study agrees with their findings in which SOD, CAT and GPx activities decreased (by 11.3, 32.5, 44.4 %, respectively) after exposure to 1 mM H_2O_2 for 24 hours, but in a lesser extent when compared with their findings. Comparing with the conditions used in the present study, Liu *et al.* used a lower concentration and exposure duration of H_2O_2 but resulted in greater inhibition of enzymes activities, this indicating that the PC12 cells were very sensitive to damage caused by H_2O_2. In the present study, the inhibition of enzymes activity (SOD, CAT and GPx) as well as decrease in CAT/SOD and GPx/SOD ratio observed in 1 mM H_2O_2 treatment in HDFa

cells implied that the capacity of cellular antioxidants defense system was not sufficient to overcome the H_2O_2 challenge and H_2O_2 concentration used (1 mM) was high enough to overwhelm the antioxidative defense, leading to the occurrence of oxidative damage as reported in section 3.4.

Similar to the results for total glutathione level, CfAa alone would not lead to enhancement of enzymes activities as the activity of CAT and GPx for treatment of CfAa alone at a concentration of 500 μg/ml was not significantly different from that of the untreated control, but co-incubation of CfAa at a concentration range 200-500 μg/ml significantly restored the 1 mM H_2O_2 -induced decrease in enzymes activities as well as CAT/SOD and GPx/SOD ratio back to about the untreated control level (Table 3.6). In view of GPx activity, the action of CfAa in two stress levels (0.2 and 1 mM H_2O_2) was similar. A number of substances such as polyphenolic compounds have been reported to exert their protective effect against oxidative damage through enhancement of antioxidant enzymes (Duthie *et al.*, 1998). The mixture of flavonoids found in red wine had also been reported to increase CAT/SOD and GPx/SOD ratios when they were simultaneously incubated with H_2O_2 (Roig *et al.*, 2006). However, whether incubation of these compounds alone would enhance the enzyme activities have not been reported.

Glutathione reducatase (GR), the enzyme that regenerates GSH from the GSSG, ensures that there is enough GSH for the action of GPx as well as maintaining the redox status in cells. GR activity would be correlated with GPx as higher activity of GPx would convert more GSH to GSSG, so higher activity of GR is required for regenerating the GSH. The results in the present study showed that there was no significant difference ($p > 0.05$) in GR activity between all treatments. This might be

due to the fact that GR activity increased only when the cellular GSSG level increased significantly. However, GPx activity was decreased by H_2O_2 while CfAa treatment restored the GPx activity back to normal level. Therefore, there was no increase in GPx activity and the change (increase) in GSSG level in cells became small, assuming that GSSG level did not affect by other factors very greatly.

Table 3.6 Total glutathione level and enzyme activities in HDFa cells treated with H_2O_2 and/or CfAa

Treatment	Total glutathione nmol/mg protein	SOD U/mg protein	CAT nmol/min/mg protein	CAT/SOD ratio	GPx nmol/min/mg protein	GPx/SOD ratio	GR nmol/min/mg protein
Control	6.54 ± 0.142^{a}	0.344 ± 0.015^{a}	39.7 ± 1.48^{a}	115	38.5 ± 2.60^{a}	112	3.60 ± 0.821^{a}
CfAa (500 μg/ml)	5.57 ± 0.351^{b}	0.324 ± 0.006^{a}	35.2 ± 0.058^{ab}	109	39.5 ± 8.41^{a}	122	3.72 ± 0.731^{a}
CfAa (500 μg/ml) + 1 mM H_2O_2	3.69 ± 0.454^{c}	0.326 ± 0.022^{a}	32.1 ± 1.40^{ab}	98.5	41.5 ± 0.643^{a}	127	3.83 ± 1.25^{a}
CfAa (400 μg/ml) + 1 mM H_2O_2	3.91 ± 0.130^{c}	0.312 ± 0.015^{a}	36.4 ± 1.08^{ab}	117	42.0 ± 1.57^{a}	135	3.81 ± 0.225^{a}
CfAa (200 μg/ml) + 1 mM H_2O_2	3.53 ± 0.122^{c}	0.318 ± 0.010^{a}	30.7 ± 5.83^{b}	96.7	40.4 ± 1.29^{a}	127	3.78 ± 0.130^{a}
H_2O_2 only (1mM)	2.24 ± 0.865^{d}	0.305 ± 0.017^{b}	26.8 ± 0.633^{c}	87.9	21.4 ± 14.5^{b}	70.2	3.63 ± 0.513^{a}
CfAa (500 μg/ml) + 0.2 mM H_2O_2	4.70 ± 0.168^{e}	0.335 ± 0.010^{a}	36.2 ± 0.388^{ab}	108	41.9 ± 2.38^{a}	125	3.38 ± 0.373^{a}
CfAa (200 μg/ml) + 0.2 mM H_2O_2	4.20 ± 0.583^{e}	0.313 ± 0.010^{a}	35.4 ± 1.24^{ab}	113	38.8 ± 0.640^{a}	124	3.32 ± 0.875^{a}
H_2O_2 only (0.2 mM)	3.70 ± 0.101^{c}	0.356 ± 0.058^{a}	36.0 ± 3.81^{ab}	101	30.7 ± 1.13^{c}	86.2	3.57 ± 0.182^{a}

Data are means ± SD (n=3). Different superscripts within the same column represent significant difference between treatments (One-way ANOVA, Tukey's multiple comparison, $p<0.05$).

3.8 Speculation on the possible components in CfAa

A variety of compounds from mushrooms have been found to be antioxidants, including melanin, polysaccharides, protein and compounds that mimic SOD. For the potential compounds in CfAa contributed to its strong H_2O_2 scavenging activity and genoprotective effect, it was not likely to be polysaccharides since their solubility in cold water is usually poor and they have higher solubility in hot water. The potential component (s) is believed to be heat-labile, so it is likely to be proteins as proteins are more sensitive to heat, which affects their structures and activities. Since the active compounds are present only in fresh mushroom, it is possible to be enzymes or compounds that mimic CAT having strong H_2O_2 scavenging activity. Further investigation is required to carried out to study the enzyme activities in CfAa to find out the nature of the antioxidative components.

Chapter 4 Conclusion and further works

For *in vitro* antioxidant activity assays, four water extracts from *A. aegerita* had no significant different in $ABTS^{\cdot+}$ scavenging activity and they had similar hydroxyl radical scavenging activity. However, the hydrogen peroxide scavenging activity of CfAa was significantly higher than the other three extracts.

For the cytotoxicity of hydrogen peroxide in HDFa cells, three variability tests (MTT, LDH assay and total cellular protein loss) did result in similar profiles. Cell viability decreased with increasing concentration of H_2O_2 with 0.5 mM being the critical H_2O_2 concentration that caused severe damage in HDFa cells as further decrease in cell viability at H_2O_2 concentration greater than 0.5 mM was small. Besides, H_2O_2 at concentration range from 0.01 to 0.1 mM caused significant DNA damage in HDFa cells.

For the protective effect of the four mushroom extracts, CfAa showed prominent protection against 1 mM H_2O_2-induced damage in both the MTT and LDH assays, as well as preventing the loss of cellular protein. The most effective concentration of CfAa for maximum protection was found to be between 200-500 g/ml. While for the other three extracts, the protective effect was insignificant. CfAa at concentration of 200-500 g/ml was found to exhibit about 50 % protection on 0.05 mM H_2O_2-induced DNA damage. It was also found that CfAa could restore the H_2O_2-induced suppression in intracellular total glutathione level and enzyme activities (SOD, CAT and GPx) back to normal level.

The prominent results found in the protective effects of CfAa on H_2O_2-induced damage in HDFa cells was in agreement with the remarkable H_2O_2 scavenging activity. It is possible that CfAa destroyed H_2O_2 directly before it could act on the cells, but it is also possible that CfAa helped maintaining the high level of cellular antioxidant defense system so that the cells could act directly against H_2O_2. Further investigation is required to confirm the underlying mechanism of this protective effect.

It has been reported that several tumor cell lines (such as colon carcinoma and ovarian carcinoma) constitutively produced large amounts of H_2O_2 (Szatrowski & Nathan, 1991). One possible explanation for this is that cancer cells could have low levels of H_2O_2-detoxifying enzymes. The H_2O_2 produced may cause further damage in the neighboring cells that favor the cancer cell development. So the strong H_2O_2 scavenging activity of CfAa may help improving this situation.

The genoprotective activity of CfAa is believed to be associated with its heat-labile component (s), but the exact nature of the protective component (s) remains to be established.

In conclusion, CfAa might contain antioxidants with strong H_2O_2 scavenging activity and protective effect on H_2O_2-induced damage. *A. aegerita* might serve as potential natural protective agents in human diets to help reduce oxidative stress.

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